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Molecular Cloning

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Chapter 1 Plasmids and Their Usefulness in Molecular Cloning

Protocol 1: Preparation of Plasmid DNA by Alkaline Lysis with SDS: Minipreparation

Plasmid DNA is isolated from small-scale (1-2 ml) bacterial cultures by treatment with alkali and SDS.

Protocol 2: Preparation of Plasmid DNA by Alkaline Lysis with SDS: Midipreparation

Plasmid DNA is isolated from intermediate-scale (20-50 ml) bacterial cultures by treatment with alkali and SDS.

Protocol 3: Preparation of Plasmid DNA by Alkaline Lysis with SDS: Maxipreparation

Plasmid DNA is isolated from large-scale (500 ml) bacterial cultures by treatment with alkali and SDS.

Protocol 4: Preparation of Plasmid DNA by Small-scale Boiling Lysis

Plasmid DNA is isolated from small-scale (1-2 ml) bacterial cultures by treatment with Triton X-100 and lysozyme, followed by heating. This method is not recommended for preparing plasmid DNA from strains of *E. coli* that express endonuclease A (*endA*$^+$ strains).

Protocol 5: Preparation of Plasmid DNA by Large-scale Boiling Lysis

Plasmid DNA is isolated from large-scale (500 ml) bacterial cultures by treatment with Triton X-100 and lysozyme, followed by heating. This method is not recommended for preparing plasmid DNA from strains of *E. coli* that express endonuclease A (*endA*$^+$ strains).

Protocol 6: Preparation of Plasmid DNA: Toothpick Minipreparation

Plasmid DNA is prepared directly from bacterial colonies plucked from the surface of agar media with toothpicks.

Protocol 7: Preparation of Plasmid DNA by Lysis with SDS

Large (>15 kb), closed circular plasmids are prepared (albeit inefficiently and in small yield) by lysing bacteria with SDS.

Protocol 8: Purification of Plasmid DNA by Precipitation with Polyethylene Glycol

Crude preparations of plasmid DNA are first treated with lithium chloride and RNase (to remove RNA). The plasmid DNA is then precipitated in a solution containing polyethylene glycol and $MgCl_2$.

Protocol 9: Purification of Plasmid DNA by Chromatography

The following table summarizes the salient features of many of the commercial resins that are currently available for plasmid purification. Individual manufacturers supply detailed instructions, which should be followed to the letter.

Protocol 10: Purification of Closed Circular DNA by Equilibrium Centrifugation in CsCl-Ethidium Bromide Gradients: Continuous Gradients

Solutions containing plasmid DNA are adjusted to a density of 1.55 g/ml with solid CsCl. The intercalating dye, ethidium bromide, which binds differentially to closed circular and linear DNAs, is then added to a concentration of 200 µg/ml. During centrifugation to equilibrium, the closed circular DNA and linear DNAs form bands at different densities.

Protocol 11: Purification of Closed Circular DNA by Equilibrium Centrifugation in CsCl-Ethidium Bromide Gradients: Discontinuous Gradients

A solution containing plasmid DNA, saturating amounts of ethidium bromide, and CsCl (44% w/v) is layered between two solutions of lesser (35% w/v CsCl) and greater density (59% w/v CsCl). During centrifugation to equilibrium, the closed circular plasmid DNA and linear DNAs form bands at different densities.

Protocol 12: Removal of Ethidium Bromide from DNA by Extraction with Organic Solvents

Ethidium bromide is removed from DNA by phase extraction with organic solvents.

Protocol 13: Removal of Ethidium Bromide from DNA by Ion-exchange Chromatography

Ethidium bromide is removed from DNA by chromatography through a cation-exchange resin.

Protocol 14: Removal of Small Fragments of Nucleic Acid from Preparations of Plasmid DNA by Centrifugation through NaCl

Contamination of plasmid DNA by fragments of DNA and RNA is reduced to an acceptable level by centrifugation through 1 M sodium chloride. This method was devised by Brian Seed when he was a graduate student at Harvard University.

Protocol 15: Removal of Small Fragments of Nucleic Acid from Preparations of Plasmid DNA by Chromatography through Sephacryl S-1000

Contamination of plasmid DNA by small fragments of nucleic acid is reduced dramatically by size-exclusion chromatography through Sephacryl S-1000.

Protocol 16: Removal of Small Fragments of Nucleic Acid from Preparations of Plasmid DNA by Precipitation with Lithium Chloride

High-molecular-weight RNA and proteins can be precipitated from preparations of plasmid DNA by high concentrations of LiCl and removed by low-speed centrifugation.

Protocol 17: Directional Cloning into Plasmid Vectors

Directional cloning requires that the plasmid vector be cleaved with two restriction enzymes that generate incompatible termini and that the fragment of DNA to be cloned carries termini that are compatible with those of the doubly cleaved vector.

Protocol 18: Attaching Adaptors to Protruding Termini

Adaptors are short double-stranded synthetic oligonucleotides that carry an internal restriction endonuclease recognition site and single-stranded tails at one or both ends. Adaptors are used to exchange restriction sites at the termini of linear DNA molecules. They may be purchased in phosphorylated and unphosphorylated forms.

Protocol 19: Blunt-ended Cloning into Plasmid Vectors

Target DNA is ligated to a blunt-ended plasmid DNA, and the products of the ligation reaction are used to transform competent *E. coli.* The maximum number of "correct" clones can generally be obtained from ligation reactions containing equimolar amounts of plasmid and target DNAs, with the total DNA concentration being <100 µg/ml. Blunt-end ligation catalyzed by bacteriophage T4 DNA ligase is suppressed by high concentrations (5 mM) of ATP and polyamines such as spermidine.

Protocol 20: Dephosphorylation of Plasmid DNA

During ligation in vitro, T4 DNA ligase will catalyze the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide carries a 5´-phosphate residue and the other carries a 3´-hydroxyl terminus. Recircularization of vector DNA can therefore be minimized by removing the 5´-phosphate residues from both termini of the linear, double-stranded plasmid DNA with alkaline phosphatase.

Protocol 21: Addition of Synthetic Linkers to Blunt-ended DNA

Linkers are small self-complementary pieces of synthetic DNA, usually 8-16 nucleotides in length, that anneal to form blunt-ended, double-stranded molecules containing a restriction site. Linkers are used to equip blunt-ended termini of DNA with restriction sites as an aid to cloning.

Protocol 22: Ligating Plasmid and Target DNAs in Low-melting-temperature Agarose

Ligation in low-melting-temperature agarose is much less efficient than ligation with purified DNA in free solution and requires a large amount of DNA ligase. The method is used chiefly for rapid subcloning of segments of DNA in dephosphorylated vectors and assembling recombinant constructs.

Protocol 23: The Hanahan Method for Preparation and Transformation of Competent *E. coli:* High-efficiency Transformation

This procedure generates competent cultures of *E. coli* that can be transformed at high frequencies (5 x 10^8 transformed colonies/mg of superhelical plasmid DNA). **IMPORTANT** All steps in this protocol should be carried out aseptically.

Protocol 24: The Inoue Method for Preparation and Transformation of Competent *E. Coli*: "Ultra-Competent" Cells

This protocol reproducibly generates competent cultures of *E. coli* that yield 1 x 10^8 to 3 x 10^8 transformed colonies/mg of plasmid DNA. The protocol works optimally when the bacterial culture is grown at 18°C. If a suitable incubator is not available, a standard bacterial shaker can be set up in a 4°C cold room and regulated to 18°C.

Protocol 25: Preparation and Transformation of Competent *E. coli* Using Calcium Chloride

This protocol, developed approx. 30 years ago, is used to prepare batches of competent bacteria that yield 5 x 10^6 to 2 x 10^7 transformed colonies/µg of supercoiled plasmid DNA.

Protocol 26: Transformation of *E. coli* by Electroporation

Electrocompetent bacteria are prepared by growing cultures to mid-log phase, washing the bacteria extensively at low temperature, and then resuspending them in a solution of low ionic strength containing glycerol. DNA is introduced during exposure of the bacteria to a short high-voltage electrical discharge.

Protocol 27: Screening Bacterial Colonies Using X-gal and IPTG: α-Complementation

α-complementation occurs when two inactive fragments of *E. coli* β-galactosidase associate to form a functional enzyme. Many plasmid vectors carry a short segment of DNA containing the coding information for the first 146 amino acids of β-galactosidase. Vectors of this type are used in host cells that express the carboxy-terminal portion of the enzyme. Although neither the host nor the plasmid-encoded fragments of β-galactosidase are themselves active, they can associate to form an enzymatically active protein. Lac^+ bacteria that result from α-complementation are easily recognized because they form blue colonies in the presence of the chromogenic substrate X-gal. However, insertion of a fragment of foreign DNA into the polycloning site of the plasmid almost invariably results in production of an amino-terminal fragment that is no longer capable of α-complementation. Bacteria carrying recombinant plasmids therefore form white colonies. The development of this simple blue-white color test has greatly simplified the identification of recombinants constructed in plasmid vectors.

Protocol 28: Screening Bacterial Colonies by Hybridization: Small Numbers

This procedure, a variant of the Grunstein and Hogness (1979) method, is used to screen a small number of bacterial colonies (<200) that are dispersed over several agar plates and are to be screened by hybridization to the same radiolabeled probe. The colonies are gridded onto a master plate and onto a nitrocellulose or nylon filter laid on the surface of a second agar plate. After a period of growth, the colonies on the filter are lysed and processed for hybridization. The master plate is stored until the results of the screening procedure become available.

Protocol 29: Screening Bacterial Colonies by Hybridization: Intermediate Numbers

Bacterial colonies growing on agar plates are transferred en masse to nitrocellulose filters. The spatial arrangement of colonies on the plates is preserved on the filters. After transfer, the filters are processed for hybridization to an appropriate radiolabeled probe while the original (master) plate is incubated for a few hours to allow the bacterial colonies to regrow in their original positions. This technique, a variant of the Grunstein and Hogness (1975) method, was developed at Cold Spring Harbor Laboratory in 1975. The procedure works best with 90-mm plates containing <2500 colonies.

Protocol 30: Screening Bacterial Colonies by Hybridization: Large Numbers

This procedure is used to plate, replicate, and subsequently screen large numbers of bacterial colonies (up to 2 x 10^4 colonies per 150-mm plate or 10^4 colonies per 90-mm plate).

Protocol 31: Lysing Colonies and Binding of DNA to Filters

In this protocol, based on the procedure of Grunstein and Hogness (1975), alkali is used to liberate DNA from bacterial colonies on nitrocellulose or nylon filters. The DNA is then fixed to the filter by UV-cross-linking or baking under vacuum.

Protocol 32: Hybridization of Bacterial DNA on Filters

This protocol describes procedures to hybridize DNA from transformed colonies immobilized on filters with radiolabeled probes and to recover from a master plate the corresponding colonies that hybridize specifically to the probe. The method is based on the procedure published by Grunstein and Hogness (1975).

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Protocol 1

Preparation of Plasmid DNA by Alkaline Lysis with SDS: Minipreparation

Plasmid DNA is isolated from small-scale (1-2 ml) bacterial cultures by treatment with alkali and SDS.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Alkaline lysis solution I
- Alkaline lysis solution II
- Alkaline lysis solution III

Antibiotic for plasmid selection

Ethanol

- ⚠ Phenol:chloroform (1:1, v/v)
- STE
- TE (pH 8.0) containing 20 µg/ml RNase A

Media

- Rich medium

METHOD

1. Inoculate 2 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37°C with vigorous shaking.
2. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at maximum speed for 30 seconds at 4°C in a microfuge. Store the unused portion of the original culture at 4°C.
3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
4. Resuspend the bacterial pellet in 100 µl of ice-cold Alkaline lysis solution I by vigorous vortexing.
5. Add 200 µl of freshly prepared Alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube rapidly five times. *Do not vortex!* Store the tube on ice.
6. Add 150 µl of ice-cold Alkaline lysis solution III. Close the tube and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 3-5 minutes.
7. Centrifuge the bacterial lysate at maximum speed for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh tube.
8. (*Optional*) Add an equal volume of phenol:chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the emulsion at maximum speed for 2 minutes at 4°C in a microfuge. Transfer the aqueous upper layer to a fresh tube.
9. Precipitate nucleic acids from the supernatant by adding 2 volumes of ethanol at room temperature. Mix the solution by vortexing and then allow the mixture to stand for 2 minutes at room temperature.
10. Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.
11. Remove the supernatant by gentle aspiration as described in Step 3 above. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a Kimwipe or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.
12. Add 1 ml of 70% ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at maximum speed for 2 minutes at 4°C in a microfuge.
13. Remove all of the supernatant by gentle aspiration as described in Step 3.Take care with this step, as the pellet sometimes does not adhere tightly to the tube.
14. Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5-10 minutes).
15. Dissolve the nucleic acids in 50 µl of TE (pH 8.0) containing 20 µg/ml DNase-free RNase A (pancreatic RNase). Vortex the solution gently for a few seconds. Store the DNA solution at -20°C.

REFERENCES

1. Birnboim H.C. and Doly J. 1979. A rapid alkaline procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523.
2. Ish-Horowicz D. and Burke J.F. 1981. Rapid and efficient cosmid cloning. *Nucleic Acids Res.* 9:2989-2998.

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Protocol 2

Preparation of Plasmid DNA by Alkaline Lysis with SDS: Midipreparation

Plasmid DNA is isolated from intermediate-scale (20-50 ml) bacterial cultures by treatment with alkali and SDS.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Alkaline lysis solution I
  For preparations of plasmid DNA that are to be subjected to further purification by chromatography (please see Chapter 1, Protocol 9), sterile Alkaline lysis solution I may be supplemented just before use with the appropriate volume of 20 mg/ml DNase-free RNase A (pancreatic RNase) to give a final concentration of 100 µg/ml.
- Alkaline lysis solution II
- Alkaline lysis solution III

Antibiotic for plasmid selection

Ethanol

Isopropanol

- ⚠ Phenol:chloroform (1:1, v/v)
- STE
- TE (pH 8.0) containing 20 µg/ml RNase A

Media

- Rich medium

METHOD

1. Inoculate 10 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37°C with vigorous shaking.
2. Transfer the culture into a 15-ml tube and recover the bacteria by centrifugation at 2000*g* (4000 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C.
3. Remove the medium by gentle aspiration, leaving the bacterial pellet as dry as possible.
4. Resuspend the bacterial pellet in 200 µl of ice-cold Alkaline lysis solution I by vigorous vortexing, and transfer the suspension to a microfuge tube.
5. Add 400 µl of freshly prepared Alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube rapidly five times. *Do not vortex!* Store the tube on ice.
6. Add 300 µl of ice-cold Alkaline lysis solution III. Close the tube and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 3-5 minutes.
7. Centrifuge the bacterial lysate at maximum speed for 5 minutes at 4°C in a microfuge. Transfer 600 µl of the supernatant to a fresh tube.
8. Add an equal volume of phenol:chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the emulsion at maximum speed for 2 minutes at 4°C in a microfuge. Transfer the aqueous upper layer to a fresh tube.
9. Precipitate nucleic acids from the supernatant by adding 600 µl of isopropanol at room temperature. Mix the solution by vortexing and then allow the mixture to stand for 2 minutes at room temperature.
10. Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at room temperature in a microfuge.
11. Remove the supernatant by gentle aspiration as described in Step 3 above. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Remove any drops of fluid adhering to the walls of the tube.
12. Add 1 ml of 70% ethanol to the pellet and recover the DNA by centrifugation at maximum speed for 2 minutes at room temperature in a microfuge.
13. Remove all of the supernatant by gentle aspiration as described in Step 3.
14. Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (2-5 minutes).
15. Dissolve the nucleic acids in 100 µl of TE (pH 8.0) containing 20 µg/ml DNase-free RNase A (pancreatic RNase). Vortex the solution gently for a few seconds and store at -20°C.

REFERENCES

1. Birnboim H.C. and Doly J. 1979. A rapid alkaline procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523.
2. Ish-Horowicz D. and Burke J.F. 1981. Rapid and efficient cosmid cloning. *Nucleic Acids Res.* 9:2989-2998.

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Protocol 3

Preparation of Plasmid DNA by Alkaline Lysis with SDS: Maxipreparation

Plasmid DNA is isolated from large-scale (500 ml) bacterial cultures by treatment with alkali and SDS.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Alkaline lysis solution I
  For preparations of plasmid DNA that are to be subjected to further purification by chromatography (please see Chapter 1, Protocol 9), sterile Alkaline lysis solution I may be supplemented just before use with the appropriate volume of 20 mg/ml DNase-free RNase A (pancreatic RNase) to give a final concentration of 100 µg/ml
- Alkaline lysis solution II
- Alkaline lysis solution III

Antibiotic for plasmid selection

- ⚠ Chloramphenicol (34 mg/ml)

Ethanol

Isopropanol

- STE
- TE (pH 8.0)

Enzymes and Buffers

- Lysozyme (10 mg/ml)

Restriction endonucleases

Media

- Rich medium

Additional Reagents

Steps 8 and 19 of this protocol require reagents listed in Chapter 5, Protocol 1 .

Step 18 of this protocol requires reagents listed in Chapter 1, Protocol 8 , Chapter 1, Protocol 9 , Chapter 1, Protocol 10 , or Chapter 1, Protocol 11 .

METHOD

1. Inoculate 30 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic either with a single colony of transformed bacteria or with 0.1-1.0 ml of a small-scale liquid culture grown from a single colony.
2. Incubate the culture at the appropriate temperature with vigorous shaking until the bacteria reach late log phase (OD_{600} = approx. 0.6).
3. Inoculate 500 ml of LB, YT, or Terrific Broth medium (prewarmed to 37°C) containing the appropriate antibiotic in a 2-liter flask with 25 ml of the late-log-phase culture. Incubate the culture for approx. 2.5 hours at 37°C with vigorous shaking (300 cycles/minute on a rotary shaker).
4. For relaxed plasmids with low or moderate copy numbers, add 2.5 ml of 34 mg/ml chloramphenicol solution. The final concentration of chloramphenicol in the culture should be 170 µg/ml.
   For high-copy-number plasmids, do not add chloramphenicol.
5. Incubate the culture for a further 12-16 hours at 37°C with vigorous shaking (300 cycles/minute on a rotary shaker).
6. Remove an aliquot (1-2 ml) of the bacterial culture to a fresh microfuge tube and store it at 4°C. Harvest the remainder of the bacterial cells from the 500-ml culture by centrifugation at 2700*g* (4100 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C. Discard the supernatant. Stand the open centrifuge bottle in an inverted position.
7. Resuspend the bacterial pellet in 200 ml of ice-cold STE. Collect the bacterial cells by centrifugation as described in Step 6. Store the pellet of bacteria in the centrifuge bottle at -20°C.
8. Use one of the methods described in Chapter 1, Protocol 1 or Chapter 1, Protocol 4 to prepare plasmid DNA from the 1-2-ml aliquot of bacterial culture set aside in Step 6. Analyze the minipreparation plasmid DNA by digestion with the appropriate restriction enzyme(s) and agarose gel electrophoresis to ensure that the correct plasmid has been propagated in the large-scale culture.
9. Allow the frozen bacterial cell pellet from Step 7 to thaw at room temperature for 5-10 minutes. Resuspend the pellet in 18 ml (10 ml) of Alkaline lysis solution I.
10. Add 2 ml (1 ml) of a freshly prepared solution of 10 mg/ml lysozyme.
11. Add 40 ml (20 ml) of freshly prepared Alkaline lysis solution II. Close the top of the centrifuge bottle and mix the contents thoroughly by gently inverting the bottle several times. Incubate the bottle for 5-10 minutes at room temperature.
12. Add 20 ml (15 ml) of ice-cold Alkaline lysis solution III. Close the top of the centrifuge bottle and mix the contents gently but well by swirling the bottle several times (there should no longer be two distinguishable liquid phases). Place the bottle on ice for 10 minutes.
13. Centrifuge the bacterial lysate at ≥20,000*g* (11,000 rpm in a Sorvall GSA rotor) for 30 minutes at 4°C in a medium-speed centrifuge. Allow the rotor to stop without braking. At the end of the centrifugation step, decant the clear supernatant into a graduated cylinder. Discard the pellet remaining in the centrifuge bottle.
14. Measure the volume of the supernatant. Transfer the supernatant together with 0.6 volume of isopropanol to a fresh centrifuge bottle. Mix the contents well and store the bottle for 10 minutes at room temperature.
15. Recover the precipitated nucleic acids by centrifugation at 12,000*g* (8000 rpm in a Sorvall GSA rotor) for 15 minutes at room temperature.
16. Decant the supernatant carefully, and invert the open bottle on a paper towel to allow the last drops of supernatant to drain away. Rinse the pellet and the walls of the bottle with 70% ethanol at room temperature. Drain off the ethanol, and use a Pasteur pipette attached to a vacuum line to remove any beads of liquid that adhere to the walls of the bottle. Place the inverted, open bottle on a pad of paper towels for a few minutes at room temperature.
17. Dissolve the damp pellet of nucleic acid in 3 ml of TE (pH 8.0).
18. Purify the crude plasmid DNA either by column chromatography (Chapter 1, Protocol 9), precipitation with polyethylene glycol (Chapter 1, Protocol 8), or equilibrium centrifugation in CsCl-ethidium bromide gradients (Chapter 1, Protocol 10 and Chapter 1, Protocol 11).
19. Check the structure of the plasmid by restriction enzyme digestion followed by gel electrophoresis.

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Protocol 4

Preparation of Plasmid DNA by Small-scale Boiling Lysis

Plasmid DNA is isolated from small-scale (1-2 ml) bacterial cultures by treatment with Triton X-100 and lysozyme, followed by heating. This method is not recommended for preparing plasmid DNA from strains of *E. coli* that express endonuclease A (*endA*$^+$ strains).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Antibiotic for plasmid selection

Ethanol

Isopropanol

- Sodium acetate (3.0 M, pH 5.2)
- STET
- TE (pH 8.0) containing 20 µg/ml RNase A

Enzymes and Buffers

- Lysozyme (10 mg/ml)

Media

- Rich medium

METHOD

1. Inoculate 2 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37°C with vigorous shaking.
2. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge the tube at maximum speed for 30 seconds at 4°C in a microfuge. Store the unused portion of the culture at 4°C.
3. Remove the medium by gentle aspiration, leaving the bacterial pellet as dry as possible.
4. Resuspend the bacterial pellet in 350 µl of STET.
5. Add 25 µl of a freshly prepared solution of lysozyme. Close the top of the tube and mix the contents by gently vortexing for 3 seconds.
6. Place the tube in a boiling water bath for *exactly* 40 seconds.
7. Centrifuge the bacterial lysate at maximum speed for 15 minutes at room temperature in a microfuge. Pour the supernatant into a fresh microfuge tube.
8. Precipitate the nucleic acids from the supernatant by adding 40 µl of 2.5 M sodium acetate (pH 5.2) and 420 µl of isopropanol. Mix the solution by vortexing, and then allow the mixture to stand for 5 minutes at room temperature.
9. Recover the precipitated nucleic acids by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge.
10. Remove the supernatant by gentle aspiration as described in Step 3 above. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a Kimwipe or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.
11. Rinse the pellet of nucleic acid with 1 ml of 70% ethanol at 4°C. Remove all of the supernatant by gentle aspiration as described in Step 3.
12. Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (2-5 minutes).
13. Dissolve the nucleic acids in 50 µl of TE (pH 8.0) containing DNase-free RNase A (pancreatic RNase). Vortex the solution gently for a brief period. Store the DNA at -20°C.

REFERENCES

1. Holmes D.S. and Quigley M. 1981. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* 114:193-197.

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Protocol 5

Preparation of Plasmid DNA by Large-scale Boiling Lysis

Plasmid DNA is isolated from large-scale (500 ml) bacterial cultures by treatment with Triton X-100 and lysozyme, followed by heating. This method is not recommended for preparing plasmid DNA from strains of *E. coli* that express endonuclease A (*endA*$^+$ strains).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Antibiotic for plasmid selection

⚠ Chloramphenicol (34 mg/ml)

Ethanol

Isopropanol

STE

STET

TE (pH 8.0)

Enzymes and Buffers

Lysozyme (10 mg/ml)

Restriction endonucleases

Media

Rich medium

Additional Reagents

Step 7 of this protocol requires the reagents listed in Chapter 1, Protocol 1 or Chapter 1, Protocol 4 .

Step 20 of this protocol requires reagents listed in Chapter 1, Protocol 8 , Chapter 1, Protocol 9 , Chapter 1, Protocol 10 , or Chapter 1, Protocol 11 .

METHOD

1. Inoculate 30 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic either with a single colony of transformed bacteria or with 0.1-1.0 ml of a small-scale liquid culture grown from a single colony.
2. Incubate the culture at the appropriate temperature with vigorous shaking (250 cycles/ minute in a rotary shaker) until the bacteria reach the late log phase of growth (i.e., an OD_{600} of approx. 0.6).
3. Inoculate 500 ml of LB, YT, or Terrific Broth (prewarmed to 37°C) containing the appropriate antibiotic in a 2-liter flask with 25 ml of the late-log-phase culture. Incubate the culture for 2.5 hours at 37°C with vigorous shaking (250 cycles/minute on a rotary shaker).
4. Add 2.5 ml of 34 mg/ml chloramphenicol. The final concentration of chloramphenicol in the culture should be 170 µg/ml. Incubate the culture for a further 12-16 hours at 37°C with vigorous shaking (250 cycles/minute on a rotary shaker).
5. Remove an aliquot (1-2 ml) of the bacterial culture to a fresh microfuge tube and store at 4°C. Harvest the remainder of the bacterial cells from the 500-ml culture by centrifugation at 2700*g* (4100 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C. Discard the supernatant. Stand the open centrifuge bottle in an inverted position to allow all of the supernatant to drain away.
6. Resuspend the bacterial pellet in 200 ml of ice-cold STE. Collect the bacterial cells by centrifugation as described in Step 5. Store the pellet of bacteria in the centrifuge bottle at -20°C.
7. Prepare plasmid DNA from the 1-2-ml aliquot of bacteria set aside in Step 5 by the minipreparation protocol (either Protocol 1 or 4). Analyze the minipreparation plasmid DNA by digestion with the appropriate restriction enzyme(s) to ensure that the correct plasmid has been propagated in the large-scale culture.
8. Allow the frozen bacterial cell pellet from Step 6 to thaw for 5-10 minutes at room temperature. Resuspend the pellet in 10 ml of ice-cold STET. Transfer the suspension to a 50-ml Erlenmeyer flask.
9. Add 1 ml of a freshly prepared solution of 10 mg/ml lysozyme.
10. Use a clamp to hold the Erlenmeyer flask over the open flame of a Bunsen burner until the liquid *just* starts to boil. Shake the flask constantly during the heating procedure.
11. Immediately immerse the bottom half of the flask in a large (2-liter) beaker of boiling water. Hold the flask in the boiling water for exactly 40 seconds.
12. Cool the flask in ice-cold water for 5 minutes.
13. Transfer the viscous contents of the flask to an ultracentrifuge tube (Beckman SW41 or its equivalent). Centrifuge the lysate at 150,000*g* (30,000 rpm in a Beckman SW41Ti rotor) for 30 minutes at 4°C.
14. Transfer as much of the supernatant as possible to a new tube. Discard the viscous liquid remaining in the centrifuge tube.
15. (*Optional*) If the supernatant contains visible strings of genomic chromatin or flocculent precipitate of proteins, filter it through 4-ply gauze before proceeding.
16. Measure the volume of the supernatant. Transfer the supernatant, together with 0.6 volume of isopropanol, to a fresh centrifuge tube(s). Store the tube(s) for 10 minutes at room temperature, after mixing the contents well.
17. Recover the precipitated nucleic acids by centrifugation at 12,000*g* (10,000 rpm in a Sorvall SS-34 rotor) for 15 minutes at *room temperature*.
    Salt may precipitate if centrifugation is carried out at 4°C.
18. Decant the supernatant carefully, and invert the open tube(s) on a paper towel to allow the last drops of supernatant to drain away. Rinse the pellet and the walls of the tube(s) with 70% ethanol at room temperature. Drain off the ethanol, and use a Pasteur pipette attached to a vacuum line to remove any beads of liquid that adhere to the walls of the tube(s). Place the inverted, open tube(s) on a pad of paper towels for a few minutes at room temperature. The pellet should still be damp.
19. Dissolve the pellet of nucleic acid in 3 ml of TE (pH 8.0).
20. Purify the crude plasmid DNA either by chromatography on commercial resins (Chapter 1, Protocol 9), precipitation with polyethylene glycol (Chapter 1, Protocol 8), or equilibrium centrifugation in CsCl-ethidium bromide gradients (Chapter 1, Protocol 10 and Chapter 1, Protocol 11).
21. Check the structure of the plasmid by restriction enzyme digestion followed by gel electrophoresis.

REFERENCES

1. Holmes D.S. and Quigley M. 1981. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.*

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Protocol 6

Preparation of Plasmid DNA: Toothpick Minipreparation

Plasmid DNA is prepared directly from bacterial colonies plucked from the surface of agar media with toothpicks.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Antibiotic for plasmid selection

- Bromophenol blue solution (0.4% w/v)
- Cresol red solution (10 mM)
- EDTA (0.5 M, pH 8.0)
- ⚠ Ethidium bromide (10 mg/ml)
- ⚠ SYBR Gold
- KCl (4 M)
- NSS solution

Media

- Rich broth
- Rich broth agar plates

Additional Reagents

Step 12 of this protocol requires the reagents listed in Chapter 8, Protocol 1 .

Step 14 of this protocol requires the reagents listed in Chapter 1, Protocol 1 or Chapter 1, Protocol 4 .

METHOD

1. Grow bacterial colonies, transformed with recombinant plasmid, on rich agar medium (LB, YT, or SOB) containing the appropriate antibiotic until they are approx. 2-3 mm in diameter (approx. 18-24 hours at 37°C for most bacterial strains).
2. Use a sterile toothpick or disposable loop to transfer a small segment of a bacterial colony to a streak or patch on a master agar plate containing the appropriate antibiotic. Transfer the remainder of the colony to a numbered microfuge tube containing 50 µl of sterile 10 mM EDTA (pH 8.0).
3. Repeat Step 2 until the desired number of colonies has been harvested.
4. Incubate the master plate for several hours at 37°C and then store it at 4°C until the results of the gel electrophoresis (Step 11 of this protocol) are available. Colonies containing plasmids of the desired size can then be recovered from the master plate.
5. While the master plate is incubating, process the bacterial suspensions as follows: To each microfuge tube in turn, add 50 µl of a freshly made solution of NSS. Close the top of the tubes and then mix their contents by vortexing for 30 seconds.
6. Transfer the tubes to a 70°C water bath. Incubate the tubes for 5 minutes and then allow them to cool to room temperature.
7. To each tube, add 1.5 µl of a solution of 4 M KCl. Vortex the tubes for 30 seconds.
8. Incubate the tubes for 5 minutes on ice.
9. Remove bacterial debris by centrifugation at maximum speed for 3 minutes at 4°C in a microfuge.
10. Transfer each of the supernatants in turn to fresh microfuge tubes. Add to each tube 0.5 µl of a solution containing 0.4% bromophenol blue if the samples are to be analyzed only by agarose gel electrophoresis *or* 2 µl of 10 mM cresol red if the samples are to be analyzed both by PCR and by agarose gel electrophoresis. Load 50 µl of the supernatant into a slot (5 mm in length x 2.5 mm in width) cast in a 0.7% agarose gel (5 mm thick).
11. After the bromophenol blue dye has migrated two-thirds to three-fourths the length of the gel, or the cresol red dye about one-half the length of the gel, stain the gel by soaking it for 30-45 minutes in a DNA-staining solution at room temperature. Examine and photograph the gel under UV illumination.
12. If cresol red has been used at Step 10, analyze the supernatants by performing PCR as described in Chapter 8, Protocol 1 , using the remainder of each sample as a template.
13. Prepare small-scale cultures of the putative recombinant clones by inoculating 2 ml of liquid medium (LB, YT, or SOB) containing the appropriate antibiotic with bacteria growing on the master plate.
14. Use the small-scale bacterial cultures to generate minipreparations (please see Chapter 1, Protocol 1 or Chapter 1, Protocol 4) of the putative recombinant plasmids. Analyze the plasmid DNAs by digestion with restriction enzymes and agarose gel electrophoresis to confirm that they have the desired size and structure.

REFERENCES

1. Barnes W.M. 1977. Plasmid detection and sizing in single colony lysates. *Science* 195:393-394.

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Protocol 7

Preparation of Plasmid DNA by Lysis with SDS

Large (>15 kb), closed circular plasmids are prepared (albeit inefficiently and in small yield) by lysing bacteria with SDS.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Antibiotic for plasmid selection

⚠ Chloramphenicol (34 mg/ml)

⚠ Chloroform

EDTA (0.5 M, pH 8.0)

Ethanol

NaCl (5 M)

⚠ Phenol:chloroform (1:1, v/v)

⚠ SDS (10% w/v)

STE, ice cold

TE (pH 8.0)

Tris-sucrose

Enzymes and Buffers

Lysozyme (10 mg/ml)

Restriction endonucleases

Media

Rich medium

Additional Reagents

Step 8 of this protocol requires the reagents listed in Chapter 1, Protocol 1 or Chapter 1, Protocol 4 .

Step 21 of this protocol requires the reagents listed in Chapter 1, Protocol 9 or Chapter 1, Protocol 10 .

METHOD

1. Inoculate 30 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic with a single transformed bacterial colony or with 0.1-1.0 ml of a late-log-phase culture grown from a single transformed colony.
2. Incubate the culture with vigorous shaking until the bacteria enters the late log phase of growth (i.e., an OD_{600} of approx. 0.6).
3. Inoculate 500 ml of LB, YT, or Terrific Broth (prewarmed to 37°C) containing the appropriate antibiotic in a 2-liter flask with 25 ml of the late-log-phase culture. Incubate the culture for approx. 2.5 hours at 37°C with vigorous shaking (250 cycles/minute on a rotary shaker).
4. For relaxed plasmids with low or moderate copy numbers, add 2.5 ml of 34 mg/ml chloramphenicol. The final concentration of chloramphenicol in the culture should be 170 µg/ml. For high-copy-number plasmids, do not add chloramphenicol.
5. Incubate the culture for a further 12-16 hours at 37°C with vigorous shaking (250 cycles/minute on a rotary shaker)
6. Remove an aliquot (1-2 ml) of the bacterial culture to a fresh microfuge tube and store it at 4°C. Harvest the remainder of the bacterial cells from the 500-ml culture by centrifugation at 2700*g* (4100 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C. Discard the supernatant. Stand the open centrifuge bottle in an inverted position.
7. Resuspend the bacterial pellet in 200 ml of ice-cold STE. Collect the bacterial cells by centrifugation as described in Step 6. Store the pellet of bacteria in the centrifuge bottle at -20°C.
8. Use one of the methods described in Chapter 1, Protocol 1 or Chapter 1, Protocol 4 to prepare plasmid DNA from the 1-2-ml aliquot of bacterial culture set aside in Step 6. Analyze the minipreparation plasmid DNA by digestion with the appropriate restriction enzyme(s) and agarose gel electrophoresis to ensure that the correct plasmid has been propagated in the large-scale culture.
9. Allow the frozen bacterial cell pellet from Step 7 to thaw at room temperature for 5-10 minutes. Resuspend the pellet in 10 ml of ice-cold Tris-sucrose solution. Transfer the suspension to a 30-ml plastic screw-cap tube.
10. Add 2 ml of a freshly prepared lysozyme solution (10 mg/ml) followed by 8 ml of 0.25 M EDTA (pH 8.0).
11. Mix the suspension by gently inverting the tube several times. Store the tube on ice for 10 minutes.
12. Add 4 ml of 10% SDS. Immediately mix the contents of the tube with a glass rod so as to disperse the solution of SDS evenly throughout the bacterial suspension. Be as gentle as possible to minimize shearing of the liberated chromosomal DNA.
13. As soon as mixing is completed, add 6 ml of 5 M NaCl (final concentration = 1 M). Use a glass rod to mix the contents of the tube gently but thoroughly. Place the tube on ice for at least 1 hour.
14. Remove high-molecular-weight DNA and bacterial debris by centrifugation at 71,000*g* (30,000 rpm in a Beckman Type 50 rotor) for 30 minutes at 4°C. Carefully transfer the supernatant to a 50-ml disposable plastic centrifuge tube. Discard the pellet.
15. Extract the supernatant once with phenol:chloroform and once with chloroform.
16. Transfer the aqueous phase to a 250-ml centrifuge bottle. Add 2 volumes (approx. 60 ml) of ethanol at room temperature. Mix the solution well. Store the solution for 1-2 hours at room temperature.
17. Recover the nucleic acids by centrifugation at 5000*g* (5500 rpm in a Sorvall GSA rotor or 5100 rpm in a Sorvall HS4 swing-out rotor) for 20 minutes at 4°C.
18. Discard the supernatant. Wash the pellet and sides of the centrifuge tube with 70% ethanol at room temperature and then centrifuge as in Step 17.
19. Discard as much of the ethanol as possible, and then invert the centrifuge bottle on a pad of paper towels to allow the last of the ethanol to drain away. Use a vacuum aspirator to remove droplets of ethanol from the walls of the centrifuge bottle. Stand the bottle in an inverted position until no trace of ethanol is visible. At this stage, the pellet should still be damp.
20. Dissolve the damp pellet of nucleic acid in 3 ml of TE (pH 8.0).
21. Purify the crude plasmid DNA either by chromatography on commercial resins (please see Chapter 1, Protocol 9) or isopycnic centrifugation in CsCl-ethidium bromide gradients (please see Chapter 1, Protocol 10 and Chapter 1, Protocol 11).
22. Check the structure of the plasmid by restriction enzyme digestion followed by gel electrophoresis.

REFERENCES

1. Godson G.N. and Vapnek D. 1973. A simple method for the large-scale purification of øX174 RFI supercoiled DNA. *Biochim. Biophys. Acta* 299:516-520.

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Protocol 8

Purification of Plasmid DNA by Precipitation with Polyethylene Glycol

Crude preparations of plasmid DNA are first treated with lithium chloride and RNase (to remove RNA). The plasmid DNA is then precipitated in a solution containing polyethylene glycol and $MgCl_2$.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform
Ethanol
Isopropanol
LiCl (5 M)
PEG-$MgCl_2$ solution
⚠ Phenol:chloroform (1:1, v/v)
Sodium acetate (3 M, pH 5.2)
TE (pH 8.0)
TE (pH 8.0) containing 20 µg/ml RNase A

Nucleic Acids

Crude plasmid preparation
Use material from either Step 17 of Chapter 1, Protocol 3 or Step 19 of Chapter 1, Protocol 5 .

METHOD

1. Transfer 3 ml of the crude large-scale plasmid preparation to a 15-ml Corex tube and chill the solution to 0°C in an ice bath.
2. Add 3 ml of an ice-cold solution of 5 M LiCl to the crude plasmid preparation, mix well, and centrifuge the solution at 12,000*g* (10,000 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C.
3. Transfer the supernatant to a fresh 30-ml Corex tube. Add an equal volume of isopropanol. Mix well. Recover the precipitated nucleic acids by centrifugation at 12,000*g* (10,000 rpm in a Sorvall SS-34 rotor) for 10 minutes at room temperature.
4. Decant the supernatant carefully, and invert the open tube to allow the last drops of supernatant to drain away. Rinse the pellet and the walls of the tube with 70% ethanol at room temperature. Carefully discard the bulk of the ethanol, and then use a vacuum aspirator to remove any beads of liquid that adhere to the walls of the tube. Place the inverted, open tube on a pad of paper towels for a few minutes. The pellet should still be damp.
5. Dissolve the damp pellet of nucleic acid in 500 µl of TE (pH 8.0) containing RNase A. Transfer the solution to a microfuge tube and store it for 30 minutes at room temperature.
6. Extract the plasmid-RNase mixture once with phenol:chloroform and once with chloroform.
7. Recover the DNA by standard ethanol precipitation.
8. Dissolve the pellet of plasmid DNA in 1 ml of sterile H_2O, and then add 0.5 ml of PEG-$MgCl_2$ solution.
9. Store the solution for ≥10 minutes at room temperature, and then collect the precipitated plasmid DNA by centrifugation at maximum speed for 20 minutes at room temperature in a microfuge.
10. Remove traces of PEG by resuspending the pellet of nucleic acid in 0.5 ml of 70% ethanol. Collect the nucleic acid by centrifugation at maximum speed for 5 minutes in a microfuge.
11. Remove the ethanol by aspiration and repeat Step 10. Following the second rinse, store the open tube on the bench for 10-20 minutes to allow the ethanol to evaporate.
12. Dissolve the damp pellet in 500 µl of TE (pH 8.0). Measure the OD_{260} of a 1:100 dilution in TE (pH 8.0) of the solution, and calculate the concentration of the plasmid DNA assuming that 1 OD_{260} = 50 µg of plasmid DNA/ml.
13. Store the DNA in aliquots at -20°C.

REFERENCES

1. Lis J.T. 1980. Fractionation of DNA-fragments by polyethylene glycol induced precipitation. *Methods Enzymol.* 65:347-353.
2. Nicoletti V.G. and Condorelli D.F. 1993. Optimized PEG-method for rapid plasmid DNA purification: High yield from "midiprep." *BioTechniques* 14:532-534, 536.

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Protocol 9

Purification of Plasmid DNA by Chromatography

The following table summarizes the salient features of many of the commercial resins that are currently available for plasmid purification. Individual manufacturers supply detailed instructions, which should be followed to the letter.

METHOD

1. **Commercially Available Resins and Their Uses**

Resin	Manufacturer	Chemistry	Use	Notes
Qiagen	Qiagen	macroporous silica	transfection of eukaryotic	some batch-to-batch variation;
	www.qiagen.com	gel, anion-exchange (DEAE)	cells	pH-sensitive
QIAprep	Qiagen	silica gel	enzymic manipulation	different columns available for
	www.qiagen.com			purification of double- or single-
				stranded DNAs
Wizard	Promega	silica particle	additional ethanol	inexpensive, reproducible
	www.promega.com		precipitation required	
			for transfection of	
			eukaryotic cells	
FlexiPrep	Pharmacia	anion exchange	enzymic manipulation	transfection requires further
	www.apbiotech.com			purification
Glass-Max	Life Technologies	silica matrix	enzymic manipulation	minipreps only
	www.lifetech.com			
GeniePrep	Ambion	hydrophobic inter-	enzymic manipulation	miniprep only; made in Texas
	www.ambion.com	actions, glass fiber		
Perfect Prep	Eppendorf 5 Prime	silica matrix	transfection of eukaryotic	very fast; miniprep only
	www.5prime.com		cells	
ClearCut	Stratagene	silica resin, hydro-	enzymic manipulation	can be used for miniprep plas-
Miniprep Kit	www.stratagene.com	phobic interaction		mid or DNA fragment purification
Concert, rapid	Life Technologies	silica gel	enzymic manipulation	mini- and maxipreps
and high purity	www.lifetech.com		and transfection of	
systems			eukaryotic cells	
NucleoBond AX	Nest Group, Inc.	macroporous silica	transfection of eukaryotic	five column sizes; resin good for
	world.std/approx. nestgrp/	gel anion exchange	cells	large DNA purification, including
				cosmids and P1 DNAs

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Protocol 10

Purification of Closed Circular DNA by Equilibrium Centrifugation in CsCl-Ethidium Bromide Gradients: Continuous Gradients

Solutions containing plasmid DNA are adjusted to a density of 1.55 g/ml with solid CsCl. The intercalating dye, ethidium bromide, which binds differentially to closed circular and linear DNAs, is then added to a concentration of 200 µg/ml. During centrifugation to equilibrium, the closed circular DNA and linear DNAs form bands at different densities.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

CsCl (solid)

Ethanol

⚠ Ethidium bromide (10 mg/ml)

Paraffin oil

Nucleic Acids

Crude plasmid preparation

Additional Reagents

Step 8 of this protocol requires the reagents listed in Chapter 1, Protocol 12 or Chapter 1, Protocol 13 .

METHOD

1. Measure the mass of the crude plasmid DNA preparation. Measurement is best done by transferring the solution into a fresh tube that has been tared on a top-loading balance. For every gram of plasmid DNA solution, add exactly 1.01 g of solid CsCl. Close the top of the tube to prevent evaporation and then warm the solution to 30°C to facilitate the dissolution of the CsCl salt. Mix the solution gently until the salt is dissolved.
2. Add 100 µl of 10 mg/ml ethidium bromide for each 5 g of original DNA solution.
3. If Corex glass tubes are used, centrifuge the solution at 7700*g* (8000 rpm in a Sorvall SS-34 rotor) for 5 minutes at room temperature. If disposable polypropylene tubes are used, centrifuge at 1100*g* (3000 rpm in a Sorvall SS-34 rotor) for 10 minutes.
4. Use a Pasteur pipette or a disposable syringe fitted with a large-gauge needle to transfer the clear, red solution under the scum and above the pellet to a tube suitable for centrifugation in an ultracentrifuge rotor. Top off the partially filled centrifuge tubes with light paraffin oil or rebanding solution. Make sure that the weights of tubes opposite each other in the rotor are equal. Seal the tubes according to the manufacturer's instructions.
5. Centrifuge the density gradients at 20°C as appropriate for the rotor:

Beckman NVT 65 rotor	366,000*g* (62,000 rpm)	for 6 hours
Beckman VTi65 rotor	194,000*g* (45,000 rpm)	for 16 hours
Beckman Type 50Ti rotor	180,000*g* (45,000 rpm)	for 48 hours
Beckman Type 65Ti rotor	314,000*g* (60,000 rpm)	for 24 hours
Beckman Type 70.1Ti rotor	331,000*g* (60,000 rpm)	for 24 hours

6. Gently remove the rotor from the centrifuge and place it on a flat surface. Carefully remove each tube and place it in a test tube rack covered with tin foil. In a dimly lit room (i.e., with the overhead fluorescent lights turned off), mount one tube in a clamp attached to a ring stand.
7. Collect the band of closed circular DNA.
   a. Use a 21-gauge hypodermic needle to make a small hole in the top of the tube to allow air to enter when fluid is withdrawn.
   b. Carefully wipe the outside of the tube with ethanol to remove any grease or oil, and then attach a piece of Scotch Tape or Time tape to the outside of the tube.
   c. Attach a 5-10-cc disposable syringe to a sterile 18-gauge hypodermic needle and insert the needle (beveled side up) into the tube through the tape so that the open, beveled side of the needle is positioned just below the lower DNA band (closed circular plasmid DNA).
   d. Slowly withdraw the plasmid DNA, taking care not to disturb the upper viscous band of chromosomal DNA.
8. Remove ethidium bromide from the DNA as described in one of the methods presented in Chapter 1, Protocol 12 or Chapter 1, Protocol 13 .

REFERENCES

1. Bauer W. and Vinograd J. 1968. The interaction of closed circular DNA with intercalative dyes. I. The superhelix density of SV40 DNA in the presence and absence of dye. *J. Mol. Biol.* 33:141-171.
2. Bauer W. and Vinograd J. 1971. The use of intercalative dyes in the study of closed circular DNA. *Progr. Mol. Subcell. Biol.* 2:181-215.
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4. Vinograd J. and Leibowitz J. 1966. Physical and topological properties of circular DNA. *J. Gen. Physiol.* 49:103-125.
5. Waring M.J. 1966. Structural requirements for the binding between ethidium bromide and nucleic acids. *Biochim. Biophys. Acta* 114:234-244.

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Protocol 11

Purification of Closed Circular DNA by Equilibrium Centrifugation in CsCl-Ethidium Bromide Gradients: Discontinuous Gradients

A solution containing plasmid DNA, saturating amounts of ethidium bromide, and CsCl (44% w/v) is layered between two solutions of lesser (35% w/v CsCl) and greater density (59% w/v CsCl). During centrifugation to equilibrium, the closed circular plasmid DNA and linear DNAs form bands at different densities.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

CsCl (solid)

⚠ Ethidium bromide (10 mg/ml)

TE (pH 8.0)

Nucleic Acids

Crude plasmid preparation
Use material from either Step 16 of Chapter 1, Protocol 3 , Step 18 of Chapter 1, Protocol 5 , or Step 19 of Chapter 1, Protocol 7.

Additional Reagents

Step 6 of this protocol requires the reagents listed in Chapter 1, Protocol 12 or Chapter 1, Protocol 13 .

METHOD

1. Prepare CsCl layers for a three-step discontinuous gradient as described in the table. Use a 3-cc hypodermic syringe equipped with an 18-gauge bone marrow (10 cm) needle to transfer 1.5 ml of the top layer (35%) CsCl solution to a 5-ml polyallomer ultracentrifuge tube (Beckman Quick-Seal or equivalent).

Three-step Discontinuous Gradient Layers

Layer	Molarity CsCl (w/w)	Refractive Index	Preparation
Top layer	2.806 (35%)	1.3670	Dissolve 4.720 g of CsCl in 8 ml of TE (pH 8.0). Adjust the volume to exactly 10 ml. Then add 120 µl of 10 mg/ml ethidium bromide.
Middle layer	3.870 (44%)	1.3792	Dissolve 0.8 g of CsCl in exactly 1 ml of the crude DNA preparation. Then add 30 µl of 10 mg/ml ethidium bromide.
Bottom layer	6.180 (59%)	1.4052	Dissolve 10.4 g of CsCl in 7 ml of TE. Adjust the volume to exactly 10 ml. Then add 120 µl of 10 mg/ml ethidium bromide.

2. Use a 1-cc tuberculin syringe equipped with an 18-gauge bone marrow (10 cm) needle to layer 0.5 ml of the middle layer (44%) CsCl solution, containing the plasmid DNA, into the bottom of the tube *under* the top layer solution.
As a rule of thumb, the crude plasmid DNA prepared from no more than 50 ml of an overnight culture should be used per gradient. The crude plasmid preparation from a 100-ml culture should be reconstituted in approx. 0.9 ml of TE (pH 8.0), which is enough to form the middle layer of two discontinuous gradients.
3. Use a 5-cc hypodermic syringe equipped with an 18-gauge bone marrow (10 cm) needle to fill the tube by layering the bottom layer (59%) CsCl solution *under* the middle layer CsCl solution.
4. Centrifuge the sealed tubes at 330,000*g* (60,000 rpm in a Beckman Type 70.1Ti rotor) for 5 hours. Make sure that the weights of tubes opposite each other in the rotor are equal. Seal the tubes according to the manufacturer's instructions.
5. Collect the band of closed circular DNA:
   a. Use a 21-gauge hypodermic needle to make a small hole in the top of the tube to allow air to enter when fluid is withdrawn.
   b. Carefully wipe the outside of the tube with ethanol to remove any grease or oil, and then attach a piece of Scotch Tape or Time tape to the outside of the tube.
   c. Attach a 5-10-cc disposable syringe to a sterile 18-gauge hypodermic needle and insert the needle (beveled side up) into the tube through the tape so that the open, beveled side of the needle is positioned just below the lower DNA band (closed circular plasmid DNA).
   d. Slowly withdraw the plasmid DNA, taking care not to disturb the upper viscous band of chromosomal DNA.
6. Remove ethidium bromide from the DNA as described in one of the methods presented in Chapter 1, Protocol 12 or Chapter 1, Protocol 13 .

REFERENCES

1. Dorin M. and Bornecque C.A. 1995. Fast separations of plasmid DNA using discontinuous gradients in the preparative ultracentrifuge. *BioTechniques* 18:90-91.

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Protocol 12

Removal of Ethidium Bromide from DNA by Extraction with Organic Solvents

Ethidium bromide is removed from DNA by phase extraction with organic solvents.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol
Isoamyl alcohol, saturated with H_2O
⚠ *n*-Butanol, saturated with H_2O
⚠ Phenol
⚠ Phenol:chloroform (1:1 v/v)
TE (pH 8.0)

Nucleic Acids

DNA sample, purified through CsCl gradient
Use material from either Step 7 of Chapter 1, Protocol 10 or Step 5 of Chapter 1, Protocol 11.

METHOD

1. To the solution of DNA in a glass or polypropylene tube, add an equal volume of either water-saturated *n*-butanol or isoamyl alcohol. Close the cap of the tube tightly.
2. Mix the organic and aqueous phases by vortexing.
3. Centrifuge the mixture at 450*g* (1500 rpm in a Sorvall RT-6000 centrifuge with an HL-4 rotor and 50-ml buckets) for 3 minutes at room temperature or stand the solution at room temperature until the organic and aqueous phases have separated.
4. Use a Pasteur pipette to transfer the upper (organic) phase, which is now a beautiful deep pink color, to an appropriate waste container.
5. Repeat the extraction (Steps 1-4) four to six times until all the pink color disappears from both the aqueous phase and organic phases.
6. Remove the CsCl from the DNA solution by ethanol precipitation (please follow Steps 7 through 12), by spin dialysis through a microconcentrator (Amicon), or by dialysis overnight (16 hours) against 2 liters of TE (pH 8.0) (change buffer frequently). If one of the latter two methods is used, then proceed to Step 13.
7. To precipitate the DNA from the CsCl-DNA solution, measure the volume of the CsCl solution, add three volumes of H_2O, and mix the solution well.
8. Add 8 volumes of ethanol (1 volume is equal to that of the CsCl-DNA solution prior to dilution with H_2O in Step 7) to the DNA solution and mix well. Store the mixture for at least 15 minutes at 4°C.
9. Collect the precipitate of DNA by centrifugation at 20,000*g* (13,000 rpm in a Sorvall SS-34 rotor) for 15 minutes at 4°C.
10. Decant the supernatant to a fresh centrifuge tube. Add an equal volume of absolute ethanol to the supernatant. Store the mixture for at least 15 minutes at 4°C and then collect the precipitate of DNA by centrifugation at 20,000*g* (13,000 rpm in a Sorvall SS-34 rotor) for 15 minutes.
11. Wash the two DNA precipitates with 70% ethanol. Remove as much of the 70% ethanol as possible and then allow any remaining fluid to evaporate at room temperature.
12. Dissolve the precipitated DNA in 2 ml of H_2O or TE (pH 8.0).
13. If the resuspended DNA contains significant quantities of ethidium bromide, as judged from its color or its emission of fluorescence when illuminated by UV light, extract the solution once with phenol and once with phenol:chloroform, and then again precipitate the DNA with ethanol.
14. Measure the OD_{260} of the final solution of DNA, and calculate the concentration of DNA. Store the DNA in aliquots at -20°C.

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2. Wang J.C. 1969. Variation of the average rotation angle of the DNA helix and the superhelical turns of covalently closed cyclic λ DNA. *J. Mol. Biol.* 43:25-39.

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Protocol 13

Removal of Ethidium Bromide from DNA by Ion-exchange Chromatography

Ethidium bromide is removed from DNA by chromatography through a cation-exchange resin.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol
⚠ HCl (1 N)
NaCl (5 M)
⚠ Phenol
⚠ Phenol:chloroform (1:1, v/v)
TE (pH 8.0)
TEN buffer
TEN buffer containing 0.2% sodium azide

Nucleic Acids

DNA sample, purified through CsCl gradient
Use material from either Step 7 of Chapter 1, Protocol 10 or Step 5 of Chapter 1, Protocol 11.

METHOD

1. Before using, equilibrate the Dowex AG50 resin:
   a. Stir approx. 20 g of Dowex AG50 in approx. 100 ml of 1 M NaCl for 5 minutes. Allow the resin to settle, and remove the supernatant by aspiration.
   b. Add approx. 100 ml of 1 N HCl, and stir the slurry for a further 5 minutes. Again allow the resin to settle, and remove the supernatant by aspiration.
   c. Continue the process with two washes with H_2O (100 ml each), followed by one wash with 100 ml of TEN buffer.
   d. Store the equilibrated resin at 4°C in TEN buffer containing 0.2% sodium azide.
2. Construct a 1-ml column of Dowex AG50 in a Pasteur pipette.
3. Remove the buffer above the resin, and rinse the column with 2 column volumes of TE (pH 8.0). Apply the solution of DNA containing ethidium bromide and CsCl directly to the resin.
4. Immediately begin collecting the effluent from the column. After all of the DNA solution has entered the column, wash the resin with 1.2 column volumes of TE (pH 8.0) and continue to collect the eluate into a 30-ml Corex tube.
5. After the column has run dry, dilute the eluate with 2.5 column volumes of H_2O.
6. Precipitate the DNA by adding eight volumes of ethanol followed by incubation for 15 minutes at 4°C. Collect the DNA by centrifugation at 17,000*g* (12,000 rpm in a Sorvall SS-34 rotor) for 15 minutes at 4°C.
7. Decant the supernatant to a fresh centrifuge tube. Add an equal volume of absolute ethanol to the supernatant. Store the mixture for at least 15 minutes at 4°C and then collect the precipitate of DNA by centrifugation at 20,000*g* (13,000 rpm in a Sorvall SS-34 rotor) for 15 minutes.
8. Wash the two DNA precipitates with 70% ethanol. Remove as much as possible of the 70% ethanol and then allow any remaining fluid to evaporate at room temperature.
9. Dissolve the precipitated DNA in 2 ml of H_2O or TE (pH 8.0).
10. If the resuspended DNA contains significant quantities of ethidium bromide, as judged from its color or its emission of fluorescence when illuminated by UV light, extract the solution once with phenol and once with phenol:chloroform, and then again precipitate the DNA with ethanol.
11. Measure the OD_{260} of the final solution of DNA, and calculate the concentration of DNA. Store the DNA in aliquots at -20°C.

REFERENCES

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2. Waring M.J. 1965. Complex formation between ethidium bromide and nucleic acids. *J. Mol. Biol.* 13:269-282.

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Protocol 14

Removal of Small Fragments of Nucleic Acid from Preparations of Plasmid DNA by Centrifugation through NaCl

Contamination of plasmid DNA by fragments of DNA and RNA is reduced to an acceptable level by centrifugation through 1 M sodium chloride. This method was devised by Brian Seed when he was a graduate student at Harvard University.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol

- NaCl (1 M) in TE (pH 8.0)
- Sodium acetate (3 M, pH 5.2)
- TE (pH 8.0)

Enzymes and Buffers

DNase-free Pancreatic RNase

Nucleic Acids

DNA sample, purified through CsCl gradient
Use material from either Step 14 of Chapter 1, Protocol 12 or Step 11 of Chapter 1, Protocol 13.

METHOD

1. Measure the volume of the plasmid preparation. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. Store the mixture for 30 minutes at 4°C.
2. Recover the precipitate of nucleic acids by centrifugation at >10,000*g* (>9100 rpm in a Sorvall SS-34 rotor) for 15 minutes at 4°C. Decant as much of the supernatant as possible, and then store the open tube on the bench for a few minutes to allow the ethanol to evaporate.
3. Dissolve the damp pellet in 0.5-1.0 ml of TE (pH 8.0).
4. Add DNase-free RNase to a final concentration fo 10 µg/ml. Incubate the mixture for 1 hour at room temperature.
5. Add 4 ml of 1 M NaCl in TE (pH 8.0) to a Beckman SW50.1 centrifuge tube (or its equivalent). Use an automatic pipette with a disposable tip to layer up to 1 ml of the plasmid preparation on top of the 1 M NaCl solution. If necessary, fill the tube with TE (pH 8.0).
6. Centrifuge the solution at 150,000*g* (40,000 rpm in a Beckman SW50.1 rotor) for 6 hours at 20°C. Carefully discard the supernatant.
7. Dissolve the pellet of plasmid DNA in 0.5 ml of TE (pH 8.0). Add 50 µl of 3 M sodium acetate (pH 5.2), and transfer the DNA solution to a microfuge tube.
8. Precipitate the DNA by addition of 2 volumes of ethanol, and store the ethanolic solution for 10 minutes at 4°C. Recover the DNA by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge. Decant as much of the supernatant as possible and then store the open tube on the bench for a few minutes to allow the ethanol to evaporate.
9. Dissolve the damp pellet of DNA in TE (pH 8.0).

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Protocol 15

Removal of Small Fragments of Nucleic Acid from Preparations of Plasmid DNA by Chromatography through Sephacryl S-1000

Contamination of plasmid DNA by small fragments of nucleic acid is reduced dramatically by size-exclusion chromatography through Sephacryl S-1000.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Bromophenol blue sucrose solution
- Ethanol
- ⚠ Phenol
- Sephacryl equilibration buffer
- Sodium acetate (3 M, pH 5.2)
- TE (pH 8.0) containing 20 µg/ml RNase A

Nucleic Acids

DNA sample, purified through CsCl gradient
Use material from either Step 14 of Chapter 1, Protocol 12 or Step 11 of Chapter 1, Protocol 13 .

METHOD

1. Prepare a 1 x 10-cm column of Sephacryl S-1000, equilibrated in Sephacryl equilibration buffer.
2. Measure the volume of the plasmid preparation. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. Store the mixture for 30 minutes at 4°C.
3. Recover the precipitate of nucleic acids by centrifugation at >10,000*g* (>9100 rpm in a Sorvall SS-34 rotor) for 15 minutes at 4°C. Drain off as much of the supernatant as possible, and then store the open tube on the bench for a few minutes to allow the ethanol to evaporate.
4. Dissolve the damp pellet of nucleic acids in a small volume (<400 µl) of TE (pH 8.0) containing RNase A at a final DNA concentration of at least 100 µg/ml.
5. Incubate the mixture for 1 hour at room temperature.
6. Extract the solution once with an equal volume of phenol equilibrated in TE (pH 8.0).
7. Recover the aqueous layer, and add 100 µl of bromophenol blue dye sucrose solution. Layer the blue aqueous phase on the column of Sephacryl S-1000.
8. Wash the DNA into the column, and apply a reservoir of Sephacryl equilibration buffer. Immediately begin collecting 0.5-ml fractions.
9. When 15 fractions have been collected, clamp off the bottom of the column. At this stage, the blue dye should have traveled about half the length of the column.
10. Analyze 10 µl of each fraction by electrophoresis through a 0.7% agarose gel or by ethidium bromide fluorescence to identify the fractions containing plasmid DNA.
11. Pool the fractions containing plasmid DNA, and recover the DNA by precipitation with 2 volumes of ethanol for 10 minutes at 4°C and centrifugation at 10,000*g* (9200 rpm in a Sorvall SS-34 rotor) for 15 minutes at 4°C.
12. Decant as much of the supernatant as possible, and then store the open tube on the bench for a few minutes to allow the ethanol to evaporate.
13. Dissolve the damp pellet in TE (pH 8.0).

REFERENCES

1. Gomez-Marquez J., Freire M., and Segade F. 1987. A simple procedure for the large-scale purification of plasmid DNA. *Gene* 54:255-259.

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Protocol 16

Removal of Small Fragments of Nucleic Acid from Preparations of Plasmid DNA by Precipitation with Lithium Chloride

High-molecular-weight RNA and proteins can be precipitated from preparations of plasmid DNA by high concentrations of LiCl and removed by low-speed centrifugation.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol

Isopropanol

- LiCl (4 M)
- Sodium acetate (3 M, pH 5.2)
- TE (pH 8.0)
- TE (pH 8.0) containing 20 µg/ml RNase A

Nucleic Acids

DNA sample, purified through CsCl gradient
Use material from either Step 14 of Chapter 1, Protocol 12 or Step 11 of Chapter 1, Protocol 13.

METHOD

1. Measure the volume of the plasmid preparation. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. Store the mixture for 30 minutes at 4°C.
2. Recover the precipitate of nucleic acids by centrifugation at >10,000*g* (>9100 rpm in a Sorvall SS-34 rotor) for 15 minutes at 4°C. Drain off as much of the supernatant as possible, and then store the open tube on the bench for a few minutes to allow the ethanol to evaporate.
3. Dissolve the damp pellet in 1 ml of TE (pH 8.0) containing RNase A at a concentration of ≥100 µg/ml.
4. Add 3 ml of 4 M LiCl solution. Incubate the mixture on ice for 30 minutes.
5. Separate the plasmid DNA from the precipitated nucleic acids by centrifugation at 12,000*g* (10,000 rpm in a Sorvall SS-34 rotor) for 15 minutes at 4°C.
6. Transfer the supernatant to a fresh centrifuge tube and add 6 ml of isopropanol. Allow the plasmid DNA to precipitate for 30 minutes at room temperature.
7. Recover the precipitated plasmid DNA by centrifugation at 12,000*g* (10,000 rpm in a Sorvall SS-34 rotor) for 15 minutes at 4°C.
8. Carefully remove the supernatant and add 5-10 ml of 70% ethanol to the tube. Vortex the tube briefly, and then recentrifuge at 12,000*g* for 10 minutes at 4°C.
9. Carefully remove the supernatant, and store the open tube on the bench top for a few minutes until the ethanol has evaporated.
10. Dissolve the damp pellet of DNA in TE (pH 8.0).

REFERENCES

1. Barlow J.J., Mathias A.P., Williamson R., and Gammack D.B. 1963. A simple method for quantitative isolation of undegraded high molecular weight ribonucleic acid. *Biochem. Biophys. Res. Commun.* 13:61-66.
2. Kondo T., Mukai M., and Kondo Y. 1991. Rapid isolation of plasmid DNA and LiCl-ethidium bromide treatment and gel filtration. *Anal. Biochem.* 198:30-35.

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Protocol 17

Directional Cloning into Plasmid Vectors

Directional cloning requires that the plasmid vector be cleaved with two restriction enzymes that generate incompatible termini and that the fragment of DNA to be cloned carries termini that are compatible with those of the doubly cleaved vector.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)
Omit ATP from the ligation reaction in Step 5 if the ligation buffer contains ATP.

Ethanol

⚠ Phenol:chloroform (1:1, v/v)

Sodium acetate (3 M, pH 5.2)

TE (pH 8.0)

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Restriction endonucleases

Nucleic Acids

Vector DNA (plasmid)

Target DNA fragment
Adaptors may be added to the target DNA as described in Chapter 1, Protocol 18 .

Additional Reagents

Step 7 of this protocol requires the reagents listed in Chapter 1, Protocol 23 , Chapter 1, Protocol 24 , Chapter 1, Protocol 25 , or Chapter 1, Protocol 26 .

METHOD

1. Digest the vector (10 µg) and foreign DNA with the two appropriate restriction enzymes.
2. Purify the digested foreign DNA by extraction with phenol:chloroform and standard ethanol precipitation.
3. Purify the vector DNA by spun-column chromatography followed by standard ethanol precipitation.
4. Reconstitute the precipitated DNAs separately in TE (pH 8.0) at a concentration of approx. 100 µg/ml. Calculate the concentration of the DNA (in pmole/ml), assuming that 1 bp has a mass of 660 daltons.
5. Transfer appropriate amounts of the DNAs to sterile 0.5-ml microfuge tubes as follows:

Tube	DNA
A and D	vector (30 fmoles [approx. 100 ng])
B	insert (foreign) (30 fmoles [approx. 10 ng])
C and E	vector (30 fmoles) plus insert (foreign) (30 fmoles)
F	superhelical vector (3 fmoles [approx. 10 ng])

The molar ratio of plasmid vector to insert DNA fragment should be approx. 1:1 in the ligation reaction. The final DNA concentration should be approx. 10 ng/µl.

a. To Tubes A, B, and C add:

10x Ligation buffer	1.0 µl
Bacteriophage T4 DNA ligase	0.1 Weiss unit
10 mM ATP	1.0 µl
H_2O	to 10 µl

b. To Tubes D and E, add:

10x Ligation buffer	1.0 µl
10 mM ATP	1.0 µl
H_2O	to 10 µl
no DNA ligase	

The DNA fragments can be added to the tubes together with the H_2O and then warmed to 45°C for 5 minutes to melt any cohesive termini that have reannealed during fragment preparation. Chill the DNA solution to 0°C before the remainder of the ligation reagents are added.

6. Incubate the reaction mixtures overnight at 16°C or for 4 hours at 20°C.
7. Transform competent *E. coli* with dilutions of each of the ligation reactions as described in Chapter 1, Protocol 23 , Chapter 1, Protocol 24 , Chapter 1, Protocol 25 , or Chapter 1, Protocol 26 . As controls, include known amounts of a standard preparation of superhelical plasmid DNA to check the efficiency of transformation.

Tube	DNA	Ligase	Expected number of transformed colonies
A	vector	+	approx. 0 (approx. 10^4 fewer than Tube F)
B	insert	+	0
C	vector and insert	+	approx. 10-fold more then Tube A or D
D	vector	-	approx. 0 (approx. 10^4 fewer than Tube F)
E	vector and insert	-	some, but fewer than Tube C
F	superhelical vector	-	>2 x 10^5

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Protocol 18

Attaching Adaptors to Protruding Termini

Adaptors are short double-stranded synthetic oligonucleotides that carry an internal restriction endonuclease recognition site and single-stranded tails at one or both ends. Adaptors are used to exchange restriction sites at the termini of linear DNA molecules. They may be purchased in phosphorylated and unphosphorylated forms.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)
Omit ATP from the ligation reaction in Step 2 if the ligation buffer contains ATP.

Ethanol

⚠ Phenol:chloroform (1:1, v/v)

Sodium acetate (3 M, pH 5.2)

TE (pH 8.0)

Enzymes and Buffers

Bacteriophage T4 DNA ligase

10x Linker kinase buffer

Polynucleotide kinase

Restriction endonucleases

Nucleic Acids and Oligonucleotides

Target DNA fragment

Synthetic oligonucleotide/adaptor dissolved in TE-(pH 8.0) at a concentration of approx. 400 µg/ml.
For a hexamer, this concentration is equivalent to a 50 µM solution

METHOD

1. To phosphorylate the adaptors, add to a sterile microfuge tube:

synthetic oligonucleotide or adaptor	0.5-2.0 µg, dissolved in TE (pH 8.0)
10x linker kinase buffer	1.0 µl
10 mM ATP	1.0 µl
H_2O	to 10 µl
Bacteriophage T4 polynucleotide kinase	1.0 unit

   Incubate the reaction for 1 hour at 37°C.
2. To ligate the phophorylated adaptors to a DNA fragment with complementary protruding ends, set up a ligation reaction as follows:

DNA fragment	100-200 ng
phosphorylated adapter	10-20-fold molar excess
10x ligation buffer	1.0 µl
bacteriophage T4 DNA ligase	0.1 Weiss unit
10 mM ATP	1.0 µl
H_2O	to 10 µl

   Incubate the ligation mixture for 6-16 hours at 4°C.
   To achieve the maximum efficiency of ligation, set up the reactions in as small a volume as possible (5-10 µl).
3. Inactivate the DNA ligase by incubating the reaction mixture for 15 minutes at 65°C.
4. Dilute the ligation reaction with 10 µl of the appropriate 10x restriction enzyme buffer. Add sterile H_2O to a final volume of 100 µl followed by 50-100 units of restriction enzyme.
5. Incubate the reaction for 1-3 hours at 37°C.
6. Extract the restriction digestion with phenol:chloroform and recover the DNA by standard ethanol precipitation.
7. Collect the precipitated DNA by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge, and resuspend the DNA in 50 µl of TE (pH 8.0).
8. Pass the resuspended DNA through a spun column to remove excess adaptors and their cleavage products.
9. The modified DNA fragment can now be ligated to a plasmid vector with protruding ends that are complementary to those of the cleaved adaptor (please see Chapter 1, Protocol 17).

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Protocol 19

Blunt-ended Cloning into Plasmid Vectors

Target DNA is ligated to a blunt-ended plasmid DNA, and the products of the ligation reaction are used to transform competent *E. coli.* The maximum number of "correct" clones can generally be obtained from ligation reactions containing equimolar amounts of plasmid and target DNAs, with the total DNA concentration being <100 µg/ml. Blunt-end ligation catalyzed by bacteriophage T4 DNA ligase is suppressed by high concentrations (5 mM) of ATP and polyamines such as spermidine.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)
Omit ATP from the ligation reaction in Step 5 if the ligation buffer contains ATP.

Ethanol

⚠ Phenol:chloroform (1:1, v/v)

⚠ PEG 8000 (30% w/v)

Sodium acetate (3 M, pH 5.2)

TE (pH 8.0)

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Restriction endonucleases

Nucleic Acids

Target DNA (blunt-end fragment)

Vector DNA (plasmid)

Additional Reagents

Step 4 of this protocol requires the reagents listed in Chapter 1, Protocol 20.

Step 7 of this protocol requires the reagents listed in Chapter 1, Protocol 23 , Chapter 1, Protocol 24 , Chapter 1, Protocol 25 , or Chapter 1, Protocol 26 .

METHOD

1. In separate reactions, digest 1-10 µg of the plasmid DNA and foreign DNA with the appropriate restriction enzyme(s) that generate blunt ends.
2. Purify the digested foreign DNA and vector DNA by extraction with phenol:chloroform and standard ethanol precipitation.
3. Reconstitute the precipitated DNAs separately in TE (pH 8.0) at a concentration of approx. 100 µg/ml. Calculate the concentration of the DNAs (in pmole/ml) assuming that 1 bp has a mass of 660 daltons.
4. Dephosphorylate the plasmid vector DNA as described in Chapter 1, Protocol 20 .
5. Transfer appropriate amounts of the DNAs to sterile 0.5-ml microfuge tubes as follows:

Tube	DNA
A and E	vector[1] (60 fmoles [approx. 100 ng])
B	foreign[2] (60 fmoles [approx. 10 ng])
C and F	vector[1] (60 fmoles) plus foreign (60 fmoles)[3]
D	linearized vector (contains 5'-terminal phosphates) (60 fmoles)
G	superhelical vector (6 fmoles [approx. 10 ng])

[1]Vector DNA is dephosphorylated as described in Chapter 1, Protocol 20 .
[2]Linkers may be ligated to foreign target DNA.
[3]The molar ratio of plasmid vector to insert DNA fragment should be approx. 1:1 in the ligation reaction. The total DNA concentration in the ligation reaction should be approx. 10 ng/µl.

a. To Tubes A, B, and C add:

10x Ligation buffer	1.0 µl
Bacteriophage T4 DNA ligase	0.5 Weiss unit
5 mM ATP	1.0 µl
H_2O	to 8.5 µl
30% PEG 8000	1-1.5 µl

b. To Tubes D, E, and F add:

10x Ligation buffer	1.0 µl
5 mM ATP	1.0 µl
H_2O	to 8.5 µl
30% PEG 8000	1-1.5 µl
no DNA ligase	

To achieve the maximum efficiency of ligation, set up the reactions in as small a volume as possible (5-10 µl). The DNA fragments can be added to the tubes together with the H_2O and then warmed to 45°C for 5 minutes to help dissociate any clumps of DNA that have formed during fragment preparation. Chill the DNA solution to 0°C before the remainder of the ligation reagents are added.

6. Incubate the reaction mixtures overnight at 16°C or for 4 hours at 20°C.
7. Transform competent *E. coli* with dilutions of each of the ligation reactions, using one of the methods described in Chapter 1, Protocol 23, Chapter 1, Protocol 24, Chapter 1, Protocol 25 , or Chapter 1, Protocol 26 . As controls, include known amounts of a standard preparation of superhelical plasmid DNA to check the efficiency of transformation.

Tube	DNA	Ligase	Expected number of transformants

A	vector[1]	+	approx. 0[3]
B	insert	+	0
C	vector[1] and insert	+	approx. 5-fold more then Tube F
D	vector[1]	-	approx. 0
E	vector[2]	-	approx. 50-fold more than Tube D
F	vector[1] and insert	-	approx. 50-fold more than Tube D
G	superhelical vector	-	2 x 10^5

[1]Dephosphorylated
[2]Not dephosphorylated
[3]Transformants arising from ligation of dephosphorylated vector DNA alone are due to failure to remove 5' residues during treatment with alkaline phosphatase.

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2. Ferretti L. and Sgaramella V. 1981. Temperature dependence of the joining by T4 DNA ligase of termini produced by type II restriction endonucleases. *Nucleic Acids Res.* 9:85-93.
3. Sgaramella V. and Ehrlich S.D. 1978. Use of the T4 polynucleotide ligase in the joining of flush-ended DNA segments generated by restriction endonucleases. *Eur. J. Biochem.* 86:531-537.

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Protocol 20

Dephosphorylation of Plasmid DNA

During ligation in vitro, T4 DNA ligase will catalyze the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide carries a 5´-phosphate residue and the other carries a 3´-hydroxyl terminus. Recircularization of vector DNA can therefore be minimized by removing the 5´-phosphate residues from both termini of the linear, double-stranded plasmid DNA with alkaline phosphatase.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- EDTA (0.5 M, pH 8.0)
- EGTA (0.5 M, pH 8.0)
- Ethanol
- ⚠ Phenol
- ⚠ Phenol:chloroform (1:1, v/v)
- ⚠ SDS (10% w/v)
- Sodium acetate (3 M, pH 5.2 and pH 7.0)
- TE (pH 8.0)
- Tris-Cl (10 mM, pH 8.3)

Enzymes and Buffers

- Calf intestinal alkaline phosphatase (CIP)
- Shrimp alkaline phosphatase (SAP)
- Proteinase K (10 mg/ml)
- Restriction endonucleases

Nucleic Acids

- Vector DNA (closed circular plasmid)

METHOD

1. Digest a reasonable quantity of closed circular plasmid DNA (10 µg) with a two- to threefold excess of the desired restriction enzyme for 1 hour.
2. Remove an aliquot (0.1 µg), and analyze the extent of digestion by electrophoresis through a 0.7% agarose gel containing ethidium bromide, using undigested plasmid DNA as a marker. If digestion is not complete, add more restriction enzyme and continue the incubation.
3. When digestion is complete, extract the sample once with phenol:chloroform and recover the DNA by standard precipitation with ethanol. Store the ethanolic solution on ice for 15 minutes.
4. Recover the DNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge, and dissolve the DNA in 110 µl of 10 mM Tris-Cl (pH 8.3).
5. To the remaining 90 µl of the linearized plasmid DNA, add 10 µl of 10x CIP or 10x SAP buffer and the appropriate amount of calf intestinal phosphatase (CIP) or shrimp alkaline phosphatase (SAP) and incubate as described in the table below.

Conditions for Dephosphorylation of 5´-phosphate Residues from DNA

Type of Terminus	Enzyme/Amount per mole DNA Ends	Incubation Temperature/Time
5´-Protruding	0.01 unit CIP[a]	37°C/30 minutes
	0.1 unit SAP	37°C/60 minutes
3´-Protruding	0.1-0.5 unit CIP[b]	37°C/15 minutes *then* 55°C/45 minutes
	0.5 unit SAP	37°C/60 minutes
Blunt	0.1-0.5 unit CIP[b]	37°C/15 minutes *then* 55°C/45 minutes
	0.2 unit SAP	37°C/60 minutes

[a]*After the initial 30-minute incubation, add a second aliquot of CIP enzyme and continue incubation for another 30 minutes at 37°C.*
[b]*Add a second aliquot of CIP just before beginning the incubation at 55°C.*

6. Inactivate the phosphatase activity:
   To inactivate CIP at the end of the incubation period: Add SDS and EDTA (pH 8.0) to final concentrations of 0.5% and 5 mM, respectively. Mix well, and add proteinase K to a final concentration of 100 µg/ml. Incubate for 30 minutes at 55°C.
   Alternatively, CIP can be inactivated by heating to 65°C for 30 minutes (or 75°C for 10 minutes) in the presence of 5 mM EDTA or 10 mM EGTA (both at pH 8.0).
   or
   To inactivate SAP: Incubate the reaction mixture for 15 minutes at 65°C in the dephosphorylation buffer.
7. Cool the reaction mixture to room temperature, and then extract it once with phenol and once with phenol:chloroform.
8. Recover the DNA by standard precipitation with ethanol. Mix the solution again and store it for 15 minutes at 0°C.
9. Recover the DNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge. Wash the pellet with 70% ethanol at 4°C and centrifuge again.
10. Carefully remove the supernatant and leave the open tube on the bench to allow the ethanol to evaporate.
11. Dissolve the precipitated DNA in TE (pH 8.0) at a concentration of 100 µg/ml. Store the DNA in aliquots at -20°C.

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bacteria of structural gene for rat growth hormone. *Nature* 270:486-494.

2. Ullrich A., Shine J., Chirgwin J., Pictet R., Tischer E., Rutter W.J., and Goodman H.M. 1977. Rat insulin genes: Construction of plasmids containing the coding sequences. *Science* 196:1313-1319.

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Protocol 21

Addition of Synthetic Linkers to Blunt-ended DNA

Linkers are small self-complementary pieces of synthetic DNA, usually 8-16 nucleotides in length, that anneal to form blunt-ended, double-stranded molecules containing a restriction site. Linkers are used to equip blunt-ended termini of DNA with restriction sites as an aid to cloning.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (4 M, pH 4.8)

⚠ ATP (5 mM and 10 mM)
Omit 5 mM from the ligation reaction in Step 2 if the ligation buffer contains ATP.

Ethanol

⚠ Phenol:chloroform (1:1, v/v)

Sodium acetate (3 M, pH 5.2)

TE (pH 8.0)

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Bacteriophage T4 polynucleotide kinase

Restriction endonucleases

Nucleic Acids and Oligonucleotides

Target DNA (blunt-end fragment)

Synthetic linkers dissolved in TE (pH 8.0) at a concentration of 400 µg/ml
For a dodecamer, this concentration is eqivalent to a 50 µM solution.

Radioactive Compounds

⚠ [γ-^{32}P]ATP (1-10 µCi)

METHOD

1. Assemble the following reaction mixture in a sterile 0.5-ml microfuge tube:

10x linker kinase buffer	1.0 µl
10 mM ATP	1.0 µl
synthetic liner dissolved in TE (pH 8.0)	2.0 µg[1]
H_2O	to 9 µl
bacteriophage T4 polynucleotide kinase	10 units

[1]*Approximately 250 pmoles of a dodecamer.*
Incubate the reaction for 1 hour at 37°C.

2. Calculate the concentration of termini in the preparation of blunt-ended DNA and then assemble the following ligation mixture in the order given in a sterile 0.5-ml microfuge tube:

50 µg of a 1 kb segment of double-stranded DNA = 78.7 nmoles or 157.4 nM of termini.

blunt-ended DNA	2 pmoles of termini
phosphorylated linkers	150-200 pmoles of termini
H_2O	to 7.5 µl
10x ligation buffer	1.0 µl
5 mM ATP (free acid)	1.0 µl
bacteriophage T4 DNA ligase	1.0 Weiss unit

Incubate the reaction mixtures for 12-16 hours at 4°C.
To achieve the maximum efficiency of ligation, set up the reactions in as small a volume as possible (5-10 µl).

3. Inactivate the bacteriophage T4 DNA ligase by heating the reaction mixture to 65°C for 15 minutes.
4. Cool the ligation mixture to room temperature and then add:

10x restriction enzyme buffer	10 µl
restriction enzyme	50 units
sterile H_2O	to a final volume of 100 µl

Incubate the reaction for 1-3 hours at 37°C.

5. Purify the restricted DNA by extraction with phenol:chloroform. Precipitate the DNA with 2 volumes of ethanol in the presence of 2 M ammonium acetate.
6. Collect the precipitated DNA by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge, and dissolve the pellet in 50 µl of TE (pH 8.0).
7. Pass the resuspended DNA through a spun column to remove excess linkers.
8. Recover the DNA by standard ethanol precipitation and dissolve the precipitate in 10-20 µl of TE (pH 8.0).
The modified DNA fragment can now be ligated as described in Chapter 1, Protocol 17 into a plasmid (or bacteriophage) vector with protruding ends that are complementary to those introduced by the linker.

REFERENCES

1. Scheller R.H., Dickerson R.E., Boyer H.W., Riggs A.D., and Itakura K. 1977. Chemical synthesis of restriction enzyme recognition sites useful for cloning. *Science* 196:177-180.

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Protocol 22

Ligating Plasmid and Target DNAs in Low-melting-temperature Agarose

Ligation in low-melting-temperature agarose is much less efficient than ligation with purified DNA in free solution and requires a large amount of DNA ligase. The method is used chiefly for rapid subcloning of segments of DNA in dephosphorylated vectors and assembling recombinant constructs.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Enzymes and Buffers

2x Bacteriophage T4 DNA ligase mixture

Restriction endonucleases

Nucleic Acids

Foreign DNA

Plasmid DNA (approx. 100 µg/ml, dephosphorylated)

METHOD

1. Use the appropriate restriction enzyme(s) to digest an amount of target DNA sufficient to yield approx. 250 ng of the desired fragment. Perform the digestion in a volume of 20 µl or less.
2. Separate the DNA fragments by electrophoresis through a low-melting/gelling-temperature agarose gel.
3. Examine the agarose gel under long-wavelength UV illumination. From the relative fluorescent intensities of the desired bands, estimate the amounts of DNA that they contain. Use a clean razor blade to cut out the desired bands in the smallest possible volume of agarose (usually 40-50 µl). Leave a small amount of each band in the gel to mark the positions of the DNA fragments and then photograph the dissected gel.
4. Place the excised and trimmed slices of gel in separate, labeled microfuge tubes.
5. Melt the agarose by heating the tubes to 70°C for 15 minutes in a heating block. Estimate the volume of the melted agarose in the tube and calculate the volume that would contain approx. 200 ng of DNA.
6. In a sterile microfuge tube warmed to 37°C, combine the following:

dephosphorylated plasmid DNA	60 fmoles
foreign DNA fragment	120-240 fmoles (in a volume of 10 µl or less)

Approximately 100 ng of dephosphorylated DNA is required for each ligation.
Mix the contents of the tube quickly with a sterile disposable pipette tip before the agarose solidifies.
7. In separate tubes, set up two additional ligations as controls, one containing only the dephosphorylated plasmid vector and the other containing only the fragment of foreign DNA.
8. Incubate the three tubes for 5-10 minutes at 37°C, and then add to each tube 10 µl of ice-cold 2x bacteriophage T4 DNA ligase mixture. Mix the contents of the tube quickly with a sterile disposable pipette tip before the agarose solidifies. Incubate the reactions for 12-16 hours at 16°C.

REFERENCES

1. Struhl K. 1985. A rapid method for creating recombinant DNA molecules. *BioTechniques* 3:452-453.

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Protocol 23

The Hanahan Method for Preparation and Transformation of Competent *E. coli:* High-efficiency Transformation

This procedure generates competent cultures of *E. coli* that can be transformed at high frequencies (5 x 10^8 transformed colonies/mg of superhelical plasmid DNA). **IMPORTANT** All steps in this protocol should be carried out aseptically.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ DMSO

DnD solution

Transformation buffers (please see Step 1)

Media

SOB agar plates containing 20 mM $MgSO_4$ and the appropriate antibiotic

SOB medium containing 20 mM $MgSO_4$

SOC medium
Approximately 1 ml of this medium is needed for each transformation reaction.

Nucleic Acids

Plasmid DNA (recombinant plasmid)
Construct using one of the methods described in Chapter 1, Protocol 17 , Chapter 1, Protocol 18 , Chapter 1, Protocol 19 , Chapter 1, Protocol 20 , Chapter 1, Protocol 21 and Chapter 1, Protocol 22 .

Vectors and Bacterial Strains

E. coli strain to be transformed (frozen stock)
The strain should be stored at -70°C in freezing medium.

METHOD

1. Prepare transformation buffer.
   Standard transformation buffer (TFB) is used when preparing competent cells for immediate use. Frozen storage buffer (FSB) is used to prepare stocks of competent cells that are to be stored at -70°C.

   To prepare standard transformation buffer

   a. Prepare 1 M MES by dissolving 19.52 g of MES in 80 ml of pure H_2O (Milli-Q, or equivalent). Adjust the pH of the solution to 6.3 with 5 M KOH, and add pure H_2O to bring the final volume to 100 ml. Sterilize the solution by filtration through a disposable prerinsed Nalgene filter (0.45-µm pore size). Divide into 10-ml aliquots and store at -20°C.

   b. Prepare TFB by dissolving all the solutes listed below in approx. 500 ml of H_2O and then add 10 ml of 1 M MES buffer (pH 6.3). Adjust the volume of the TFB to 1 liter with pure H_2O.

Reagent	Amount per liter	Final concentration
1 M MES (pH 6.3)	10 ml	10 mM
$MnCL_2 \cdot 4H_2O$	8.91 g	45 mM
$CaC_2 \cdot 2H_2O$	1.47 g	10 mM
KCl	7.46 g	100 mM
Hexamminecobalt chloride	0.80 g	3 mM
H_2O	to 1 liter	

   c. Sterilize the TFB by filtration through a disposable prerinsed Nalgene filter (0.45-µm pore size). Divide the solution into 40-ml aliquots in tissue-culture flasks (e.g., Corning, or equivalent) and store them at 4°C.

   To prepare frozen storage buffer

   a. Prepare 1 M potassium acetate by dissolving 9.82 g of potassium acetate in 90 ml of pure H_2O (Milli-Q, or equivalent). Adjust the pH of the solution to 7.5 with 2 M acetic acid, add pure H_2O to bring the final volume to 100 ml. Divide the solution into aliquots and store at -20°C.

   b. Prepare FSB by dissolving all of the solutes listed below in approx. 500 ml of pure H_2O. After the components are dissolved, adjust the pH of the solution to 6.4 with 0.1 N HCl. Too high a pH cannot be adjusted by adding base; instead, discard the solution and and begin again. Adjust the volume of the final solution to 1 liter with pure H_2O.

Reagent	Amount per liter	Final concentration
1 M potassium acetate (pH 7.5)	10 ml	10 mM
$MnCL_2 \cdot 4H_2O$	8.91 g	45 mM
$CaCl_2 \cdot 2H_2O$	1.47 g	10 mM
KCl	7.46 g	10 mM
Hexamminecobalt chloride	0.80 g	100 mM
Glycerol	100 ml	10% (v/v)
H_2O	to 1 liter	

   c. Sterilize the solution by filtration through a disposable prerinsed Nalgene filter (0.45-µm pore size). Dispense the solution into 40-ml aliquots and store the aliquots in tissue culture flasks (e.g., Corning, or equivalent) at 4°C. During storage, the pH of the solution drifts down to a final value of 6.1-6.2 but then stabilizes.

2. Use an inoculating loop to streak *E. coli* of the desired strain directly from a frozen stock onto the surface of an SOB agar plate. Incubate the plate for 16 hours at 37°C.
3. Transfer four or five well-isolated colonies into 1 ml of SOB containing 20 mM $MgSO_4$. Disperse the bacteria by vortexing at moderate speed, and then dilute the culture in 30-100 ml of SOB containing 20 mM $MgSO_4$ in a 1-liter flask.
4. Grow the cells for 2.5-3.0 hours at 37°C, monitoring the growth of the culture.
5. Transfer the cells to sterile, disposable, ice-cold 50-ml polypropylene tubes. Cool the cultures to 0°C by storing the

tubes on ice for 10 minutes.

6. Recover the cells by centrifugation at 2700*g* (4100 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C.
7. Decant the medium from the cell pellets. Stand the tubes in an inverted position for 1 minute to allow the last traces of medium to drain away.
8. Resuspend the pellets by swirling or gentle vortexing in approx. 20 ml (per 50-ml tube) of ice-cold TFB or FSB transformation buffer. Store the resuspended cells on ice for 10 minutes.
9. Recover the cells by centrifugation at 2700*g* (4100 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C.
10. Decant the buffer from the cell pellets. Stand the tubes in an inverted position for 1 minute to allow the last traces of buffer to drain away.
11. Resuspend the pellets by swirling or gentle vortexing in 4 ml (per 50-ml tube) of ice-cold TFB or FSB. Proceed either with Step 12a if the competent cells are to be used immediately or with Step 12b if the competent cells are to be stored at -70°C and used at a later date.
12. Prepare competent cells for transformation.

To prepare fresh competent cells

a. Add 140 µl of DnD solution into the center of each cell suspension. Immediately mix by swirling gently, and then store the suspension on ice for 15 minutes.

b. Add an additional 140 µl of DnD solution to each suspension. Mix by swirling gently, and then store the suspension on ice for a further 15 minutes.

c. Dispense aliquots of the suspensions into chilled, sterile 17 x 100-mm polypropylene tubes. Store the tubes on ice.

To prepare frozen stocks of competent cells

a. Add 140 µl of DMSO per 4 ml of resuspended cells. Mix gently by swirling, and store the suspension on ice for 15 minutes.

b. Add an additional 140 µl of DMSO to each suspension. Mix gently by swirling, and then return the suspensions to an ice bath.

c. Working quickly, dispense aliquots of the suspensions into chilled, sterile microfuge tubes or tissue culture vials. Immediately snap-freeze the competent cells by immersing the tightly closed tubes in a bath of liquid nitrogen. Store the tubes at -70°C until needed.

d. When needed, remove a tube of competent cells from the -70°C freezer. Thaw the cells by holding the tube in the palm of the hand. Just as the cells thaw, transfer the tube to an ice bath. Store the cells on ice for 10 minutes.

e. Use a chilled, sterile pipette tip to transfer the competent cells to chilled, sterile 17 x 100-mm polypropylene tubes. Store the cells on ice.

Include all of the appropriate positive and negative controls.

13. Add the transforming DNA (up to 25 ng per 50 µl of competent cells) in a volume not exceeding 5% of that of the competent cells. Swirl the tubes gently several times to mix their contents. Set up at least two control tubes for each transformation experiment, including a tube of competent bacteria that receives a known amount of a standard preparation of superhelical plasmid DNA and a tube of cells that receives no plasmid DNA at all. Store the tubes on ice for 30 minutes.
14. Transfer the tubes to a rack placed in a preheated 42°C circulating water bath. Store the tubes in the rack for exactly 90 seconds. Do not shake the tubes.
15. Rapidly transfer the tubes to an ice bath. Allow the cells to cool for 1-2 minutes.
16. Add 800 µl of SOC medium to each tube. Warm the cultures to 37°C in a water bath, and then transfer the tubes to a shaking incubator set at 37°C. Incubate the cultures for 45 minutes to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.
17. Transfer the appropriate volume (up to 200 µl per 90-mm plate) of transformed competent cells onto agar SOB medium containing 20 mM $MgSO_4$ and the appropriate antibiotic.
18. Store the plates at room temperature until the liquid has been absorbed.
19. Invert the plates and incubate them at 37°C. Transformed colonies should appear in 12-16 hours.

REFERENCES

1. Hanahan D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557-580.
2. Hanahan D., Jessee J., and Bloom F.R. 1991. Plasmid transformation of *E. coli* and other bacteria. *Methods Enzymol.* 204:63-113.

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Protocol 24

The Inoue Method for Preparation and Transformation of Competent *E. Coli*: "Ultra-Competent" Cells

This protocol reproducibly generates competent cultures of *E. coli* that yield 1 x 10^8 to 3 x 10^8 transformed colonies/mg of plasmid DNA. The protocol works optimally when the bacterial culture is grown at 18°C. If a suitable incubator is not available, a standard bacterial shaker can be set up in a 4°C cold room and regulated to 18°C.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ DMSO

Inoue transformation buffer (please see Step 1)
Chilled to 0°C before use.

Nucleic Acids

Plasmid DNA (recombinant plasmid)
Construct using one of the methods described in Chapter 1, Protocol 17 , Chapter 1, Protocol 18 , Chapter 1, Protocol 19 , Chapter 1, Protocol 20 , Chapter 1, Protocol 21 and Chapter 1, Protocol 22 .

Media

- SOB medium for initial growth of culture
- SOB agar plates containing 20 mM $MgSO_4$ and the appropriate antibiotic
- SOB medium, for growth of culture to be transformed
- SOC medium

METHOD

1. Prepare Inoue transformation buffer (chilled to 0°C before use).
   a. Prepare 0.5 M PIPES (pH 6.7) (piperazine-1,2-bis[2-ethanesulfonic acid]) by dissolving 15.1 g of PIPES in 80 ml of pure H_2O (Milli-Q, or equivalent). Adjust the pH of the solution to 6.7 with 5 M KOH, and then add pure H_2O to bring the final volume to 100 ml. Sterilize the solution by filtration through a disposable prerinsed Nalgene filter (0.45-µm pore size). Divide into aliquots and store frozen at -20°C.
   b. Prepare Inoue transformation buffer by dissolving all of the solutes listed below in 800 ml of pure H_2O and then add 20 ml of 0.5 M PIPES (pH 6.7). Adjust the volume of the Inoue transformation buffer to 1 liter with pure H_2O.

Reagent	Amount per liter	Final concentration
$MnCl_2 \cdot 4H_2O$	10.88 g	55 mM
$CaCl_2 \cdot 2H_2O$	2.20 g	15 mM
KCl	18.65 g	250 mM
PIPES (0.5 M, pH 6.7)	10 ml	10 mM
H_2O	to 1 liter	

   c. Sterilize Inoue transformation buffer by filtration through a prerinsed 0.45-µm Nalgene filter. Divide into aliquots and store at -20°C.
2. Pick a single bacterial colony (2-3 mm in diameter) from a plate that has been incubated for 16-20 hours at 37°C. Transfer the colony into 25 ml of SOB medium (LB may be used instead) in a 250-ml flask. Incubate the culture for 6-8 hours at 37°C with vigorous shaking (250-300 rpm).
3. At about 6 o'clock in the evening, use this starter culture to inoculate three 1-liter flasks, each containing 250 ml of SOB. The first flask receives 10 ml of starter culture, the second receives 4 ml, and the third receives 2 ml. Incubate all three flasks overnight at 18-22°C with moderate shaking.
4. The following morning, read the OD_{600} of all three cultures. Continue to monitor the OD every 45 minutes.
5. When the OD_{600} of one of the cultures reaches 0.55, transfer the culture vessel to an ice-water bath for 10 minutes. Discard the two other cultures.
6. Harvest the cells by centrifugation at 2500*g* (3900 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C.
7. Pour off the medium and store the open centrifuge bottle on a stack of paper towels for 2 minutes. Use a vacuum aspirator to remove any drops of remaining medium adhering to walls of the centrifuge bottle or trapped in its neck.
8. Resuspend the cells gently in 80 ml of ice-cold Inoue transformation buffer.
9. Harvest the cells by centrifugation at 2500*g* (3900 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C.
10. Pour off the medium and store the open centrifuge tube on a stack of paper towels for 2 minutes. Use a vacuum aspirator to remove any drops of remaining medium adhering to the walls of the centrifuge tube or trapped in its neck.
11. Resuspend the cells gently in 20 ml of ice-cold Inoue transformation buffer.
12. Add 1.5 ml of DMSO. Mix the bacterial suspension by swirling and then store it in ice for 10 minutes.
13. Working quickly, dispense aliquots of the suspensions into chilled, sterile microfuge tubes. Immediately snap-freeze the competent cells by immersing the tightly closed tubes in a bath of liquid nitrogen. Store the tubes at -70°C until needed.
14. When needed, remove a tube of competent cells from the -70°C freezer. Thaw the cells by holding the tube in the palm of the hand. Just as the cells thaw, transfer the tube to an ice bath. Store the cells on ice for 10 minutes.
15. Use a chilled, sterile pipette tip to transfer the competent cells to chilled, sterile 17 x 100-mm polypropylene tubes. Store the cells on ice.
    Include all of the appropriate positive and negative controls.
16. Add the transforming DNA (up to 25 ng per 50 µl of competent cells) in a volume not exceeding 5% of that of the competent cells. Swirl the tubes gently several times to mix their contents. Set up at least two control tubes for each transformation experiment, including a tube of competent bacteria that receives a known amount of a standard preparation of superhelical plasmid DNA and a tube of cells that receives no plasmid DNA at all. Store the tubes on ice for 30 minutes.
17. Transfer the tubes to a rack placed in a preheated 42°C circulating water bath. Store the tubes in the rack for exactly 90 seconds. Do not shake the tubes.
18. Rapidly transfer the tubes to an ice bath. Allow the cells to cool for 1-2 minutes.
19. Add 800 µl of SOC medium to each tube. Warm the cultures to 37°C in a water bath, and then transfer the tubes to a shaking incubator set at 37°C. Incubate the cultures for 45 minutes to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.
20. Transfer the appropriate volume (up to 200 µl per 90-mm plate) of transformed competent cells onto agar SOB medium containing 20 mM $MgSO_4$ and the appropriate antibiotic.
21. Store the plates at room temperature until the liquid has been absorbed.
22. Invert the plates and incubate them at 37°C. Transformed colonies should appear in 12-16 hours.

REFERENCES

1. Inoue H., Nojima H., and Okayama H. 1990. High efficiency transformation of *Escherichia coli* with plasmids. *Gene* 96:23-28.

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Protocol 25

Preparation and Transformation of Competent *E. coli* Using Calcium Chloride

This protocol, developed approx. 30 years ago, is used to prepare batches of competent bacteria that yield 5 x 10^6 to 2 x 10^7 transformed colonies/µg of supercoiled plasmid DNA.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

$CaCl_2 \cdot 2H_2O$ (1 M)

Standard transformation buffer (TFB) (please see Chapter 1, Protocol 23 , Step 1) may be used in Step 8.

$MgCl_2$-$CaCl_2$ solution, ice cold

Media

- SOB medium for initial growth of culture
- SOB agar plates containing 20 mM $MgSO_4$ and the appropriate antibiotic
- SOC medium

Nucleic Acids

Plasmid DNA (recombinant plasmid)
Construct using one of the methods described in Chapter 1, Protocol 17 , Chapter 1, Protocol 18 , Chapter 1, Protocol 19 , Chapter 1, Protocol 20 , Chapter 1, Protocol 21 and Chapter 1, Protocol 22 .

METHOD

1. Pick a single bacterial colony (2-3 mm in diameter) from a plate that has been incubated for 16-20 hours at 37°C. Transfer the colony into 100 ml SOB medium (LB-may be used) in a 1-liter flask. Incubate the culture for 3 hours at 37°C with vigorous agitation, monitoring the growth of the culture. As a guideline, 1 OD_{600} of a culture of *E. coli* strain DH1 contains approx. 10^9 bacteria/ml.
2. Transfer the bacterial cells to sterile, disposable, ice-cold 50-ml polypropylene tubes. Cool the cultures to 0°C by storing the tubes on ice for 10 minutes.
3. Recover the cells by centrifugation at 2700*g* (4100 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C.
4. Decant the medium from the cell pellets. Stand the tubes in an inverted position on a pad of paper towels for 1 minute to allow the last traces of media to drain away.
5. Resuspend each pellet by swirling or gentle vortexing in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution.
6. Recover the cells by centrifugation at 2700*g* (4100 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C.
7. Decant the medium from the cell pellets. Stand the tubes in an inverted position on a pad of paper towels for 1 minute to allow the last traces of media to drain away.
8. Resuspend the pellet by swirling or gentle vortexing in 2 ml of ice-cold 0.1 M $CaCl_2$ (or TFB) for each 50 ml of original culture.
   When preparing competent cells, thaw a 10-ml aliquot of the $CaCl_2$ stock solution and dilute it to 100 ml with 90 ml of pure H_2O. Sterilize the solution by filtration through a prerinsed Nalgene filter (0.45-µm pore size), and then chill it to 0°C.
   For many strains of E. coli*, standard TFB (Chapter 1, Protocol 23) may be used instead of $CaCl_2$ with equivalent or better results.*
9. At this point, either use the cells directly for transformation as described in Steps 10 through 16 below or dispense into aliquots and freeze at -70°C (please see Chapter 1, Protocol 23 , Step 12).
   Include all of the appropriate positive and negative controls.
10. To transform the $CaCl_2$-treated cells directly, transfer 200 µl of each suspension of competent cells to a sterile, chilled 17 x 100-mm polypropylene tube using a chilled micropipette tip. Add DNA (no more than 50 ng in a volume of 10 µl or less) to each tube. Mix the contents of the tubes by swirling gently. Store the tubes on ice for 30 minutes.
11. Transfer the tubes to a rack placed in a preheated 42°C circulating water bath. Store the tubes in the rack for exactly 90 seconds. Do not shake the tubes.
12. Rapidly transfer the tubes to an ice bath. Allow the cells to chill for 1-2 minutes.
13. Add 800 µl of SOC medium to each tube. Incubate the cultures for 45 minutes in a water bath set at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.
14. Transfer the appropriate volume (up to 200 µl per 90-mm plate) of transformed competent cells onto agar SOB medium containing 20 mM $MgSO_4$ and the appropriate antibiotic.
15. Store the plates at room temperature until the liquid has been absorbed.
16. Invert the plates and incubate at 37°C. Transformed colonies should appear in 12-16 hours.

REFERENCES

1. Cohen S.N., Chang A.C.Y., and Hsu L. 1972. Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci.* 69:2110-2114.

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Protocol 26

Transformation of *E. coli* by Electroporation

Electrocompetent bacteria are prepared by growing cultures to mid-log phase, washing the bacteria extensively at low temperature, and then resuspending them in a solution of low ionic strength containing glycerol. DNA is introduced during exposure of the bacteria to a short high-voltage electrical discharge.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Glycerol (10% v/v) (molecular biology grade), ice cold

  Pure H_2O

  Milli-Q or equivalent, sterilized by filtration through prerinsed 0.45-µm filters. Store at 4°C.

Nucleic Acids

Plasmid DNA (recombinant plasmid)
Construct using one of the methods described in Chapter 1, Protocol 17 , Chapter 1, Protocol 18 , Chapter 1, Protocol 19 , Chapter 1, Protocol 20 , Chapter 1, Protocol 21 and Chapter 1, Protocol 22 .

Media

- GYT medium, ice cold
- LB medium, prewarmed to 37°C
- SOB agar plates containing 20 mM $MgSO_4$ and the appropriate antibiotic
- SOC medium

METHOD

1. Inoculate a single colony of *E. coli* from a fresh agar plate into a flask containing 50 ml of LB medium. Incubate the culture overnight at 37°C with vigorous aeration (250 rpm in a rotary shaker).
2. Inoculate two aliquots of 500 ml of prewarmed LB medium in separate 2-liter flasks with 25 ml of the overnight bacterial culture. Incubate the flasks at 37°C with agitation (300 cycles/minute in a rotary shaker). Measure the OD_{600} of the growing bacterial cultures every 20 minutes.
3. When the OD_{600} of the cultures reaches 0.4, rapidly transfer the flasks to an ice-water bath for 15-30 minutes. Swirl the culture occasionally to ensure that cooling occurs evenly. In preparation for the next step, place the centrifuge bottles in an ice-water bath.
4. Transfer the cultures to ice-cold centrifuge bottles. Harvest the cells by centrifugation at 1000*g* (2500 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C. Decant the supernatant and resuspend the cell pellet in 500 ml of ice-cold pure H_2O.
5. Harvest the cells by centrifugation at 1000*g* (2500 rpm in a Sorvall GSA rotor) for 20 minutes at 4°C. Decant the supernatant and resuspend the cell pellet in 250 ml of ice-cold 10% glycerol.
6. Harvest the cells by centrifugation at 1000*g* (2500 rpm in a Sorvall GSA rotor) for 20 minutes at 4°C. Decant the supernatant and resuspend the pellet in 10 ml of ice-cold 10% glycerol.
7. Harvest cells by centrifugation at 1000*g* (2500 rpm in a Sorvall GSA rotor) for 20 minutes at 4°C. Carefully decant the supernatant and use a Pasteur pipette attached to a vacuum line to remove any remaining drops of buffer. Resuspend the pellet in 1 ml of ice-cold GYT medium.
8. Measure the OD_{600} of a 1:100 dilution of the cell suspension. Dilute the cell suspension to a concentration of 2 x 10^{10} to 3 x 10^{10} cells/ml (1.0 OD_{600} = approx. 2.5 x 10^8 cells/ml) with ice-cold GYT medium.
9. Transfer 40 µl of the suspension to an ice-cold electroporation cuvette (0.2-cm gap) and test whether arcing occurs when an electrical discharge is applied (please see Step 16 below). If so, wash the remainder of the cell suspension once more with ice-cold GYT medium to ensure that the conductivity of the bacterial suspension is sufficiently low (<5 mEq).
10. To use the electrocompetent cells immediately, proceed directly to Step 12. Otherwise, store the cells at -70°C until required. For storage, dispense 40-µl aliquots of the cell suspension into sterile, ice-cold 0.5-ml microfuge tubes, drop into a bath of liquid nitrogen, and transfer to a -70°C freezer.
11. To use frozen electrocompetent cells, remove an appropriate number of aliquots of cells from the -70°C freezer. Store the tubes at room temperature until the bacterial suspensions are thawed and then transfer the tubes to an ice bath.
12. Pipette 40 µl of the freshly made (or thawed) electrocompetent cells into ice-cold sterile 0.5-ml microfuge tubes. Place the cells on ice, together with an appropriate number of bacterial electroporation cuvettes.
13. Add 10 pg to 25 ng of the DNA to be electroporated in a volume of 1-2 µl to each microfuge tube and incubate the tube on ice for 30-60 seconds. Include all of the appropriate positive and negative controls.
14. Set the electroporation apparatus to deliver an electrical pulse of 25 µF capacitance, 2.5 kV, and 200 ohm resistance.
15. Pipette the DNA/cell mixture into a cold electroporation cuvette. Tap the solution to ensure that the suspension of bacteria and DNA sits at the bottom of the cuvette. Dry condensation and moisture from the outside of the cuvette. Place the cuvette in the electroporation device.
16. Deliver a pulse of electricity to the cells at the settings indicated above. A time constant of 4-5 milliseconds with a field strength of 12.5 kV/cm should register on the machine.
17. As quickly as possible after the pulse, remove the electroporation cuvette and add 1 ml of SOC medium at room temperature.
18. Transfer the cells to a 17 x 100-mm or 17 x 150-mm polypropylene tube and incubate the cultures with gentle rotation for 1 hour at 37°C.
19. Plate different volumes (up to 200 µl per 90-mm plate) of the electroporated cells onto SOB agar medium containing 20 mM $MgSO_4$ and the appropriate antibiotic.
20. Store the plates at room temperature until the liquid has been absorbed.
21. Invert the plates and incubate them at 37°C. Transformed colonies should appear in 12-16 hours.

REFERENCES

1. Chassy B.M. and Flickinger J.L. 1987. Transformation of *Lactobacillus casei* by electroporation. *FEMS Microbiol. Lett.* 44:173-177.
2. Chassy B.M., Mercenier A., and Flickinger J. 1988. Transformation of bacteria by electroporation. *Trends Biotechnol.* 6:303-309.
3. Dower W.J., Miller J.F., and Ragsdale C.W. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* 16:6127-6145.

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Protocol 27

Screening Bacterial Colonies Using X-gal and IPTG: ∝-Complementation

∝-complementation occurs when two inactive fragments of *E. coli* β-galactosidase associate to form a functional enzyme. Many plasmid vectors carry a short segment of DNA containing the coding information for the first 146 amino acids of β-galactosidase. Vectors of this type are used in host cells that express the carboxy-terminal portion of the enzyme. Although neither the host nor the plasmid-encoded fragments of β-galactosidase are themselves active, they can associate to form an enzymatically active protein. Lac$^+$ bacteria that result from ∝-complementation are easily recognized because they form blue colonies in the presence of the chromogenic substrate X-gal. However, insertion of a fragment of foreign DNA into the polycloning site of the plasmid almost invariably results in production of an amino-terminal fragment that is no longer capable of ∝-complementation. Bacteria carrying recombinant plasmids therefore form white colonies. The development of this simple blue-white color test has greatly simplified the identification of recombinants constructed in plasmid vectors.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- IPTG solution (20% w/v)
- ⚠ X-gal solution (2% w/v)

Media

- Rich broth agar plates containing the appropriate antibiotic
- Rich broth top agar

Vectors and Bacterial Strains

E. coli culture, transformed with recombinant plasmids
Use bacteria transformed by one of the methods described in Chapter 1, Protocol 23 , Chapter 1, Protocol 24 , Chapter 1, Protocol 25 , Chapter 1, Protocol 26 .

METHOD

1. Dispense aliquots of molten top agar into 17 x 100-mm tubes. Place the tubes in a 45°C heating block until they are needed.
2. Remove the first tube from the heating block. Working quickly, add 0.1 ml of bacterial suspension containing <3000 viable bacteria for a 90-mm plate and <10,000 for a 150-mm plate. Close the top of the tube and invert it several times to disperse the bacteria through the molten agar.
3. Open the tube and add the appropriate amounts of X-gal and IPTG (if required) as shown in the table below. Close the top of the tube and gently invert it several times to mix the contents.

Components for Top Agar

		Amount of Reagent	
Size of Plate	**Molten Top Agar**	**X-gal**	**IPTG[a]**
90 mm	3 ml	40 µl	7 µl
150 mm	7 ml	100 µl	20 µl

[a]*May not be required; please see the entry on IPTG in the Materials list.*

4. Quickly pour the molten top agar into the center of a hardened agar plate containing the appropriate antibiotic and distribute the solution by swirling.
5. Repeat Steps 2-4 until all of the samples have been plated.
6. Allow the soft agar to harden at room temperature, wipe any condensation from the lid of the plates, and then incubate the plates in an inverted position for 12-16 hours at 37°C.
7. Remove the plates from the incubator and store them for several hours at 4°C, to allow the blue color to develop.
8. Identify colonies carrying recombinant plasmids.
   - Colonies that carry wild-type plasmids contain active β-galactosidase. These colonies are pale blue in the center and dense blue at their periphery.
   - Colonies that carry recombinant plasmids do not contain active β-galactosidase. These colonies are creamy-white or eggshell blue, sometimes with a faint blue spot in the center.
9. Select and culture colonies carrying recombinant plasmids.

REFERENCES

1. Davies J. and Jacob F. 1968. Genetic mapping of the regulator and operator genes of the lac operon. *J. Mol. Biol.* 36:413-417.
2. Ullmann A., Jacob F., and Monod J. 1967. Characterization by in vitro complementation of a peptide corresponding to an operator-proximal segment of the beta-galactosidase structural gene of *Escherichia coli*. *J. Mol. Biol.* 24:339-343.

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Protocol 28

Screening Bacterial Colonies by Hybridization: Small Numbers

This procedure, a variant of the Grunstein and Hogness (1979) method, is used to screen a small number of bacterial colonies (<200) that are dispersed over several agar plates and are to be screened by hybridization to the same radiolabeled probe. The colonies are gridded onto a master plate and onto a nitrocellulose or nylon filter laid on the surface of a second agar plate. After a period of growth, the colonies on the filter are lysed and processed for hybridization. The master plate is stored until the results of the screening procedure become available.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Media

- Rich broth agar plates containing the appropriate antibiotic
- Rich broth agar plates containing chloramphenicol

Additional Reagents

Step 8 of this protocol requires the reagents listed in Chapter 1, Protocol 31 and Chapter 1, Protocol 32 .

Vectors and Bacterial Strains

E. coli strain, transformed with recombinant plasmids
Use bacteria transformed by one of the methods described in Chapter 1, Protocol 23 , Chapter 1, Protocol 24 , Chapter 1, Protocol 25 and Chapter 1, Protocol 26 .

E. coli strain, transformed with nonrecombinant plasmid (e.g., pUC, used as a negative control)

METHOD

1. Place a nitrocellulose or nylon filter on an agar plate (test plate) containing the selective antibiotic.
2. Draw a numbered grid on a piece of graph paper (1-cm-square grid). Number the base of each agar master plate and place the plate on the grid. Draw a mark on the side of the plate at the 6 o'clock position.
3. Use sterile toothpicks or inoculating loops to transfer bacterial colonies one by one onto the filter on the test plate and then onto the master agar plate that contains the selective antibiotic but no filter. Make small streaks 2-3 mm in length (or dots) arranged according to the grid pattern under the dish. Streak each colony in an identical position on both plates.
4. Finally, streak a colony containing a nonrecombinant plasmid (e.g., pUC) onto the filter and the master plate.
5. Invert the plates and incubate them at 37°C until the bacterial streaks have grown to a width of 0.5-1.0 mm (typically 6-16 hours).
6. Mark the filter in three or more asymmetric locations by stabbing through it and into the agar of the test plate with an 18-gauge needle, attached to a syringe, dipped in waterproof black drawing ink (India Ink). Mark the master plate in approximately the same locations.
7. Seal the master plate with Parafilm and store it at 4°C in an inverted position until the results of the hybridization reaction are available.
8. Lyse the bacteria adhering to the filter and bind the liberated DNA to the nitrocellulose or nylon filter using the procedures described in Chapter 1, Protocol 31 . Proceed with hybridization as described in Chapter 1, Protocol 32 .

REFERENCES

1. Grunstein M. and Hogness D.S. 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci.* 72:3961-3965.

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Protocol 29

Screening Bacterial Colonies by Hybridization: Intermediate Numbers

Bacterial colonies growing on agar plates are transferred en masse to nitrocellulose filters. The spatial arrangement of colonies on the plates is preserved on the filters. After transfer, the filters are processed for hybridization to an appropriate radiolabeled probe while the original (master) plate is incubated for a few hours to allow the bacterial colonies to regrow in their original positions. This technique, a variant of the Grunstein and Hogness (1975) method, was developed at Cold Spring Harbor Laboratory in 1975. The procedure works best with 90-mm plates containing <2500 colonies.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Media

- Rich broth agar plates (90-mm) containing appropriate antibiotics
- Rich broth agar plates containing chloramphenicol

Additional Reagents

Step 6 of this protocol requires the reagents listed in Chapter 1, Protocol 31 and Chapter 1, Protocol 32 .

Vectors and Bacterial Strains

E. coli strain, transformed with recombinant plasmids, as culture
Use bacteria transformed by one of the methods described in Chapter 1, Protocol 23 , Chapter 1, Protocol 24 , Chapter 1, Protocol 25 and Chapter 1, Protocol 26 .

METHOD

1. Plate out the transformed *E. coli* culture onto 90-mm LB or SOB agar plates, at dilutions calculated to generate up to 2500 transformed colonies. When the colonies reach an average size of 1.5 mm, transfer the plates from the incubator to a cold room.
2. Number the dry filters with a soft-lead pencil or a ball-point pen, wet them with water, and interleave them between dry Whatman 3MM filters. Wrap the stack of filters loosely in aluminum foil, and sterilize them by autoclaving (15 psi [1.05 kg/cm^2] for 10 minutes on liquid cycle).
3. Place a dry, sterile detergent-free nitrocellulose filter, numbered side down, on the surface of the LB (or SOB) agar medium, in contact with the bacterial colonies (plated in Step 1), until it is completely wet.
4. Once the filter is in place, key the filter to the underlying medium by stabbing in three or more asymmetric locations through the filter with a 23-gauge needle attached to a syringe, dipped in waterproof black drawing ink.
5. Grip the edge of the filter with blunt-ended forceps and, in a single smooth movement, peel the filter from the surface of the agar.
6. Proceed with one of the following options as appropriate:
   - Lyse the bacteria adhering to the filter and bind the liberated DNA to the nitrocellulose or nylon filter using the procedures described in Chapter 1, Protocol 31 . Proceed with hybridization as described in Chapter 1, Protocol 32 .
   - Lyse the bacteria and immobilize the DNA as described in the alternative protocol on p. 1.131 of the print version of the manual.
   - Place the filter, colony side up, on the surface of a fresh LB (or SOB) agar plate containing the appropriate antibiotic. After incubation for a few hours, when the colonies have grown to a size of 2-3 mm, remove the filter and proceed with lysis and hybridization as described in Chapter 1, Protocol 31 and Chapter 1, Protocol 32 .
   - Amplify the colonies on the filter by transferring the filter to an agar plate containing chloramphenicol (170-200 µg/ml) and incubating for 12 hours at 37°C. Proceed with lysis and hybridization (Chapter 1, Protocol 31 and Chapter 1, Protocol 32).
   - Use the filter to prepare a second replica:
     a. Place the filter colony side up on the surface of a fresh LB (or SOB) agar plate containing the appropriate antibiotic.
     b. Lay a dry nitrocellulose filter carefully on top of the first and key to it as described in Step 4 above.
     c. Incubate the "filter sandwich" for several hours at 37°C.
     d. Proceed with lysis and hybridization (Chapter 1, Protocol 31 and Chapter 1, Protocol 32), keeping the filters as a sandwich during the lysis and neutralization steps, but peeling them apart before the final wash.
7. Incubate the master plate for 5-7 hours at 37°C until the colonies have regrown. Seal the plate with Parafilm, and store it at 4°C in an inverted position.

REFERENCES

1. Grunstein M. and Hogness D.S. 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci.* 72:3961-3965.

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Protocol 30

Screening Bacterial Colonies by Hybridization: Large Numbers

This procedure is used to plate, replicate, and subsequently screen large numbers of bacterial colonies (up to 2 x 10^4 colonies per 150-mm plate or 10^4 colonies per 90-mm plate).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Media

- Rich broth agar plates containing appropriate antibiotics
  Plates that are 2-3 days old give the best results in this protocol because they absorb fluid from the bacterial inoculum more readily.
- Rich broth agar plates containing appropriate antibiotics and 25% (v/v) glycerol
- Rich broth agar plates containing chloramphenicol

Additional Reagents

Step 14 of this protocol requires the reagents listed in Chapter 1, Protocol 31 and Chapter 1, Protocol 32 .

Vectors and Bacterial Strains

E. coli strain, transformed with recombinant plasmids, as culture
Use bacteria transformed by one of the methods described in Chapter 1, Protocol 23 , Chapter 1, Protocol 24 , Chapter 1, Protocol 25 and Chapter 1, Protocol 26

or

Amplified aliquot of cDNA library, grown as culture
Please see Chapter 11, Protocol 1.

METHOD

1. Number the dry filters with a soft-lead pencil or a ball-point pen, wet them with water, and interleave them between dry Whatman 3MM filters. Wrap the stack of filters loosely in aluminum foil, and sterilize them by autoclaving (15 psi [1.05 kg/cm^2] for 10 minutes on liquid cycle).
2. Use sterile, blunt-ended forceps to lay a sterile filter, numbered side down, on a 2-3-day-old LB (or SOB) agar plate containing the appropriate antibiotic. When the filter is thoroughly wet, peel it from the plate and replace it, numbered side up, on the surface of the agar.
3. Apply the bacteria, in a small volume of liquid, to the center of the filter on the surface of the agar plate. Use a sterile glass spreader to disperse the fluid evenly, leaving a border 2-3 mm wide around the circumference of the filter free of bacteria.
4. Incubate the plate (noninverted) with the lid ajar for a few minutes in a laminar flow hood to allow the inoculum to evaporate. Then close the lid, invert the plate, and incubate at 37°C until small colonies (0.1-0.2-mm diameter) appear (approx. 8-10 hours).
5. If desired, replica filters may be prepared at this stage (proceed with Step 6). Otherwise, prepare the bacterial colonies for storage at -20°C:
   a. Transfer the filter colony side up to a labeled LB (or SOB) agar plate containing the appropriate antibiotic and 25% glycerol.
   b. Incubate the plate for 2 hours at 37°C.
   c. Seal the plate well with Parafilm, and store it in an inverted position in a sealed plastic bag at -20°C.
6. Lay the master nitrocellulose or nylon filter colony side up on a sterile Whatman 3MM paper.
7. Number a damp, sterile nitrocellulose or nylon filter, and lay it on the master filter. Take care to prevent air bubbles from becoming trapped between the two filters.
8. Cover the filter sandwich with a second 3MM circle and place the bottom of a Petri dish on top of the 3MM paper. Press down firmly on the Petri dish with the palm of the hand to facilitate transfer of bacteria from the master filter to the replica.
9. Dismantle the Petri dish bottom and top 3MM paper, and orient the two filters by making a series of holes with an 18-gauge needle attached to a syringe.
10. Peel the filters apart. Lay the replica on a fresh LB (or SOB) agar plate containing the appropriate antibiotic.
11. Place the second replica filter (if made) and the master filter on a fresh LB (or SOB) agar plate containing the appropriate antibiotic and incubate all plates at 37°C until colonies appear (4-6 hours).
12. At this stage, when the bacteria are still growing rapidly, the filter may be transferred to an agar plate containing chloramphenicol (170-200 µg/ml) and incubated for 12 hours at 37°C.
13. Move the master nitrocellulose filter to a fresh LB (or SOB) agar plate containing the appropriate antibiotic and 25% glycerol. Then freeze it as described in Step 5.
14. Lyse the bacteria adhering to the replica filters and bind the liberated DNA to the nitrocellulose or nylon filter using the procedures described in Chapter 1, Protocol 31 . Proceed with hybridization as described in Chapter 1, Protocol 32 .

REFERENCES

1. Hanahan D. and Meselson M. 1980. Plasmid screening at high colony density. *Gene* 10:63-67.

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Protocol 31

Lysing Colonies and Binding of DNA to Filters

In this protocol, based on the procedure of Grunstein and Hogness (1975), alkali is used to liberate DNA from bacterial colonies on nitrocellulose or nylon filters. The DNA is then fixed to the filter by UV-cross-linking or baking under vacuum.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Denaturation solution
- Neutralizing solution
- ⚠ SDS (10% w/v)
- 2X SSPE

Vectors and Bacterial Strains

E. coli transformants immoblized on filters
Use transformants prepared by one of the methods described in Chapter 1, Protocol 28 , Chapter 1, Protocol 29 and Chapter 1, Protocol 30 .

METHOD

1. Cut four pieces of Whatman 3MM paper (or an equivalent) to an appropriate size and shape and fit them neatly onto the bottoms of four glass or plastic trays. Saturate each of the pieces of 3MM paper with one of the following solutions:
   10% SDS (optional)
   denaturizing solution
   neutralizing solution
   2x SSPE
2. Pour off any excess liquid and roll a 10-ml pipette along the sheet to smooth out any air bubbles that occur between the 3MM paper and the bottom of the container.
3. Use blunt-ended forceps to peel the nitrocellulose or nylon filters from their plates and place them colony side up on the SDS-impregnated 3MM paper for 3 minutes.
4. After the first filter has been exposed to the SDS solution for 3 minutes, transfer it to the second sheet of 3MM paper saturated with denaturing solution. Transfer the remainder of the filters in the same order in which they were removed from their agar plates. Expose each filter to the denaturing solution for 5 minutes.
5. Transfer the filters to the third sheet of 3MM paper, which has been saturated with neutralizing solution. Leave the filters for 5 minutes.
   Optional: Repeat this step once.
6. Transfer the filters to the last sheet of 3MM paper, which has been saturated with 2x SSPE. Leave the filters for 5 minutes.
7. Dry the filters using one of the methods below.
   If the DNA is to be fixed to the filters by baking: Lay the filters, colony side up, on a sheet of dry 3MM paper and allow them to dry at room temperature for at least 30 minutes.
   If the DNA is to be fixed to the filters by cross-linking with UV light: Lay the filters on a sheet of 2x SSPE-impregnated 3MM paper or on dry paper, depending on the manufacturer's recommendation.
8. Fix the DNA to the filters using one of the methods below.
   For baking: Sandwich the filters between two sheets of dry 3MM paper, and fix the DNA to the filters by baking for 1-2 hours at 80°C in a vacuum oven.
   For cross-linking with UV light: Follow the manufacturer's instructions for fixing DNA to filters using a commercial device for this purpose.
9. Hybridize the DNA immobilized on the filters to a labeled probe as described in Chapter 1, Protocol 32 .

REFERENCES

1. Grunstein M. and Hogness D.S. 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci.* 72:3961-3965.

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Protocol 32

Hybridization of Bacterial DNA on Filters

This protocol describes procedures to hybridize DNA from transformed colonies immobilized on filters with radiolabeled probes and to recover from a master plate the corresponding colonies that hybridize specifically to the probe. The method is based on the procedure published by Grunstein and Hogness (1975).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Formamide

Prehybridization/hybridization solution (for Plaque/Colony Lifts)

Prewashing solution (1-32)

Wash solution 1 (1-32)

Wash solution 2 (1-32)

Wash solution 3 (1-32)

Media

Rich medium containing the appropriate antibiotic

Nucleic Acids and Oligonucleotides

Filters with immobilized DNA from transformed colonies
Use filters prepared as described in Chapter 1, Protocol 31 .

Probes

^{32}P-labeled double-stranded DNA probe or Synthetic oligonucleotide probes

Additional Reagents

Step 15 of this protocol requires the reagents listed in Chapter 1, Protocol 1 or Chapter 1, Protocol 4 .

Step 15 may also require the reagents listed in Chapter 8, Protocol 12 .

METHOD

1. Float the baked or cross-linked filters on the surface of a tray of 2x SSC until they have become thoroughly wetted from beneath. Submerge the filters for 5 minutes.
2. Transfer the filters to a glass baking dish containing at least 200 ml of prewashing solution. Stack the filters on top of one another in the solution. Cover the dish with Saran Wrap and transfer it to a rotating platform in an incubator. Incubate the filters for 30 minutes at 50°C.
3. Gently scrape the bacterial debris from the surfaces of the filters using Kimwipes soaked in prewashing solution. This scraping ensures removal of colony debris and does not affect the intensity or sharpness of positive hybridization signals.
4. Transfer the filters to 150 ml of prehybridization solution in a glass baking dish. Incubate the filters with agitation for 1-2 hours or more at the appropriate temperature (i.e., 68°C when hybridization is to be carried out in aqueous solution; 42°C when hybridization is to be carried out in 50% formamide).
5. Denature ^{32}P-labeled double-stranded DNA by heating to 100°C for 5 minutes. Chill the probe rapidly in ice water. *Single-stranded probes need not be denatured.*
6. Add the probe to the prehybridization solution covering the filters. Incubate at the appropriate temperature until 1-3 $C_o t_{1/2}$ is achieved. During the hybridization, keep the containers holding the filters tightly closed to prevent the loss of fluid by evaporation.
   Use between 2 x 10^5 and 1 x 10^6 cpm of ^{32}P-labeled probe (specific activity approx. 5 x 10^7 cpm/µg) per milliliter of prehybridization solution. Using more probe will cause the background of nonspecific hybridization to increase, whereas using less will reduce the rate of hybridization.
   Hybridization mixtures containing radiolabeled single-stranded probes may be stored at 4°C for several days and reused without further treatment. In some cases, hybridization probes prepared from double-stranded DNA templates can be reused after freezing the solution, thawing, and boiling for 5 minutes in a chemical fume hood.
7. When the hybridization is complete, remove the hybridization solution and immediately immerse the filters in a large volume (300-500 ml) of Wash solution 1 at room temperature. Agitate the filters gently and turn them over at least once during washing. After 5 minutes, transfer the filters to a fresh batch of wash solution and continue to agitate them gently. Repeat the washing procedure twice more.
8. Wash the filters twice for 0.5-1.5 hours in 300-500 ml of Wash solution 2 at 68°C.
9. Dry the filters in the air at room temperature on 3MM paper. Streak the underside of the filters with a water-soluble glue stick and arrange the filters (numbered side up) on a clean, dry, flat sheet of 3MM paper. Press the filters firmly against the 3MM paper to ensure sticking.
10. Apply adhesive dot labels marked with either radioactive ink or chemiluminescent markers to several asymmetric locations on the 3MM paper. Cover the filters and labels with Saran Wrap. Use tape to secure the wrap to the back of the 3MM paper and stretch the wrap over the paper to remove wrinkles.
11. Analyze the filters by phosphorimaging or exposing them to X-ray film (Kodak XAR-2, XAR-5, or their equivalents) for 12-16 hours at -70°C with an intensifying screen.
12. Develop the film and align it with the filters using the marks left by the radioactive ink. Use a nonradioactive fiber-tip pen in a nonblack color to mark the film with the positions of the asymmetrically located dots on the numbered filters.
13. Tape a piece of clear Mylar or other firm transparent sheet to the film. Mark on the clear sheet the positions of positive hybridization signals. Also mark (in a different color) the positions of the asymmetrically located dots. Remove the clear sheet from the film. Identify the positive colonies by aligning the dots on the clear sheet with those on the agar plate.
14. Use a sterile toothpick or inoculating needle to transfer each positive bacterial colony into 1-2 ml of rich medium (e.g., LB, YT, or Terrific Broth) containing the appropriate antibiotic.
15. After a period of growth, plasmid DNA can be isolated from the culture by one of the minipreparation methods described in Chapter 1, Protocol 1 and Chapter 1, Protocol 4 and can be further analyzed by restriction endonuclease digestion or by PCR.

REFERENCES

1. Grunstein M. and Hogness D.S. 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci.* 72:3961-3965.

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Chapter 2 Bacteriophage λ and Its Vectors

Protocol 18: Partial Digestion of Eukaryotic DNA for Use in Genomic Libraries: Preparative Reactions

The results of the pilot experiment (Chapter 2, Protocol 17) are used to establish conditions for partial digestion of eukaryotic DNA in the large-scale reactions described here.

Protocol 19: Ligation of Bacteriophage λ Arms to Fragments of Foreign Genomic DNA

Pilot ligations and packaging reactions are used to establish the amounts of fragmented genomic DNA and bacteriophage λ arms that yield the maximum number of recombinants. Additional ligation and packaging reactions may then be set up to yield a comprehensive library of genomic DNA.

Protocol 20: Amplification of Genomic Libraries

Libraries of recombinant bacteriophages may be amplified by growing plate stocks directly from the packaging mixtures generated in Chapter 2, Protocol 18 .

Protocol 21: Transfer of Bacteriophage DNA from Plaques to Filters

This protocol and Chapter 2, Protocol 22 , which are based on a method described by Benton and Davis (1977), are used to identify and isolate recombinants containing DNA sequences of interest.

Protocol 22: Hybridization of Bacteriophage DNA on Filters

Using hybridization, it is possible to identify a single recombinant that carries the desired target sequence on a filter that carries the imprint of 15,000 or more plaques.

Protocol 23: Rapid Analysis of Bacteriophage λ Isolates: Purification of λ DNA from Plate Lysates

This protocol is used to purify small amounts of bacteriophage λ DNA that are suitable for use as substrates for restriction enzymes and templates for DNA and RNA polymerases.

Protocol 24: Rapid Analysis of Bacteriophage λ Isolates: Purification of λ DNA from Liquid Cultures

This protocol is used to purify small amounts of recombinant DNAs cloned in robust strains of bacteriophage λ such as λgt10, λgt11, λZAP, or ZipLox. The DNAs are suitable for use as substrates for restriction enzymes and templates for DNA and RNA polymerases.

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Protocol 1

Plating Bacteriophage λ

This protocol describes a method for generating isolated plaques from a stock of bacteriophage λ. Each plaque derives from infection of a single bacterium by a single bacteriophage particle. Because each plaque contains the progeny of a single virus particle, the bacteriophages derived from a single plaque are essentially genetically identical to one another.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- $MgSO_4$ (10 mM)
- SM
- SM plus gelatin

Media

- Rich λ medium
- Rich λ agar medium
  Freshly poured plates are too wet for use in plaque assays. To prevent running and smearing of plaques, store the plates for 2 days at room temperature before use. They can then be transferred to plastic sleeves and stored at 4°C. Plates stored at 4°C should be placed at room temperature for 1-2 hours before use in Step 10 below. Warming the plates in this way reduces problems of condensation and allows the top agar subsequently to spread across the entire surface of the plate before it sets. For further information, please see note to Step 12 on p. 2.30 in the print version of the manual.
- Rich λ top agarose (0.7%)
  Agarose is preferred to agar in this protocol in order to minimize contamination with inhibitors that can interfere with subsequent enzymatic analysis of bacteriophage DNA.

Vectors and Bacterial Strains

Bacteriophage λ stocks

E. coli strain
Use strains of E. coli *that have been appropriately designed to support the growth of the vector.*

METHOD

1. Inoculate rich λ medium (50 ml of NZCYM or LB in a 250-ml conical flask) with a single bacterial colony of the appropriate *E. coli* strain. Grow the culture overnight at 37°C with moderate agitation.
2. Centrifuge the cells at 4000*g* (5800 rpm in a Sorvall SS-34 rotor) for 10 minutes at room temperature.
3. Discard the supernatant, and resuspend the cell pellet in 20 ml of 10 mM $MgSO_4$. Measure the OD_{600} of a 1/100 dilution of the resuspended cells and dilute the cells to a final concentration of 2.0 OD_{600} with 10 mM $MgSO_4$.
4. Store the suspension of plating bacteria at 4°C.
5. Melt top agar or agarose by heating it in a microwave oven for a short period of time. Store aliquots of the melted agar or agarose (3 ml for 100-mm plates, 7 ml for 200-mm plates) on a heating block or in a water bath at 47°C to keep the solution molten.
6. Prepare tenfold serial dilutions of the bacteriophage stocks (in SM plus gelatin). Mix each dilution by *gentle* vortexing or by tapping on the side of the tube.
7. Dispense 0.1 ml of plating bacteria from Step 4 into a series of sterile tubes (13 or 17 x 100 mm).
8. Add 0.1 ml of each dilution of bacteriophage stock to a tube of plating bacteria. Mix the bacteria and bacteriophages by shaking or gently vortexing.
9. Incubate the mixture for 20 minutes at 37°C to allow the bacteriophage particles to adsorb to the bacteria. Remove the tubes from the water bath and allow them to cool to room temperature.
10. Add an aliquot of molten agar or agarose to the first tube. Mix the contents of the tube by gentle tapping or vortexing for five seconds and, *without delay*, pour the entire contents of the tube onto the center of a labeled agar plate. Try to avoid creating air bubbles. Swirl the plate gently to ensure an even distribution of bacteria and top agarose. Repeat the procedure until the contents of all the tubes have been transferred to separate labeled plates.
11. Replace the lids on the plates. Allow the top agar/agarose to harden by standing the plates for 5 minutes at room temperature. Invert the closed plates and incubate them at 37°C.
    With some E. coli *strains and bacteriophage vectors, better plaques are formed when the plates are incubated at temperatures other than 37°C. For example, when using the Stratagene* E. coli *strains SRB℘ and SRB(P2)℘ as hosts, an incubation temperature of 39°C is recommended. In addition, λgt10 vectors produce better plaques on* E. coli hfl *strains when incubated at 39°C.*
12. Continue incubating the plates overnight, then count or select (pick) individual plaques.

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Protocol 2

Picking Bacteriophage λ Plaques

This protocol describes a general method to pick and store plaques.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

SM

Vectors and Bacterial Strains

Bacteriophage λ, grown as well-isolated plaques on bacterial lawn (please see Chapter 2, Protocol 1)

METHOD

1. Place 1 ml of SM in a sterile microfuge tube or polypropylene test tube. Add 1 drop (approx. 50 µl) of chloroform.
2. Use a borosilicate Pasteur pipette equipped with a rubber bulb, or a micropipette, to stab through the chosen plaque of bacteriophage λ into the hard agar beneath. Apply mild suction so that the plaque, together with the underlying agar, is drawn into the pipette.
3. Wash out the fragments of agar from the borosilicate Pasteur pipette into the tube containing SM/chloroform (prepared in Step 1). Let the capped tube stand for 1-2 hours at room temperature to allow the bacteriophage particles to diffuse from the agar. To assist the elution of the virus, rock the tube gently on a rocking platform. Store the bacteriophage suspension at 4°C.
   An average bacteriophage plaque yields approx. 10^6 infectious bacteriophage particles, which can be stored indefinitely at 4°C in SM/chloroform without loss of viability. The virus recovered from a plaque can be used as described in Chapter 2, Protocol 3 and Chapter 2, Protocol 4 to prepare larger stocks of bacteriophages by the plate lysis or the liquid culture methods.

REFERENCES

1. Crowe J.S., Cooper H.J., Smith M.A., Sims M.J., Parker D., and Gewert D. 1991. Improved cloning efficiency of polymerase chain reaction (PCR) products after proteinase K digestion. *Nucleic Acids Res.* 19:184.
2. Wybranietz W.A. and Lauer U. 1998. Distinct combination of purification methods dramatically improves cohesive-end subcloning of PCR products. *BioTechniques* 24:578-580.

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Protocol 3

Preparing Stocks of Bacteriophage λ by Plate Lysis and Elution

A reliable method to prepare plate lysates and to recover infectious bacteriophages by elution from the top agar.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

SM

Media

Rich λ agar medium
Freshly poured plates (10-cm or 15-cm diameter) that have been equilibrated to room temperature give the best results in these methods. The older the plates, the lower the titer of the resulting plate stock.

Rich λ top agarose (0.7%)
Agarose is preferred to agar in this protocol in order to minimize contamination with inhibitors that can interfere with subsequent enzymatic analysis of bacteriophage DNA.

Additional Reagents

Step 9 of this protocol requires the reagents listed in Chapter 2, Protocol 1.

Vectors and Bacterial Strains

Bacteriophage λ stock
Prepared as described in Chapter 2, Protocol 2.

E. coli plating bacteria
Prepared as described in Chapter 2, Protocol 1.

METHOD

1. Prepare infected cultures for plating:
   For a 10-cm diameter Petri dish: Mix 10^5 pfu of bacteriophage (usually approx. 1/10 of a resuspended individual plaque or 1/100 of a macroplaque with 0.1 ml of plating bacteria).
   For a 15-cm Petri dish: Mix 2 x 10^5 pfu with 0.2 ml of plating cells.
   Always set up at least one control tube containing uninfected cells. Incubate the infected and control cultures for 20 minutes at 37°C to allow the virus to attach to the cells.
   When preparing stocks of isolated bacteriophage λ that grow poorly, increase the inoculum to 10^6 pfu per 0.1 ml of plating bacteria.
2. Add 3 ml of molten top agarose (47°C) (10-cm plate) or 7.0 ml of molten top agarose (47°C) (15-cm plate) to the first tube of infected cells. Mix the contents of the tube by gentle tapping or vortexing for a few seconds, and, *without delay*, pour the entire contents of the tube onto the center of a labeled agar plate. Try to avoid creating air bubbles. Swirl the plate gently to ensure an even distribution of bacteria and top agarose. Repeat this step until the contents of each of the tubes have been transferred onto separate plates.
3. Incubate the plates *without inversion* for approx. 12-16 hours at 37°C.
4. Remove the plates from the incubator and add SM (5 ml to each 10-cm plate or 10 ml to each 15-cm plate). Store the plates for several hours at 4°C on a shaking platform.
5. Using a separate Pasteur pipette for each plate, transfer as much of the SM as possible into sterile screw- or snap-cap polypropylene tubes.
6. Add 1 ml of fresh SM to each plate, swirl the fluid gently, and store the plates for 15 minutes in a tilted position to allow all of the fluid to drain into one area. Again remove the SM and combine it with the first harvest. Discard the plate.
7. Add 0.1 ml of chloroform to each of the tubes containing SM, vortex the tubes briefly, and then remove the bacterial debris by centrifugation at 4000*g* (5800 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C.
8. Transfer the supernatants to fresh polypropylene tubes, and add 1 drop of chloroform to each tube. Store the resulting bacteriophage plate stocks at 4°C.
9. Measure the concentration of infectious virus particles in each stock by plaque assay as described in Chapter 2, Protocol 1.
   The titer of plate stocks should be approx. 10^9 to 10^{10} pfu/ml and should remain stable as long as the stock is stored properly.

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Protocol 4

Preparing Stocks of Bacteriophage λ by Small-scale Liquid Culture

High-titer stocks of bacteriophage λ are easily prepared by infecting small-scale bacterial cultures.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

SM

Media

Rich λ medium prewarmed to 37°C

Vectors and Bacterial Strains

Bacteriophage λ stock
Prepared as described in Chapter 2, Protocol 2 .

METHOD

1. Inoculate a single colony of an appropriate *E. coli* strain into 5 ml of rich λ medium in a sterile polypropylene culture tube. Incubate the culture overnight with vigorous shaking at 30°C.
2. Transfer 0.1 ml of the fresh overnight bacterial culture (prepared in Step 1) to a sterile 17 x 100-mm polypropylene culture tube with a loose-fitting cap. Infect the culture with approx. 10^6 pfu of bacteriophage λ in 50-100 µl of SM.
3. Incubate the infected culture for 20 minutes at 37°C to allow the bacteriophage particles to adsorb to the bacteria.
4. Add 4 ml of rich λ medium, prewarmed to 37°C, and incubate the culture with vigorous agitation until lysis occurs (usually 8-12 hours at 37°C).
5. After lysis has occurred, add 2 drops (approx. 100 µl) of chloroform and continue incubation for 15 minutes at 37°C.
6. Centrifuge the culture at 4000*g* (5800 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C.
7. Recover the supernatant, add 1 drop (approx. 50 µl) of chloroform, and store the virus stock.

REFERENCES

1. Leder P., Tiemeier D., and Enquist L. 1977. EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms: The λ gt*WES* system. *Science* 196:175-177.

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Protocol 5

Large-scale Growth of Bacteriophage λ: Infection at Low Multiplicity

After infection with bacteriophage λ at low multiplicity, a bacterial culture is transferred to a large volume of medium and incubated until complete lysis of the host cells occurs.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

SM

Media

NZCYM

Vectors and Bacterial Strains

High-titer stock of bacteriophage λ

Prepared as described in Chapter 2, Protocol 3 or Chapter 2, Protocol 4. The titer should be 10^9 to 10^{10} pfu/ml.

METHOD

1. Inoculate 100 ml of NZCYM in a 500-ml conical flask with a single colony of an appropriate bacterial host. Incubate the culture overnight at 37°C with vigorous agitation.
   For a starter culture, prepare 100 ml of NZCYM in a 500-ml conical flask. For subsequent large-scale culture, prepare 4 x 500-ml aliquots of NZCYM in 2-liter flasks, prewarmed to 37°C. Four additional 500-ml aliquots may be needed for Step 9.
2. Measure the OD_{600} of the culture. Calculate the cell concentration assuming that 1 OD_{600} = 1 x 10^9 cells/ml.
3. Withdraw four aliquots, each containing 10^{10} cells. Centrifuge each aliquot at 4000*g* (5800 rpm in a Sorvall SS-34 rotor) for 10 minutes at room temperature. Discard the supernatants.
4. Resuspend each bacterial pellet in 3 ml of SM.
5. Add the appropriate number of infectious bacteriophage particles and swirl the culture to ensure that the inoculum is dispersed rapidly throughout the culture.
   The number of bacteriophage particles used is crucial. For strains of bacteriophage λ that grow well (e.g., EMBL3 and 4 and λgt10), add 5 x 10^7 pfu to each suspension of 10^{10} cells; for bacteriophages that grow relatively poorly (e.g., the Charon series), it is better to increase the starting inoculum to 5 x 10^8 pfu.
6. Incubate the infected cultures for 20 minutes at 37°C with intermittent swirling.
7. Add each infected aliquot to 500 ml of NZCYM, prewarmed to 37°C in 2-liter flasks. Incubate the cultures at 37°C with vigorous shaking (300 cycles/minute in a rotary shaker).
8. Begin to monitor the cultures for lysis after 8 hours. Concomitant growth of bacteria and bacteriophages should occur, resulting in lysis of the culture after 8-12 hours. If lysis is observed, proceed to Step 10.
9. If lysis is not apparent after 12 hours, check a small sample of the cultures for evidence of bacteriophage growth.
   a. Transfer two aliquots (1 ml each) of the infected culture into glass tubes.
   b. Add 1 or 2 drops of chloroform (approx. 50-100 µl) to one of the tubes, and incubate both tubes for 5-10 minutes at 37°C with intermittent shaking.
   c. Compare the appearance of the two cultures by holding the tubes up to a light. If infection is near completion but the cells have not yet lysed, the chloroform will cause the cells to burst and the turbid culture will clear to the point where it is translucent. In this case, proceed to Step 10.
10. Add 10 ml of chloroform to each flask, and continue the incubation for a further 10 minutes at 37°C with shaking.
11. Cool the cultures to room temperature and proceed to precipitate the bacteriophage particles as described in Chapter 2, Protocol 6 .

REFERENCES

1. Blattner F.R., Williams B.G., Blechl A.E., Denniston-Thompson K., Faber H.E., Furlong L.-A., Grunwald D.J., Kiefer D.O., Moore D.D., Schumm J.W., Sheldon E.L., and Smithies O. 1977. Charon phages: Safer derivatives of bacteriophage lambda for DNA cloning. *Science* 196:161-169.

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Protocol 6

Precipitation of Bacteriophage λ Particles from Large-scale Lysates

Bacteriophage λ particles are recovered from bacterial lysates by precipitation with polyethylene glycol at high ionic strength.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

NaCl (solid)

⚠ PEG 8000
Use approx. 50 g for each 500 ml of culture.

SM

Enzymes and Buffers

Pancreatic DNase I (1 mg/ml)

Pancreatic RNase (1 mg/ml) in TE (pH 7.6)

Vectors and Bacterial Strains

E. coli culture, infected with bacteriophage λ and lysed
Prepared as described in Chapter 2, Protocol 5.

METHOD

1. Cool the lysed cultures containing bacteriophage λ to room temperature. Add pancreatic DNase I and RNase, each to a final concentration of 1 µg/ml. Incubate the lysed cultures for 30 minutes at room temperature.
2. To each 500-ml culture, add 29.2 g of solid NaCl (final concentration, 1 M). Swirl the cultures until the salt has dissolved. Store the cultures for 1 hour on ice.
3. Remove debris by centrifugation at 11,000*g* (8300 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C. Combine the supernatants from the four cultures into a clean 2-liter graduated cylinder.
4. Measure the volume of the pooled supernatants and then transfer the preparation to a clean 2-liter flask. Add solid PEG 8000 to a final concentration of 10% w/v (i.e., 50 g per 500 ml of supernatant). Dissolve the PEG by slow stirring on a magnetic stirrer at room temperature.
5. Transfer the solution to polypropylene centrifuge bottles, cool the bacteriophage/PEG solution in ice water, and store the centrifuge bottles for at least 1 hour on ice to allow the bacteriophage particles to precipitate.
6. Recover the precipitated bacteriophage particles by centrifugation at 11,000*g* (8300 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C. Discard the supernatants, and stand the inverted centrifuge bottles in a tilted position for 5 minutes to allow the remaining fluid to drain away from the pellet. Remove any residual fluid with a pipette.
7. Use a wide-bore pipette equipped with a rubber bulb to resuspend the bacteriophage pellet gently in SM (8 ml for each 500 ml of supernatant from Step 3). Place the centrifuge bottles on their sides at room temperature for 1 hour so that the SM covers and soaks the pellets.
8. Extract the PEG and cell debris from the bacteriophage suspension by adding an equal volume of chloroform. Vortex the mixture gently for 30 seconds. Separate the organic and aqueous phases by centrifugation at 3000*g* (4300 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C. Recover the aqueous phase, which contains the bacteriophage particles.

REFERENCES

1. Yamamoto K.R., Alberts B.M., Benzinger R., Lawhorne L., and Treiber G. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* 40:734-744.

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Protocol 7

Assaying the DNA Content of Bacteriophage λ Stocks and Lysates by Gel Electrophoresis

The DNA content of bacteriophage λ stocks is easily and rapidly estimated.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 2.5x SDS-EDTA dye mix

Enzymes and Buffers

- DNase I dilution buffer
- Pancreatic DNase I (1 mg/ml)

Nucleic Acids and Oligonucleotides

Bacteriophage λ DNA (control DNA)

Vectors and Bacterial Strains

Bacteriophage λ lysates or stocks
Prepared by using one of the methods described in Chapter 2, Protocol 3 and Chapter 2, Protocol 4 (for small-scale lysates) or Chapter 2, Protocol 5 (for large-scale lysates).

METHOD

1. Make a working solution of pancreatic DNase I (1 µg/ml) as follows: Dilute 1 µl of the stock solution of DNase I with 1 ml of ice-cold DNase I dilution buffer.
2. Mix the solution by gently inverting the closed tube several times. Take care to avoid bubbles and foam. Store the solution in ice until needed. Discard the working solution after use.
3. Transfer 10 µl of crude bacteriophage lysate or stock to a microfuge tube. Add 1 µl of the working solution of pancreatic DNase and incubate the mixture for 30 minutes at 37°C.
4. Add 4 µl of 2.5x SDS-EDTA dye mixture and incubate the closed tube for 5 minutes at 65°C.
5. Load the sample onto an 0.7% agarose gel containing 0.5 µg/ml ethidium bromide.
   As controls, use samples containing 5, 25, and 100 ng of bacteriophage λ DNA.
6. Perform electrophoresis at <5 V/cm until the bromophenol blue has migrated 3-4 cm.
7. Examine the gel under UV illumination. Use the intensity of fluorescence of the DNA standards as a guide to estimate the amount of bacteriophage λ DNA in the test sample.
   A high-titer lysate (10 µl) should contain between 10 ng and 50 ng of bacteriophage DNA.

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Protocol 8

Purification of Bacteriophage λ Particles by Isopycnic Centrifugation through CsCl Gradients

Isopycnic centrifugation through CsCl gradients is used to prepare infectious bacteriophage λ particles of the highest purity that are essentially free of contaminating bacterial nucleic acids.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

CsCl (solid)

Ethanol

SM

Vectors and Bacterial Strains

Suspension of bacteriophage λ particles
Prepared as described in Chapter 2, Protocol 6.

METHOD

1. Use a high quality (molecular biology grade) of solid CsCl to prepare three solutions of different densities by adding solid CsCl to SM, as indicated below. Store the solutions at room temperature.
   CsCl Solutions Prepared in SM (100 ml) for Step Gradients

Density ρ (g/ml)	CsCl (g)	SM (ml)	Refractive Index η
1.45	60	85	1.3768
1.50	67	82	1.3815
1.70	95	75	1.3990

   Measure the volume of the bacteriophage suspension, and add 0.5 g of solid CsCl per ml of bacteriophage suspension. Place the suspension on a rocking platform until the CsCl is completely dissolved.
2. Pour enough CsCl step gradients to fractionate the bacteriophage suspension. Each gradient can accommodate approx. 16 ml of bacteriophage suspension. The number of step gradients required equals the final aqueous volume (Step 1) divided by 0.4 x tube volume. Use clear plastic centrifuge tubes (e.g., Beckman Ultra-Clear tubes) that fit the Beckman SW41 or SW28 rotor (or equivalent).
   The step gradients may be made either by carefully layering three CsCl solutions of decreasing density on top of one another or by layering solutions of increasing density under one another.
3. Make a mark with a permanent felt-tipped marker pen on the outside of the tube opposite the position of the interface between the ρ = 1.50 g/ml layer and the ρ = 1.45 g/ml layer.
   The CsCl step ingredients should occupy approx. 60% of the volume of the ultracentrifuge tube. For example, in a Beckman SW28 tube (or equivalent), which hold 38 ml, the step gradients consist of 7.6 ml of each of the three CsCl solutions. Balance tubes should be poured with the same CsCl density solutions.
4. Carefully layer the bacteriophage suspension over the step gradients. Centrifuge the gradients at 87,000*g* (22,000 rpm in a Beckman SW28 rotor) for 2 hours at 4°C.
5. Collect the bacteriophage particles by puncturing the side of the tube as follows.
   a. Carefully wipe the outside of the tube with ethanol to remove any grease or oil, and then attach a piece of Scotch Tape to the outside of the tube, level with the band of bacteriophage particles.
   b. Use a 21-gauge hypodermic needle (no syringe-barrel required) to puncture the tube through the tape and collect the band of bacteriophage particles.
6. Place the suspension of bacteriophage particles in an ultracentrifuge tube that fits a Beckman Ti50 or SW50.1 rotor (or equivalent) and fill the tube with CsCl solution (ρ = 1.5 g/ml in SM). Centrifuge at 150,000*g* (41,000 rpm in a Beckman Ti50 rotor) for 24 hours at 4°C or at 160,000*g* (36,000 rpm in a Beckman SW50.1 rotor) for 24 hours at 4°C.
7. Collect the band of bacteriophage particles as described in Step 5. Store the bacteriophage suspension at 4°C in the CsCl solution in a tightly capped tube.
8. (*Optional*) If necessary, the bacteriophage particles can be further purified and concentrated by a second round of equilibrium centrifugation in CsCl. Transfer the bacteriophage suspension to one or more ultracentrifuge tubes that fit a Beckman SW50.1 rotor (or equivalent). Fill the tubes with a solution of CsCl in SM (ρ = 1.5) and centrifuge the tubes at 160,000*g* (36,000 rpm in a Beckman SW50.1 rotor)) for 24 hours at 4°C. When centrifugation is complete, collect the bacteriophage particles.

REFERENCES

1. Yamamoto K.R., Alberts B.M., Benzinger R., Lawhorne L., and Treiber G. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* 40:734-744.

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Protocol 9

Purification of Bacteriophage λ Particles by Centrifugation through a Glycerol Step Gradient

DNA extracted from bacteriophage λ particles purified by pelleting/centrifugation through glycerol step gradients can be used for subcloning or for preparing bacteriophage λ arms.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- EDTA (0.5 M, pH 8.0)
- Glycerol (5% and 40% v/v) in SM
- SM

Enzymes and Buffers

- Pancreatic DNase I (1 mg/ml)
- Pancreatic RNase in TE (1 mg/ml, pH 7.6)

Vectors and Bacterial Strains

Suspension of bacteriophage λ particles
Prepared as described in Chapter 2, Protocol 6.

METHOD

1. Prepare a glycerol step gradient in a Beckman SW41 polycarbonate tube (or its equivalent; one tube is needed for each 5 ml of bacteriophage suspension):
   a. Pipette 3 ml of a solution consisting of 40% glycerol in SM into the bottom of the tube.
   b. Carefully layer 4 ml of a solution consisting of 5% glycerol in SM on top of the 40% glycerol solution.
   c. Carefully layer the bacteriophage suspension on top of the 5% glycerol layer. Fill the tube with SM.
2. Centrifuge the step gradient at 151,000*g* (35,000 rpm in a Beckman SW41 or SW28 rotor) for 60 minutes at 4°C.
3. Discard the supernatant, and resuspend the bacteriophage pellet in 1 ml of SM per liter of original culture.
4. Add pancreatic DNase I and RNase to final concentrations of 5 µg/ml and 1 µg/ml, respectively. Incubate the reaction mixture for 30 minutes at 37°C.
5. Add EDTA from a 0.5 M stock solution (pH 8.0) to a final concentration of 20 mM.

REFERENCES

1. Vande Woude G.F., Oskarsson M., Enquist L.W., Nomura S., Sullivan M., and Fischinger P.J. 1979. Cloning of integrated Moloney sarcoma proviral DNA sequences in bacteriophage lambda. *Proc. Natl. Acad. Sci.* 76:4464-4468.

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Protocol 10

Purification of Bacteriophage λ Particles by Pelleting/Centrifugation

This protocol is used to prepare bacteriophage λ particles that yield DNA suitable for subcloning or for preparing bacteriophage λ arms.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

SM

Vectors and Bacterial Strains

Suspension of bacteriophage λ particles
Prepared as described in Chapter 2, Protocol 6.

METHOD

1. Transfer the bacteriophage suspension into a tube for use in a Beckman SW28 rotor (or equivalent).
2. Collect the bacteriophage particles by centrifugation at 110,000*g* (25,000 rpm in a Beckman SW28 rotor) for 2 hours at 4°C.
3. Carefully pour off and discard the supernatant.
4. Add 1-2 ml of SM to the pellet, and store it overnight at 4°C, preferably on a slowly rocking platform.
5. The following morning, pipette the solution gently up and down to ensure that all of the bacteriophage particles have been resuspended.

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Protocol 11

Extraction of Bacteriophage λ DNA from Large-scale Cultures Using Proteinase K and SDS

DNA is best isolated from bacteriophage λ particles by digesting the viral coat proteins with a powerful protease such as proteinase K, followed by extraction with phenol:chloroform. This procedure is easily adapted for use with smaller-scale preparations of bacteriophage λ.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform
EDTA (0.5 M, pH 8.0)
Ethanol
λ Dialysis buffer
⚠ Phenol
⚠ Phenol:chloroform (1:1, v/v)
⚠ SDS (10% w/v)
Sodium acetate (3 M, pH 7.0)
TE (pH 7.6 and pH 8.0)

Enzymes and Buffers

Proteinase K
Restriction endonucleases

Vectors and Bacterial Strains

Bacteriophage λ particles in CsCl suspension
Purify as described in Chapter 2, Protocol 8 , Chapter 2, Protocol 9 , or Chapter 2, Protocol 10 .

METHOD

1. Transfer the prepared bacteriophage suspension to a section of dialysis tubing sealed at one end with a knot or a plastic closure. Close the other end of the dialysis tube. Place the sealed tube in a flask containing a 1000-fold volume excess of dialysis buffer and a magnetic stir bar. Dialyze the bacteriophage suspension for 1 hour at room temperature with slow stirring.
2. Transfer the dialysis tube to a fresh flask of buffer and dialyze the bacteriophage suspension for an additional hour.
3. Transfer the bacteriophage suspension into a polypropylene centrifuge tube.
4. To the dialyzed bacteriophage suspension, add 0.5 M EDTA (pH 8.0) to a final concentration of 20 mM.
5. To the suspension, add proteinase K to a final concentration of 50 µg/ml.
6. Add SDS to a final concentration of 0.5%, and mix the solution by gently inverting the tube several times.
7. Incubate the digestion mixture for 1 hour at 56°C and then cool the mixture to room temperature.
8. Add an equal volume of equilibrated phenol to the digestion mixture, and mix the organic and aqueous phases by gently inverting the tube several times until a complete emulsion has formed.
9. Separate the phases by centrifugation at 3000*g* (5000 rpm in a Sorvall SS-34 rotor) for 5 minutes at room temperature. Use a wide-bore pipette to transfer the aqueous phase to a clean tube.
10. Extract the aqueous phase once with a 1:1 mixture of equilibrated phenol and chloroform.
11. Recover the aqueous phase as described above (Step 9), and repeat the extraction with an equal volume of chloroform. For large-scale preparations, proceed to Step 12. For smaller-scale quantities (bacteriophage from 50- to 100-ml cultures):
    a. Recover the bacteriophage DNA by standard ethanol precipitation.
    b. Store the solution for 30 minutes at room temperature.
    c. Redissolve the DNA in an appropriate volume of TE (pH 7.6), and proceed to Step 14.
12. Transfer the aqueous phase to a dialysis sac.
13. Dialyze the preparation of bacteriophage DNA overnight at 4°C against three changes of a 1000-fold volume of TE (pH 8.0).
14. Measure the absorbance of the solution at 260 nm and calculate the concentration of the DNA.
    1 OD_{260} = 50 µg/ml of double-stranded DNA. A single particle of bacteriophage contains approx. 5 x 10^{-11} µg of DNA. The yield of bacteriophage DNA usually ranges from 500 µg to several mg per liter, depending on the titer of the bacteriophage in the lysed culture.
15. Check the integrity of the DNA by analyzing aliquots (0.5 µg) that are undigested or have been cleaved by appropriate restriction enzyme(s). Analyze the DNAs by electrophoresis through a 0.7% agarose gel, using markers of an appropriate size.
16. Store the stock of bacteriophage DNA at 4°C.

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Protocol 12

Extraction of Bacteriophage λ DNA from Large-scale Cultures Using Formamide

Formamide can be used instead of proteinase K to dissociate bacteriophage λ coat proteins from the viral λ. The procedure is rapid and works best with large-scale preparations of bacteriophage λ.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- EDTA (0.5 M, pH 8.0)

Ethanol

⚠ Formamide, deionized

- NaCl (5 M)
- TE (pH 8.0)
- Tris-Cl (2 M, pH 8.5)

Enzymes and Buffers

Restriction endonucleases

Vectors and Bacterial Strains

Bacteriophage λ particles
Purify as described in Chapter 2, Protocol 9 or Chapter 2, Protocol 10 .

METHOD

1. If necessary, remove CsCl from the preparation of bacteriophage particles as described in Steps 1-4 of Chapter 2, Protocol 11 .
2. Measure the volume of the preparation of bacteriophage particles.
3. Add 0.1 volume of 2 M Tris (pH 8.5), 0.05 volume of 0.5 M EDTA (pH 8.0), and 1 volume of deionized formamide. Incubate the solution for 30 minutes at 37°C.
4. Precipitate the bacteriophage λ DNA by adding 1 volume (equal to the final volume in Step 3) of H_2O and 6 volumes (each equal to the final volume in Step 3) of ethanol.
5. Hook the precipitate of bacteriophage λ DNA onto the end of a sealed borosilicate Pasteur pipette or Shepherd's crook and transfer it to microfuge tube containing 70% ethanol.
6. Collect the DNA pellet by brief centrifugation (10 seconds) in a microfuge.
7. Discard the supernatant and store the open tube on the bench for a few minutes to allow the ethanol to evaporate. Redissolve the damp pellet of DNA in 300 µl of TE by tapping on the side of the tube. Try to avoid vortexing.
8. Reprecipitate the DNA by adding 6 µl of 5 M NaCl and 750 µl of ethanol. Collect the precipitated DNA and redissolve it as described in Steps 6 and 7.
9. Check the integrity of the DNA by analyzing aliquots (0.5 µg) that are undigested or have been cleaved by appropriate restriction enzyme(s). Analyze the DNAs by electrophoresis through a 0.7% agarose gel using markers of an appropriate size.
10. Store the stock of bacteriophage DNA at 4°C.

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Protocol 13

Preparation of Bacteriophage λ DNA Cleaved with a Single Restriction Enzyme for Use as a Cloning Vector

Bacteriophage λ vectors that allow genetic selection of recombinant bacteriophages (e.g., the EMBL series, λ2001, λDASH, λZAP, and λgt10) can be prepared for cloning by simple digestion by one or more restriction enzymes.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)
Omit ATP from Step 7 if the ligation buffer contains ATP.

⚠ Chloroform

EDTA (0.5 M, pH 8.0)

Ethanol

⚠ Phenol:chloroform (1:1, v/v)

Sodium acetate (3 M, pH 7.0)

Gel-loading buffer IV

TE (pH 7.6 and pH 8.0)

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Restriction endonucleases with appropriate buffers

Nucleic Acids and Oligonucleotides

Bacteriophage λ DNA
Prepared as described in Chapter 2, Protocol 11 or Chapter 2, Protocol 12 .

Additional Reagents

Step 7 (part e) of this protocol requires the reagents listed in Chapter 2, Protocol 1 .

Vectors and Bacterial Strains

Bacteriophage λ packaging mixtures

METHOD

1. Mix 25-50 µg of bacteriophage λ DNA with TE (pH 8.0) to give a final volume of 170 µl.
2. Add 20 µl of the appropriate 10x restriction enzyme buffer. Remove two aliquots, each containing 0.2 µg of undigested bacteriophage λ DNA. Store the aliquots of undigested DNA on ice.
3. Add a threefold excess (75-150 units) of the appropriate restriction enzyme and incubate the digestion mixture for 1 hour at the temperature recommended by the manufacturer.
4. Cool the reaction to 0°C on ice. Remove another aliquot (0.2 µg). Incubate this aliquot and one of the two aliquots of undigested DNA (Step 2 above) for 10 minutes at 68°C to disrupt the cohesive termini of the bacteriophage DNA. Add a small amount (10 µl) of sucrose gel-loading buffer and immediately load the samples onto an 0.7% agarose gel.
   This step is not as easy as it sounds. The left and right arms of bacteriophage λ DNA carry complementary termini 12 bases in length that can reanneal with one another. The resulting hydrogen-bonded DNA species can be easily confused with uncleaved bacteriophage λ DNA. For this reason, it is important to load and run the gel immediately after the DNA samples have been removed from the 68°C water bath.
   If digestion is incomplete, warm the reaction to the appropriate temperature, add more restriction enzyme (50-100 units), and continue the incubation at the optimal temperature recommended by the manufacturer.
5. When digestion is complete, add 0.5 M EDTA (pH 8.0) to a final concentration of 5 mM, and extract the digestion mixture once with phenol:chloroform and once with chloroform.
6. Recover the DNA from the aqueous phase by ethanol precipitation in the presence of 0.3 M sodium acetate (pH 7.0). Collect the precipitate by centrifuging at maximum speed for 2 minutes at 4°C in a microfuge. Wash the pellet in 70% ethanol and redissolve the DNA in 100 µl of TE (pH 7.6). Determine the concentration by measuring absorbance at 260 nm.
7. Remove an aliquot of DNA (0.5 µg), and test for its ability to be ligated as follows:
   a. Adjust the volume of the DNA solution to 17 µl with H_2O.
   b. Add 2 µl of 10x ligation buffer and, if necessary, 1 µl of 10 mM ATP.
   c. Remove 5 µl of the mixture prepared in Step b and store on ice.
   d. Add 0.2-0.5 Weiss unit of bacteriophage T4 DNA ligase to the remainder of the mixture (Step b), and incubate the reaction for 2 hours at 16°C.
   e. Use a commercially available bacteriophage λ packaging reaction to package 0.1 µg of the ligated and unligated samples and 0.1 µg of the undigested vector DNA from Step 2. Determine the titer (pfu/ml) of each packaged reaction as described in Chapter 2, Protocol 1 .

   The packaging efficiency of the digested vector should increase by nearly three orders of magnitude after ligation. The packaging efficiency of the ligated sample should be approx. 10% of that of undigested vector DNA.

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Protocol 14

Preparation of Bacteriophage λ DNA Cleaved with Two Restriction Enzymes for Use as a Cloning Vector

Many replacement vectors (e.g., the EMBL series, λ2001, and λDASH) contain a series of restriction sites, arranged in opposite orientations, at each end of the central stuffer fragment. Digestion of these vectors with two different restriction enzymes yields left and right arms, a stuffer fragment, and short segments of the polycloning sites. These can easily be removed from the arms by differential precipitation with isopropanol or spun-column chromatography.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)
⚠ Chloroform
Ethanol
⚠ Phenol:chloroform (1:1, v/v)
Sodium acetate (3 M, pH 5.2)
Gel-loading buffer IV
TE (pH 7.6 and pH 8.0)

Enzymes and Buffers

Bacteriophage T4 DNA ligase
Restriction endonucleases

Nucleic Acids and Oligonucleotides

Bacteriophage λ (replacement vector) DNA
Prepared as described in Chapter 2, Protocol 11 or Chapter 2, Protocol 12.

Additional Reagents

Step 10 (part e) of this protocol requires the reagents listed in Chapter 2, Protocol 1 .

Vectors and Bacterial Strains

Bacteriophage λ packaging mixtures

METHOD

1. Mix 25-50 µg of bacteriophage λ DNA purified from a replacement vector with TE (pH 8.0) to give a final volume of 170 µl.
2. Add 20 µl of one of the two appropriate 10x restriction enzyme buffers. Remove two aliquots, each containing 0.2 µg of undigested bacteriophage λ DNA to serve as controls. Store the aliquots of undigested DNA on ice.
3. Add a fourfold excess (100-200 units) of one of the two appropriate restriction enzymes and incubate the digestion mixture for 4 hours at the temperature recommended by the manufacturer.
4. Cool the reaction to 0°C on ice. Remove two aliquots (0.2 µg). Incubate one of these aliquots (save the other for analysis in Step 10 below) and one of the two aliquots of undigested DNA (Step 2 above) for 10 minutes at 68°C to disrupt the cohesive termini of the bacteriophage DNA. Add a small amount (approx. 10 µl) of sucrose gel-loading buffer and and *immediately* electrophorese the samples through an 0.7% agarose gel.
   If the restriction enzyme digestion is complete, no DNA will migrate at the position of the undigested control bands. Instead, three or more (depending on the number of cleavage sites in the vector) smaller DNA fragments will be seen. The number and yield of these smaller fragments should be examined carefully to ensure that no partial digestion products are present.
5. Purify the DNA by extracting twice with phenol:chloroform and once with chloroform.
6. Recover the DNA by standard ethanol precipitation.
7. Redissolve the DNA in TE (pH 8.0) at a concentration of 250 µg/ml. Add the appropriate 10x restriction buffer and digest the DNA with the second restriction enzyme. Use a fourfold excess of enzyme and incubate the reaction for 4 hours.
8. Purify the DNA by extracting twice with phenol:chloroform and once with chloroform. Recover the DNA by standard ethanol precipitation.
9. Redissolve the DNA in TE (pH 7.6) at a concentration of 300-500 µg/ml. Store an aliquot (0.2 µg) at -20°C.
10. To determine the effectiveness of the digestion procedure, set up trial ligation reactions using 0.2 µg of the vector digested with only the first enzyme (the aliquot set aside at Step 4 above) and 0.2 µg of the final preparation (Step 9). Package equivalent amounts of DNA (0.1 µg) from each ligation mixture and titrate the infectivity of the resulting bacteriophage particles.
    a. Adjust the volumes of the two DNA solutions to 17 µl with H_2O.
    b. Add to each sample 2 µl of 10x ligation buffer and, if necessary, 1 µl of 10 mM ATP.
       Omit ATP if using a commercial ligase buffer that contains ATP.
    c. Remove 10-µl aliquots of each of the mixtures prepared in Step b and store the aliquots on ice.
    d. Add 0.2-0.5 Weiss units of bacteriophage T4 DNA ligase to the remainder of the mixtures (Step b) and incubate the reactions for 2 hours at 16°C.
    e. Use a commercial bacteriophage λ packaging reaction to package 0.1 µg of the ligated and unligated samples and 0.1 µg of the undigested vector DNA from Step 2. Determine the titer (pfu/ml) of each packaged reaction as described in Chapter 2, Protocol 1 .

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Protocol 15

Alkaline Phosphatase Treatment of Bacteriophage λ Vector DNA

Removal of the 5'-phosphate groups from the internal termini of bacteriophage λ arms is used to prevent self-ligation and suppress the background of nonrecombinant bacteriophages during cloning.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- λ Annealing buffer
- ⚠ ATP (10 mM)
  Omit ATP from Step 2 if the ligation buffer contains ATP.
- ⚠ Chloroform
- EDTA (0.5 M, pH 8.0)
- Ethanol
- ⚠ Phenol:chloroform (1:1, v/v)
- ⚠ SDS (10% w/v)
- Sodium acetate (3 M, pH 5.2 and pH 7.0)
- TE (pH 7.6 and pH 8.0)
- Tris-Cl (10 mM, pH 8.3)

Enzymes and Buffers

- Bacteriophage T4 DNA ligase
- Calf intestinal alkaline phosphatase
- 10x CIP Dephosphorylation buffer
- Proteinase K
- Restriction endonucleases

Nucleic Acids and Oligonucleotides

Bacteriophage λ DNA
Prepared as described in Chapter 2, Protocol 11 or Chapter 2, Protocol 12 .

Additional Reagents

Step 13 of this protocol requires the reagents listed in Chapter 2, Protocol 1 and Chapter 2, Protocol 14 .

Vectors and Bacterial Strains

Bacteriophage λ packaging mixtures

METHOD

1. Dissolve 50-60 µg of DNA of the appropriate bacteriophage λ vector in a final volume of 150 µl of λ annealing buffer. Incubate the DNA for 1 hour at 42°C to allow the ends of the viral DNA containing the *cos* sites to anneal.
2. Add 20 µl of 10x ligase buffer, 20 µl of 10 mM ATP (if necessary), and 0.2-0.5 Weiss unit of bacteriophage T4 DNA ligase/µg of DNA. Incubate the reaction for 1-2 hours at 16°C.
   Omit ATP if using a commercial ligase buffer that contains ATP.
3. Extract the ligation reaction with phenol:chloroform.
   During ligation, the λ DNA will form closed circles and long concatemers and become sensitive to shearing. Handle the ligated DNA carefully! Do not vortex. Carry out the phenol:chloroform extraction by gently inverting the tube to elicit emulsion formation.
4. Separate the organic and aqueous phases by centrifugation for 1 minute at room temperature in a microfuge. Remove the aqueous phase containing the viral DNA to a new tube using an automatic pipetting device equipped with a disposable tip that has been snipped with dog toe-nail clippers to increase the diameter of the hole.
5. Recover the DNA by standard ethanol precipitation. Rinse the pellet with 1 ml of 70% ethanol and recentrifuge for 2 minutes. Remove the 70% ethanol supernatant and store the open tube on the bench to allow the ethanol to evaporate. Redissolve the damp pellet of DNA in 150 µl of TE (pH 8.0).
   Check that the ligation of cos *termini has succeeded by heating an aliquot (0.2 µg) of the ligated DNA for 5 minutes at 68°C in TE. Chill the DNA in ice water and then electrophorese the DNA immediately through a 0.6% agarose gel. As controls, use (i) bacteriophage λ DNA that has been heated but not ligated and (ii) bacteriophage λ DNA that has been ligated but not heated.*
   Ligation should convert the bacteriophage λ DNA to closed circular and concatenated forms that show no change in migration after heating. The unligated, heated control DNA should migrate as a linear molecule, approx. 50 kb in length.
6. Digest the ligated DNA with one or more restriction enzymes as described in Chapter 2, Protocol 13 or Chapter 2, Protocol 14 .
7. Repeat Steps 3 and 4 (above).
8. Add 0.1 volume of 3 M sodium acetate (pH 7.0) and 2 volumes of ethanol. Recover the precipitate of DNA by centrifugation for 10 minutes at 4°C in a microfuge. Rinse the pellet with 1 ml of 70% ethanol and recentrifuge for 2 minutes. Remove the 70% ethanol supernatant and store the open tube on the bench to allow the ethanol to evaporate.
9. Dissolve the digested and ethanol-precipitated DNA at a concentration of 100 µg/ml in 10 mM Tris-Cl (pH 8.3), and store an aliquot (0.2 µg) on ice. Treat the remainder of the DNA with an excess of CIP for 1 hour at 37°C as follows:
   a. Add 0.1 volume of 10x dephosphorylation buffer and 0.01 unit of CIP for every 10 µg of bacteriophage λ DNA.
   b. Mix, and incubate the reaction for 30 minutes at 37°C. Add a second aliquot of CIP and continue incubation for an additional 30 minutes.
10. Add SDS and EDTA (pH 8.0) to final concentrations of 0.5% and 5 mM, respectively. Mix the solution by gentle vortexing and add proteinase K to a final concentration of 100 µg/ml. Incubate the mixture for 30 minutes at 56°C.
11. Cool the reaction mixture to room temperature, and purify the bacteriophage λ DNA by extracting once with phenol:chloroform and once with chloroform. Recover the DNA by ethanol precipitation in the presence of 0.3 M sodium acetate (pH 7.0).
12. Dissolve the DNA in TE (pH 7.6) at a concentration of 300-500 µg/ml. Store the dephosphorylated DNA at -20°C in aliquots of 1-5 µg.
13. Measure the efficiency of dephosphorylation by ligating a portion (0.2 µg) of the digested vector before and after treatment with CIP (for ligation conditions, please see Chapter 2, Protocol 13). Package the DNA into bacteriophage particles (for packaging conditions, please see Chapter 2, Protocol 14), and titrate the infectivity.

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Protocol 16

Purification of Bacteriophage λ Arms: Centrifugation through Sucrose Density Gradients

This method, derived from Maniatis et al. (1978), is used to prepare the arms of any bacteriophage λ vector.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- EDTA (0.5 M, pH 8.0)
  Ethanol
  $MgCl_2$ (1 M)
- NaCl (1 M)
- ⚠ *n*-Butanol
- Sodium acetate (3 M, pH 5.2)
- Gel-loading buffer IV
- TE (pH 7.6 and pH 8.0)

Nucleic Acids and Oligonucleotides

Bacteriophage λ DNA
Prepared as described in Chapter 2, Protocol 11 or Chapter 2, Protocol 12 .

Additional Reagents

Step 3 of this protocol requires the reagents listed in Chapter 2, Protocol 13 or Chapter 2, Protocol 14 .

METHOD

1. This step serves as an optional preliminary sequence of steps that may be performed before Step 3 for purifying bacteriophage λ arms. In this "Ligation First" method, the cohesive termini of the vector DNA are ligated together before digesting with restriction enzyme(s). The resulting concatemers are then cleaved by the appropriate restriction enzymes into left and right arms (which remain joined together) and the stuffer fragment. Ligation, followed by restriction endonuclease digestion, ensures that a majority of the purified vector has intact *cos* sites. These, in turn, increase the efficiency of packaging in the subsequent cloning steps. If this step is omitted, begin the protocol at Step 2.
   i. Incubate the undigested bacteriophage λ DNA for 1 hour at 42°C in 150 µl of 0.1 M Tris-Cl (pH 7.6), 10 mM $MgCl_2$ to allow the cohesive termini to anneal.
   ii. Add 20 µl of 10x ligation buffer (please see Chapter 2, Protocol 13), 20 µl of 10 mM ATP (if necessary), and 0.2-0.5 Weiss unit of bacteriophage T4 DNA ligase/µg of DNA. Incubate the reaction mixture for 1-2 hours at 16°C.
   iii. Extract the ligated DNA once with phenol:chloroform.
   During ligation, the bacteriophage λ DNA forms closed circles and long concatemers and will be more sensitive to shearing. Handle the ligated DNA carefully! Do not vortex. Carry out the phenol:chloroform extraction by gently inverting the tube to emulsify the two phases.
   iv. Centrifuge the emulsion for 1 minute at room temperature to separate the organic and aqueous phases. Transfer the aqueous phase containing the viral DNA to a new tube using an automatic pipetting device equipped with a large-bore tip.
   v. Recover the DNA by standard ethanol precipitation.
   vi. Proceed with Step 2 of this protocol.
2. Prepare two sucrose solutions, one containing 10% (w/v) sucrose and another containing 40% (w/v) sucrose in a buffer of 1 M NaCl, 20 mM Tris-Cl (pH 8.0), and 5 mM EDTA (pH 8.0). Sterilize the two solutions by filtration through 0.22-µm nitrocellulose filters. Prepare one or more 38-ml (10-40% w/v) sucrose gradients in clear ultracentrifuge tubes. Store the gradients for 1-2 hours at 4°C in a quiet place until they are needed (Step 5).
   Continuous sucrose density gradients are best made in a gradient-making device such as those supplied by Bio-Rad or Techware. Each gradient should take 10-20 minutes to pour at room temperature using a gradient maker. Each gradient can accommodate 60-75 µg of digested bacteriophage λ DNA.
3. Digest and analyze approx. 60 µg of the bacteriophage λ vector DNA as described in Chapter 2, Protocol 13 or Chapter 2, Protocol 14 . After standard ethanol precipitation, dissolve the DNA in TE (pH 7.6) at a concentration of 150 µg/ml. Set aside an aliquot (0.2 µg) for use as an electrophoretic control (Step 8).
4. Add $MgCl_2$ (1 M) to a final concentration of 10 mM, and incubate the solution of bacteriophage DNA for 1 hour at 42°C to allow the cohesive termini of bacteriophage λ DNA to anneal. Analyze an aliquot (0.2 µg) by electrophoresis through an 0.7% agarose gel to determine whether annealing has occurred.
5. Load onto each gradient no more than 75 µg of annealed, digested bacteriophage λ DNA in a volume of 500 µl or less.
   More DNA can cause the gradient to be overloaded and lead to poor separation of the stuffer fragments from the arms.
6. Centrifuge the gradients at 120,000*g* (26,000 rpm in a Beckman SW28 rotor) for 24 hours at 15°C.
7. Collect 0.5-ml fractions through a 21-gauge needle inserted through the bottom of the centrifuge tube.
8. Take two 15-µl aliquots from every third fraction and dilute each with 35 µl of H_2O. Add 8 µl of sucrose gel-loading buffer, heat one aliquot from each fraction to 68°C for 5 minutes, and leave the second aliquot untreated. Analyze all of the samples by electrophoresis through a thick 0.5% agarose gel. Use as markers intact bacteriophage λ DNA and the aliquot of digested DNA set aside in Step 3.
   Adjust the sucrose and salt concentrations of the markers to match those of the samples; otherwise, their electrophoretic mobilities will not be comparable.
9. After photographing the gel, locate and pool the fractions that contain the annealed arms.
   Be careful not to include fractions that are visibly contaminated with undigested bacteriophage λ DNA or fractions that contain significant quantities of unannealed left or right arms or stuffer fragment(s).
10. Dialyze the pooled fractions against a 1000-fold excess of TE (pH 8.0) for 12-16 hours at 4°C, with at least one change of buffer.
    Be sure to allow for a two- to threefold increase in volume during dialysis.
11. Extract the dialyzed sample several times with *n*-butanol to reduce its volume to less than 3 ml.
12. Recover the dialyzed DNA by standard ethanol precipitation.
13. Dissolve the DNA in TE (pH 7.6) at a concentration of 300-500 µg/ml.
14. Measure the concentration of the DNA spectrophotometrically (1 OD_{260} = approx. 50 µg/ml), and analyze an aliquot by electrophoresis through a 0.5% agarose gel to assess its purity. Store the DNA at -20°C in aliquots of 1-5 µg.

REFERENCES

1. Maniatis T., Hardison R.C., Lacy E., Lauer J., O'Connell C., Quon D., Sim G.K., and Efstratiadis A. 1978. The isolation of structural genes from libraries of eukaryotic DNA. *Cell* 15:687-701.

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Protocol 17

Partial Digestion of Eukaryotic DNA for Use in Genomic Libraries: Pilot Reactions

This protocol is used to establish conditions for restriction enzyme digestion of eukaryotic genomic DNA that will generate fragments of a size appropriate for construction of genomic libraries. To construct a genomic library, the average length of the starting genomic DNA should be at least eight times the capacity of the vector. This size range ensures that the majority of DNA molecules created by partial digestion with restriction enzyme(s) are derived from internal segments of the high-molecular-weight DNA and therefore carry termini that are compatible with those of the vector arms.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Gel-loading buffer IV
- Tris-Cl (10 mM, pH 8.0)

Enzymes and Buffers

Restriction endonucleases
The best results are obtained if the same batch of 10x buffer is used in both the pilot reactions and the large-scale reaction.

Nucleic Acids and Oligonucleotides

Genomic DNA, high molecular weight

Oligomers of bacteriophage λ DNA and plasmids
Use as DNA size standards during gel electrophoresis.

METHOD

1. Set up pilot reactions using the same batch of genomic DNA that will be used to prepare fragments for cloning.
   a. Dilute 30 µg of high-molecular-weight eukaryotic DNA to 900 µl with 10 mM Tris-Cl (pH 8.0) and add 100 µl of the appropriate 10x restriction enzyme buffer.
   b. Use a sealed glass capillary to mix the solution gently. This mixing ensures that the high-molecular-weight DNA is distributed evenly throughout the restriction enzyme buffer.
   c. After mixing, store the diluted DNA for 1 hour at room temperature to allow any residual clumps of DNA to disperse (please also see the note to Step 8, below).

   If the concentration of the high-molecular-weight DNA is low, it is best to increase the volume of the pilot reactions and concentrate the DNA after digestion by standard ethanol precipitation. This approach minimizes the possibility of shearing the high-molecular-weight DNA, which can occur if it is concentrated before digestion. Each pilot reaction should contain at least 1 µg of DNA to allow the heterogeneous products of digestion to be detected by staining with ethidium bromide.
2. Label a series of microfuge tubes 1 through 10. Use a wide-bore glass capillary or disposable plastic pipette tip to transfer 60 µl of the DNA solution to a microfuge tube (Tube 1). Transfer 30 µl of the DNA solution to each of nine additional labeled microfuge tubes. Incubate the tubes on ice.
3. Add 2 units of the appropriate restriction enzyme to Tube 1.
   Use a sealed glass capillary to mix the restriction enzyme with the DNA. Do not allow the temperature of the reaction to rise above 4°C.
4. Use a fresh pipette tip to transfer 30 µl of the reaction from Tube 1 to the next tube in the series. Mix as before, and continue transferring the reaction to successive tubes. Do not add anything to the tenth tube (the no enzyme control), but discard 30 µl from the ninth tube.
5. Incubate the reactions for 1 hour at 37°C.
6. Inactivate the restriction enzyme by heating the reactions to 70°C for 15 minutes.
7. Cool the reactions to room temperature and add the appropriate amount of sucrose gel-loading buffer.
   Use a sealed glass capillary to mix the solutions gently.
8. Use wide-bore plastic pipette tip or a disposable wide-bore glass capillary to transfer the solutions to the wells of a 0.6% agarose gel or, even better, to the lanes of an agarose gel for pulsed-field electrophoresis (please see Chapter 5). Perform electrophoresis.
   When separating the partial digestion products by agarose gel electrophoresis, it is essential to run the gel under conditions of maximum resolution. Use the same batch of buffer to cast the gel and to fill the gel tank prior to electrophoresis. The gel should be run slowly (<1 V/cm) at 4°C to prevent smearing of the fragments of DNA.
9. Compare the size of the digested eukaryotic DNA with that of DNA standards composed of oligomers of bacteriophage λ DNA and plasmids. Identify the partial digestion conditions that result in a majority of the genomic DNA migrating in the desired size range.

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Protocol 18

Partial Digestion of Eukaryotic DNA for Use in Genomic Libraries: Preparative Reactions

The results of the pilot experiment (Chapter 2, Protocol 17) are used to establish conditions for partial digestion of eukaryotic DNA in the large-scale reactions described here.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
Ethanol
⚠ *n*-Butanol
⚠ Phenol:chloroform (1:1, v/v)
Sodium acetate (3 M, pH 5.2)
Gel-loading buffer IV
TE (pH 8.0)
Tris-Cl (10 mM, pH 8.0)

Enzymes and Buffers

Restriction endonucleases
The best results are obtained if the same batch of 10x buffer is used in both the pilot reactions and the large-scale reaction.

Nucleic Acids and Oligonucleotides

Genomic DNA, high molecular weight
Oligomers of bacteriophage λ DNA and plasmids
Use as DNA size standards during gel electrophoresis.

METHOD

1. Prepare two sucrose solutions, one containing 10% (w/v) sucrose and another containing 40% (w/v) sucrose in a buffer of 1 M NaCl, 20 mM Tris-Cl (pH 8.0), and 5 mM EDTA (pH 8.0). Sterilize the two solutions by filtration through 0.22-µm nitrocellulose filters. Prepare one or more 38-ml (10-40% w/v) sucrose gradients in clear ultracentrifuge tubes. Store the gradients for 1-2 hours at 4°C in a quiet place until they are needed (Step 5).
2. Set up a series of digestions, each containing 100 µg of high-molecular-weight DNA.
   a. Use three different concentrations of restriction enzyme that straddle the optimal concentration determined in the pilot experiments (Chapter 2, Protocol 17).
   b. Incubate the reactions for the appropriate time with the restriction enzyme.
   c. Analyze an aliquot of the partially digested DNA by gel electrophoresis to ensure that the digestion has worked according to prediction. Until the results are available, store the remainder of the sample at 0°C.
3. Gently extract the digested DNA twice with phenol:chloroform.
4. Recover the DNA by standard precipitation with ethanol and dissolve it in 200 µl of TE (pH 8.0).
5. Heat the DNA sample (100 µg) for 10 minutes at 68°C, cool to 20°C, and gently layer the sample on the top of the gradient. Centrifuge the gradients at 83,000*g* (25,000 rpm in a Beckman SW28 rotor) for 22 hours at 20°C.
6. Use a 21-gauge needle or a gradient fractionation device to puncture the bottom of the tube and collect 350-µl fractions.
7. Mix 10 µl of every other fraction with 10 µl of H_2O and 5 µl of sucrose gel-loading buffer. Analyze the size of the DNA in each fraction by electrophoresis through a 0.6% agarose gel, using oligomers of plasmid DNA or other high-molecular-weight standards as markers. Adjust the sucrose and salt concentrations of the markers to correspond to those of the samples.
8. Following electrophoresis, pool the gradient fractions containing DNA fragments of the desired size (e.g., 35-45 kb for construction of libraries in cosmids and 20-25 kb for construction of libraries in bacteriophage λ vectors).
9. Dialyze the pooled fractions against 2 liters of TE (pH 8.0) for 12-16 hours at 4°C, with a change of buffer after 4-6 hours.
   Leave space in the dialysis sac for the sample to expand two- to threefold in volume.
10. Extract the dialyzed DNA several times with an equal volume of *n*-butanol until the volume is reduced to approx. 1 ml.
11. Precipitate the DNA with ethanol at room temperature in the presence of 2 M ammonium acetate (from a 10 M stock solution).
12. Recover the DNA by centrifugation and dissolve the DNA in TE (pH 8.0) at a concentration of 300-500 µg/ml. Analyze an aliquot of the DNA (0.5 µg) by electrophoresis through a conventional 0.6% agarose gel or by pulsed-field electrophoresis to check that the size distribution of the digestion products is correct. Store the DNA at 4°C.
13. To establish genomic DNA libraries, ligate the fractionated DNA to the arms of bacteriophage λ vectors as described in Chapter 2, Protocol 19 . For the preparation of cosmid libraries, please see Chapter 4, Protocol 1 .

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Protocol 19

Ligation of Bacteriophage λ Arms to Fragments of Foreign Genomic DNA

Pilot ligations and packaging reactions are used to establish the amounts of fragmented genomic DNA and bacteriophage λ arms that yield the maximum number of recombinants. Additional ligation and packaging reactions may then be set up to yield a comprehensive library of genomic DNA.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)
Omit ATP from Step 1 if the ligation buffer contains ATP.

SM

SM plus gelatin

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Nucleic Acids and Oligonucleotides

Genomic DNA, of an appropriate size for the vector
Prepared as described in Chapter 2, Protocol 18 .

Media

Rich λ agar medium

Rich λ top agarose
Agarose is preferred to agar in this protocol in order to minimize contamination with inhibitors that can interfere with subsequent enzymatic analysis of bacteriophage.

Additional Reagents

Step 4 of this protocol requires the reagents listed in Chapter 2, Protocol 1.

Vectors and Bacterial Strains

Bacteriophage λ packaging mixtures

Bacteriophage λ DNA arms
Prepared by one of the methods described in Chapter 2, Protocol 11 , Chapter 2, Protocol 12 , Chapter 2, Protocol 13 , Chapter 2, Protocol 14 and Chapter 2, Protocol 15 .

E. coli plating bacteria
Prepared as described in Chapter 2, Protocol 1 .

METHOD

1. Use the table below as a guide to set up a series of ligation reactions that contain the following:

bacteriophage λ arms	0.5-1.0 µg
partially digested genomic DNA	6-1200 ng
10x ligation buffer	0.5-1.0 µl
10 mM ATP (if necessary)	0.5-1.0 µl
bacteriophage T4 DNA ligase	0.5-1.0 µl
H_2O	to 5 or 1.0 µl

Set up two control reactions in which the vector and insert DNAs are each ligated in the absence of the other. Incubate the ligation reactions for 4-16 hours at 16°C.

Amounts of Insert DNA Used in Trial Ligations Containing 1 µg of Bacteriophage λ Arms

Size of Potential Insert DNA (kb)	Amount of Insert DNA (ng)
2-4	6-200
4-8	12-400
8-12	24-600
12-16	36-800
16-20	48-1000
20-24	60-1200

2. Package an aliquot (10-25%) of each of the ligation reactions into bacteriophage particles, following the instructions provided by the manufacturer of the packaging extract.
3. Make a series of tenfold dilutions (10^{-1} to 10^{-5}) of the packaging reactions, using as a diluent SM plus gelatin or an equivalent buffer recommended by the manufacturer of the packaging extract.
4. Assay the number of plaque-forming units in 1 µl and 10 µl of each dilution as described in Chapter 2, Protocol 1 .
5. From the ligation reaction yielding the largest number of infectious bacteriophage particles, pick 6-12 plaques and prepare a small amount of recombinant DNA from each as described in Chapter 2, Protocol 23 .
6. Check the size of the inserts of genomic DNA by digestion with the appropriate restriction enzymes, followed by electrophoresis through a 0.7% agarose gel, using appropriate size markers.
7. If the bacteriophages are recombinants and contain inserts of the desired size, establish a genomic DNA library by setting up multiple ligation and packaging reactions. The ratio of insert to vector DNA in these reactions should be that which generated the greatest number of recombinant plaques in the trial reactions.
8. Estimate the total number of recombinant plaques generated in the large-scale ligation and packaging reactions. Calculate the depth to which a library of this size would cover the target genome.
To provide fivefold coverage of a mammalian genome (3×10^9 bp), a bacteriophage λ library containing inserts whose average size is 20 kb would contain 2×10^6 independent recombinants.

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Protocol 20

Amplification of Genomic Libraries

Libraries of recombinant bacteriophages may be amplified by growing plate stocks directly from the packaging mixtures generated in Chapter 2, Protocol 18 .

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

SM

Media

Rich λ agar medium plates (150 mm)

Rich λ top agarose
Agarose is preferred to agar in this protocol in order to minimize contamination with inhibitors that can interfere with subsequent enzymatic analysis of bacteriophage.

Vectors and Bacterial Strains

Bacteriophage λ library
Prepared as described in Chapter 2, Protocol 19.

E. coli plating bacteria
Prepared as described in Chapter 2, Protocol 1.

METHOD

1. To amplify a bacteriophage λ library, mix aliquots of the packaging mixture containing 10,000-20,000 recombinant bacteriophages in a volume of 50 µl or less with 0.2 ml of plating bacteria in a 13 x 100-mm tube. Incubate the infected culture for 20 minutes at 37°C.
2. Add 6.5 ml of melted top agar/agarose (47°C) to the first aliquot of infected bacteria. Mix the contents of the tube by tapping or by gentle vortexing, and spread the infected bacteria onto the surface of a freshly poured 150-mm plate of bottom agar. Repeat the procedure with the remaining infected cultures.
   Alternatively, as many as 450,000 bacteriophages may be mixed with 1.4 ml of bacteria and plated in 75 ml of top agar/agarose on 500 ml of bottom agar in a 23 x 33-cm glass baking dish.
3. Incubate the plates for a maximum of 8-10 hours at 37°C.
   Do not allow the plaques to grow so large that they touch one another.
4. Overlay the plates with 12 ml of SM (or 150 ml of SM if baking dishes are used). Store the plates overnight at 4°C on a level surface.
5. Harvest the SM from all of the plates into a single, sterile polypropylene centrifuge tube or bottle. Wash each plate with an additional 4 ml of SM, and combine the washings with the primary harvest. Add 0.2 ml of chloroform to the resulting amplified bacteriophage stock. Store the stock for 15 minutes at room temperature with occasional gentle shaking to allow time for the chloroform to lyse all of the infected cells.
6. Remove cell and agarose debris by centrifugation at 4000*g* (5800 rpm in a Sorvall SS-34 rotor) for 5 minutes at 4°C.
7. Transfer the supernatant to a sterile glass tube or bottle. Divide the amplified bacteriophage library into aliquots and store them at 4°C. Measure the titer of the library by plaque assay.

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Protocol 21

Transfer of Bacteriophage DNA from Plaques to Filters

This protocol and Chapter 2, Protocol 22 , which are based on a method described by Benton and Davis (1977), are used to identify and isolate recombinants containing DNA sequences of interest.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Denaturation solution
  Depending on the number of filters to be processed, 1 liter or more may be required.
- Neutralizing solution
  Depending on the number of filters to be processed, 1 liter or more may be required.
- SM plus gelatin
- 2x SSPE
  Depending on the number of filters to be processed, 1 liter or more may be required.

Media

- Rich λ agar plates
  If the plates are not well-dried, the layer of top agarose will peel off the agar base when the filter is removed. Usually, 2-day-old plates that have been dried for several additional hours at 37°C with the lids slightly open work well. In humid weather, however, incubation for 1 day or more at 41°C may be necessary.
- Rich λ top agarose

Vectors and Bacterial Strains

Bacteriophage λ library
Prepared as described in Chapter 2, Protocol 19 or purchase from a commercial source.

E. coli plating bacteria
Prepared as described in Chapter 2, Protocol 1 .

METHOD

1. Prepare the filters for transfer:
   a. Number the dry filters with a soft-lead pencil or a ball-point pen.
   Prepare enough filters to make one or two (duplicate) replicas from the starting agar plate. In the latter case, number two sets of filters 1A, 1B, 2A, 2B, etc.
   b. Soak the filters in water for 2 minutes.
   c. Arrange the filters in a stack with each filter separated from its neighbor by an 85-mm-diameter Whatman 3MM filter paper.
   d. Wrap the stack of filters in aluminum foil, and sterilize them by autoclaving for 15 minutes at 15 psi (1.05 kg/cm^2) on liquid cycle.
2. Make a dilution (in SM plus gelatin) of the packaging mixture, bacteriophage stock, or library. Mix aliquots of the diluted bacteriophage stock with the appropriate amount of freshly prepared plating bacteria (please see the table below). Incubate the infected bacterial cultures for 20 minutes at 37°C.
   The diluted stock (100 µl) should contain approx. 15,000 infectious bacteriophages when using 100-mm plates or 50,000 infectious particles when using 150-mm plates.
   When screening bacteriophage libraries, it is best to infect the cells as a single pool. For example, when using ten 150-mm plates, 1 ml of diluted bacteriophage stock containing 500,000 pfu would be added to 3 ml of freshly prepared plating bacteria. After 20 minutes at 37°C, equal volumes of the infected culture are then distributed into 17 x 100-mm tubes for plating. This procedure ensures that each plate contains approximately the same number of plaques.
   To find a bacteriophage that carries a particular genomic sequence, it may be necessary to screen one million or more recombinants in a genomic DNA library. The table shows the maximum number of plaques that can be efficiently screened in culture dishes of different sizes.

Number of Plaques in Culture Dishes of Various Sizes

Size of Petri dish	90 mm	150 mm
Total area of dish	63.9 cm^2	176.7 cm^2
Volume of bottom agar	30 ml	80 ml
Volume of plating bacteria	0.1 ml	0.3 ml
Volume of top agarose	2.5 ml	6.5-7.5 ml
Maximum number of plaques/dish	15,000	50,000

3. Add to each aliquot of infected cells 3 ml or 6.5 ml of molten (47°C) top agarose. Pour the contents of each tube onto separate, numbered 90-mm or 150-mm agar plates.
4. Close the plates, allow the top agarose to harden, and incubate at 37°C in an inverted position until plaques appear and are just beginning to make contact with one another (10-12 hours).
   To minimize plate-to-plate variation, it is crucial that each plate be heated to the same extent when placed in the incubator.
5. Chill the plates for at least 1 hour at 4°C to allow the top agarose to harden.
6. Remove the plates from the cold room or refrigerator. Make imprints of the plaques on each plate using the first set of labeled filters. Place a dry, labeled circular nitrocellulose or nylon filter neatly onto the surface of the top agarose so that it comes into direct contact with the plaques. Handle the filter with gloved hands.
7. Mark the filter in three or more asymmetric, peripheral locations by stabbing through it and into the agar beneath with a 21-gauge needle attached to a syringe containing waterproof black drawing ink.
8. After 1-2 minutes, use blunt-ended forceps (e.g., Millipore forceps) to peel the filters from each plate in turn.
9. Transfer each filter, plaque side up, to a sheet of Whatman 3MM paper (or equivalent) impregnated with denaturing solution in a plastic cafeteria tray or Pyrex dish for 1-5 minutes.
   Make sure that excess denaturing solution does not rinse over the sides of the nitrocellulose or nylon filters. When transferring the filters, use the edge of cafeteria tray or dish to remove as much fluid as possible from the underside of the filters.
10. Transfer the filters, plaque side up, to a sheet of Whatman 3MM paper impregnated with neutralizing solution for five minutes.
    If nitrocellulose filters are used, repeat the neutralizing step using a fresh impregnated sheet of 3MM paper.
11. Prepare the filters for fixing.
    To fix the DNA to the filters by microwaving or baking: If using a microwave oven, proceed directly to Step 13. If baking in a vacuum oven, transfer the filters, plaque side up, to a sheet of dry 3MM paper or a stack of paper towels. Allow

filters to dry for at least 30 minutes at room temperature.
To fix the DNA to nylon filters by cross-linking with UV light: Place the filters on a sheet of Whatman 3MM paper impregnated with 2x SSPE, and move the tray of 2x SSPE containing the filters to the vicinity of the UV light cross-linker.

12. After the first set of filters has been processed, use the second set of filters to take another imprint of the plaques, if required. Make sure that both sets of filters are keyed to the plate at the same positions.
Generally, the second set of filters is left in contact with the plaques for 3 minutes, or until the filter is completely wet.
13. Fix the DNA from the plaques to the filter.
To fix by treatment in a microwave oven: Place the damp filters on a sheet of dry Whatman 3MM paper and irradiate them for 2-3 minutes at full power in a microwave oven.
To fix by baking: Arrange the dried filters (Step 10) in a stack with adjacent filters separated by a sheet of dry Whatman 3MM paper. Bake the stack of filters for 1-2 hours at 80°C in a vacuum oven.
Check the oven after 30 minutes and wipe away any condensation from the door. The filters must be baked under vacuum rather than stewed.
To fix by cross-linking with UV light: Carry out the procedure using a commercial device for this purpose and follow the manufacturer's instructions.
IMPORTANT *Do not allow the filters to dry out prior to cross-linking.*
14. After baking or cross-linking, loosely wrap the dry filters in aluminum foil and store them at room temperature. Alternatively, if hybridization is to be carried out within a day or so, wash the filters for 30 minutes at 65°C in 0.1x SSC or SSPE, 0.5% SDS and store them wet in sealed plastic bags.

REFERENCES

1. Benton W.D. and Davis R.W. 1977. Screening lambda gt recombinant clones by hybridization to single plaques in situ. *Science* 196:180-182.

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Protocol 22

Hybridization of Bacteriophage DNA on Filters

Using hybridization, it is possible to identify a single recombinant that carries the desired target sequence on a filter that carries the imprint of 15,000 or more plaques.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

Prehybridization/hybridization solution (for Plaque/Colony Lifts)

SM

2x SSPE
Depending on the number of filters to be processed, 1 liter or more may be required.

Wash solution 1 (2-22)

Wash solution 2 (2-22)

Wash solution 3 (2-22)

Nucleic Acids and Oligonucleotides

Filters immobilized with bacteriophage DNA
Prepared as described in Chapter 2, Protocol 21 .

⚠ Radiolabeled probe

METHOD

1. If the filters are dry, float the baked or cross-linked filters on the surface of 2x SSPE until they have become thoroughly wetted from beneath. Submerge the filters for 5 minutes.
   Make sure that no air bubbles are trapped under the filters. The filters should change from white to a bluish color as the aqueous solvent penetrates the pores of the filter. Make sure that there are no white spots or patches remaining on the filters before proceeding to Step 2.
2. Transfer the filters to a Pyrex dish or other hybridization chamber containing prehybridization solution. Use 3 ml of prehybridization solution per 82-mm filter or 5 ml per 132-mm filter. Incubate the filters with gentle agitation on a rocking platform for 1-2 hours or more at the appropriate temperature (i.e., 68°C when hybridization is to be carried out in aqueous solution; 42°C when hybridization is to be carried out in 50% formamide).
   Whatever type of container is used, the important point is that the filters are completely covered by the prehybridization solution.
3. Denature ^{32}P-labeled double-stranded probes by heating for 5 minutes at 100°C. Chill the probe rapidly in ice water. Single-stranded probes need not be denatured.
   Between 2 x 10^5 and 1 x 10^6 cpm of ^{32}P-labeled probe (specific activity 5 x 10^7 cpm/µg) should be used per milliliter of hybridization solution. Using more probe causes the background of nonspecific hybridization to increase; using less reduces the rate of hybridization.
4. Add the denatured probe to the prehybridization solution covering the filters. Incubate the filters for 12-16 hours at the appropriate temperature (please see Chapter 6, Protocol 10).
   Keep the containers holding the filters tightly closed to prevent the loss of fluid by evaporation.
   To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6x SSC or 6x SSPE) at a temperature that is 20-25°C below the melting temperature (please see Chapter 1, Protocol 28 , Chapter 1, Protocol 29 and Chapter 1, Protocol 30 or Chapter 6, Protocol 10). Both SSPE and SSC work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6x SSPE is preferred because of its greater buffering capacity.
5. When the hybridization is completed, *quickly* remove filters from the hybridization solution and *immediately* immerse them in a large volume (300-500 ml) of Wash solution 1 at room temperature. Agitate the filters gently, turning them over at least once during washing. After 5 minutes, transfer the filters to a fresh batch of wash solution and continue to agitate them gently. Repeat the washing procedure twice more.
   At no stage during the washing procedure should the filters be allowed to dry or to stick together.
6. Wash the filters twice for 1-1.5 hours in 300-500 ml of Wash solution 2 at 68°C.
   With experience, it is possible to use a hand-held monitor to test whether washing is complete. If the background is still too high or if the experiment demands washing at high stringencies, immerse the filters for 60 minutes in 300-500 ml of Wash solution 3 at 68°C.
7. Dry the filters in the air at room temperature on sheets of Whatman 3MM paper or stacks of paper towels. Streak the underside of the filters with a water-soluble glue stick and arrange the filters (numbered side up) on a clean, dry, flat sheet of 3MM paper. Firmly press the filters against the 3MM paper to ensure that they do not move. Apply adhesive labels marked with radioactive ink or chemiluminescent markers to several asymmetric locations on the 3MM paper. These markers serve to align the autoradiograph with the filters. Cover the filters and labels with Saran Wrap/Cling Film. Use tape to secure the wrap to the back of the 3MM paper and stretch the wrap over the paper to remove wrinkles.
8. Expose the filters to X-ray film (Kodak XAR-2, XAR-5, or their equivalent) for 12-16 hours at -70°C with an intensifying screen.
9. Develop the film and align it with the filters using the marks left by the radioactive ink or fluorescent marker. Use a nonradioactive red fiber-tip pen to mark the film with the positions of the asymmetrically located dots on the numbered filters.
10. Identify the positive plaques by aligning the orientation marks with those on the agar plate.
    When duplicate sets of filters are hybridized to the same probe, there is less chance of confusing a background smudge with a positive plaque. Pick only those plaques that yield convincing hybridization signals on both sets of filters for further analysis. When screening a genomic library for a single-copy gene, expect to find no more than one positive clone per 10^5 plaques screened. When screening cDNA libraries, the number of positives depends on the abundance of the mRNA of interest.
11. Pick each positive plaque as described in Chapter 2, Protocol 2 and store in 1 ml of SM containing a drop (50 µl) of chloroform.
12. To purify a hybridization-positive plaque, plate an aliquot (usually 50 µl of a 10^{-2} dilution) of the bacteriophages that are recovered from the cored agar plug and proceed with subsequent rounds of screening by hybridization.

REFERENCES

1. Benton W.D. and Davis R.W. 1977. Screening lambda gt recombinant clones by hybridization to single plaques in situ.

Science 196:180-182.

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Protocol 23

Rapid Analysis of Bacteriophage λ Isolates: Purification of λ DNA from Plate Lysates

This protocol is used to purify small amounts of bacteriophage λ DNA that are suitable for use as substrates for restriction enzymes and templates for DNA and RNA polymerases.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform
Ethanol
λ High-salt buffer
Isopropanol
λ Low-salt buffer
⚠ Phenol:chloroform (1:1, v/v)
SM
TE (pH 8.0)
TM

Media

NZCYM agarose plates
Freshly poured plates (150-mm diameter) that have been equilibrated to room temperature give the best results in this method. Agarose is preferred to agar in order to minimize contaminants that can interfere with enzymatic analysis of DNA prepared from plate lysates of bacteriophage λ DNA.

NZCYM top agarose
For details on using top agarose, please see Chapter 2, Protocol 1 . Agarose is preferred to agar in this protocol in order to minimize contamination with inhibitors that can interfere with subsequent enzymatic analysis of bacteriophage.

Vectors and Bacterial Strains

Bacteriophage λ recombinant, grown as single well-isolated plaques on a lawn of bacteria
Prepared as described in Chapter 2, Protocol 1 .

E. coli plating bacteria
Prepared as described in Chapter 2, Protocol 1 .

METHOD

1. Use a borosilicate Pasteur pipette to pick 8-10 well-isolated bacteriophage plaques from a plate derived from a genetically pure, plaque-purified bacteriophage stock. Place the plaques in 1 ml of SM and 50 µl of chloroform. Store the suspension for 4-6 hours at 4°C to allow the bacteriophage particles to diffuse from the top agarose.
2. In a small, sterile culture tube, mix 50-100 µl of the bacteriophage suspension (approx. 10^5 pfu) with 150 µl of plating bacteria. Incubate the infected culture for 20 minutes at 37°C. Add 7.0 ml of molten (47°C) top agarose (0.7%), and spread the bacterial suspension on the surface of a freshly poured 150-mm plate containing NZCYM agarose.
3. Incubate the inverted plate at 37°C until the plaques cover almost the entire surface of the plate (7-9 hours).
   Do not incubate the plates for too long, otherwise confluent lysis will occur, which reduces the yield of bacteriophage DNA.
4. Add 7 ml of TM directly onto the surface of the top agarose. Allow the bacteriophage particles to elute during 4 hours of incubation at 4°C with constant, gentle shaking.
5. Transfer the bacteriophage λ eluate to a centrifuge tube, and remove the bacterial debris by centrifugation at 4000*g* (58,000 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C. A small aliquot of cleared lysate can be set aside at this step as a bacteriophage stock solution. Store the stock at 4°C over a small volume of chloroform.
6. Dispense 10 ml of a 2:1 slurry of DE52 resin into a centrifuge tube and sediment the resin by centrifugation at 500*g* (2000 rpm in a Sorvall SS-34 rotor) for 5 minutes at room temperature. Remove the supernatant from the resin pellet and place the centrifuge tube on ice.
7. Resuspend the DE52 in the cleared TM and allow the bacteriophage particles to absorb to the resin by rocking the centrifuge tube for 3 minutes at room temperature.
8. Centrifuge the TM/DE52 slurry at 4000*g* (5800 rpm in a Sorvall SS-34 rotor) for 5 minutes. Carefully transfer the supernatant to a fresh centrifuge tube and repeat the centrifugation step. Discard the pellet after each centrifugation.
9. Transfer the supernatant from the second centrifugation to a fresh centrifuge tube. Extract the supernatant, which contains the bacteriophage λ particles, once with phenol:chloroform.
10. Transfer the aqueous phase, which contains the bacteriophage λ DNA, to a fresh polypropylene tube and add an equal volume of isopropanol. Store the mixture for 10 minutes at -70°C.
11. Collect the precipitated bacteriophage DNA by centrifugation at 16,500*g* (12,000 rpm in a Sorvall SS-34 rotor) for 20 minutes at 4°C.
12. Drain the isopropanol from the centrifuge tube and allow the pellet of DNA to dry in air.
13. Redissolve the DNA pellet in 2 ml of low-salt buffer.
14. Purify the bacteriophage DNA by chromatography on an Elutip-d column:
    a. Use a syringe to push 1-2 ml of high-salt buffer through the Elutip-d column.
    b. Push 5 ml of low-salt buffer through the column.
    c. Attach the 0.45-µm prefilter to the column and slowly push the DNA sample (Step 13) through the column.
    d. Rinse the column with 2-3 ml of low-salt buffer.
    e. Remove the prefilter and elute the DNA with 0.4 ml of high-salt buffer. Collect the eluate at this step in a 1.5-ml microfuge tube.
15. Add 1 ml of ethanol to the solution of eluted DNA, invert the tube several times, and incubate the mixture on ice for 20 minutes. Collect the precipitated DNA by centrifugation in a microfuge, discard the supernatant, and rinse the pellet of DNA with 0.5 ml of 70% ethanol. Discard the supernatant and allow the pellet of DNA to dry in the air. Dissolve the pellet of DNA in 50 µl of TE (pH 8.0).
    Resuspend the DNA by tapping on the side of the tube. Try to avoid vortexing. If the DNA proves difficult to dissolve, incubate the tube for 15 minutes at 50°C.
    If minipreparations are working well, expect to isolate approx. 5 µg of purified bacteriophage DNA from 5 x 10^{10} infectious particles.

REFERENCES

1. Xu S.-Y. 1986. A rapid method for preparing phage lambda DNA from agar plate lysates. *Gene Anal. Tech.* 3:90-91.

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Protocol 24

Rapid Analysis of Bacteriophage λ Isolates: Purification of λ DNA from Liquid Cultures

This protocol is used to purify small amounts of recombinant DNAs cloned in robust strains of bacteriophage λ such as λgt10, λgt11, λZAP, or ZipLox. The DNAs are suitable for use as substrates for restriction enzymes and templates for DNA and RNA polymerases.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

Ethanol

λ High-salt buffer

Isopropanol

λ Low-salt buffer

⚠ Phenol:chloroform (1:1, v/v)

SM

TE (pH 8.0)

Media

NZCYM medium

Vectors and Bacterial Strains

Bacteriophage λ recombinant, grown as single well-isolated plaques on a lawn of bacteria
Prepared as described in Chapter 2, Protocol 1 .

E. coli strain
Inoculate a single colony of an appropriate E. coli *strain into 25 ml of NZCYM medium and incubate overnight at 30°C. Measure the OD_{600} of the overnight culture and calculate the number of cells/ml using the conversion factor: 1 OD_{600} = 1 x 10^9 cells/ml.*

METHOD

1. Use a borosilicate Pasteur pipette to pick a single well-isolated bacteriophage plaque into 1 ml of SM containing a drop of chloroform in a small sterile polypropylene tube. Store the suspension for 4-6 hours at 4°C to allow the bacteriophage particles to diffuse from the top agarose.
2. In a 25-ml tube, mix 0.5 ml of the bacteriophage suspension (approx. 3 x 10^6 bacteriophages) with 0.1 ml of an overnight culture of bacteria. Incubate the culture for 15 minutes at 37°C.
3. Add 4 ml of NZCYM medium, and incubate the culture for approx. 9 hours at 37°C with vigorous agitation.
   The culture should be clear, but very little debris should be evident.
4. Add 0.1 ml of chloroform to the culture and continue incubation for a further 15 minutes at 37°C with vigorous agitation. Transfer the lysate to a 5-ml polypropylene centrifuge tube. Centrifuge at 800*g* (2600 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C.
5. Transfer the supernatant to a fresh tube, and remove the bacterial debris by centrifugation at 4000*g* (5800 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C. A small aliquot of cleared lysate can be set aside at this step as a bacteriophage stock solution. Store the stock at 4°C over chloroform.
6. Dispense 10 ml of a 2:1 slurry of DE52 resin into a fresh centrifuge tube and sediment the resin by centrifugation at 500*g* (2000 rpm in a Sorvall SS-34 rotor) for 5 minutes at room temperature. Remove the supernatant from the resin pellet and place the centrifuge tube on ice.
7. Resuspend the DE52 in the cleared bacteriophage λ supernatant and allow the bacteriophage particles to absorb to the resin by rocking the centrifuge tube for 3 minutes at room temperature.
8. Centrifuge the bacteriophage λ supernatant/DE52 slurry at 4000*g* (5800 rpm in a Sorvall SS-34 rotor) for 5 minutes. Carefully transfer the supernatant to a fresh centrifuge tube and repeat the centrifugation step. Discard the pellet after each centrifugation.
9. Transfer the supernatant from the second centrifugation to a fresh centrifuge tube. Extract the supernatant, which contains the bacteriophage λ particles, once with phenol:chloroform.
10. Transfer the aqueous phase, which contains the bacteriophage λ DNA, to a fresh polypropylene tube and add an equal volume of isopropanol. Store the mixture for 10 minutes at -70°C.
11. Collect the precipitated bacteriophage DNA by centrifugation at 16,500*g* (12,000 rpm in a Sorvall SS-34 rotor) for 20 minutes at 4°C.
12. Drain the isopropanol from the centrifuge tube and allow the pellet of DNA to dry in the air.
13. Dissolve the DNA pellet in 2 ml of low-salt buffer.
14. Purify the bacteriophage DNA by chromatography on an Elutip-d column as described in Chapter 2, Protocol 23 .
15. Mix 1 ml of ethanol with the solution of eluted DNA and incubate the mixture on ice for 20 minutes. Collect the precipitated DNA by centrifugation in a microfuge, discard the supernatant, and rinse the pellet of DNA with 0.5 ml of 70% ethanol. Discard the supernatant and allow the ethanol to evaporate. Dissolve the damp pellet of DNA in 50 µl of TE (pH 8.0).
    Resuspend the DNA by tapping on the side of the tube. Try to avoid vortexing. If the DNA proves difficult to dissolve, incubate the tube for 15 minutes at 50°C.
    If minipreparations are working well, expect to isolate approx. 5 µg of purified bacteriophage DNA from 5 X 10^{10} infectious particles. It is possible to estimate the quantity of bacteriophage DNA present in a plate lysate or liquid culture lysate by direct agarose gel electrophoresis (please see Chapter 2, Protocol 7).
    For restriction analysis and agarose gel electrophoresis, digest a 5-10-µl aliquot of the resuspended DNA.

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Chapter 3 Working with Bacteriophage M13 Vectors

Protocol 1: Plating Bacteriophage M13

Bacteriophage M13 forms turbid plaques on lawns of male strains of *E. coli*.

Protocol 2: Growing Bacteriophage M13 in Liquid Culture

Most manipulations with M13, including preparations of viral stocks and isolation of single- and double-stranded DNAs, begin with small-scale liquid cultures that are infected with an M13 plaque, picked from an agar plate.

Protocol 3: Preparation of Double-stranded (Replicative Form) Bacteriophage M13 DNA

The double-stranded replicative form (RF) of bacteriophage M13 is isolated from infected cells using methods similar to those used to purify plasmid DNA. Several micrograms of RF DNA can be isolated from a 1-2-ml culture of infected cells.

Protocol 4: Preparation of Single-stranded Bacteriophage M13 DNA

Bacteriophage M13 single-stranded DNA is prepared from virus particles secreted by infected cells into the surrounding medium. The filamentous particles are concentrated by precipitation from a high-ionic-strength buffer with polyethylene glycol. Subsequent extraction with phenol releases the single-stranded DNA, which is then collected by precipitation with ethanol. This protocol is generally used to prepare single-stranded DNA from a small number of M13 isolates. Typically, the yield of single-stranded DNA is 5-10 µg/ml infected culture.

Protocol 5: Large-scale Preparation of Single-stranded and Double-stranded Bacteriophage M13 DNA

This protocol, a scaled-up version of Chapter 3, Protocol 3 and Chapter 3, Protocol 4 , is used chiefly to generate large stocks of double-stranded DNA of strains of M13 that are routinely used as cloning vectors. Large amounts of single-stranded DNA of an individual recombinant may occasionally be needed for specific purposes, e.g., to generate many preparations of a particular radiolabeled probe or to construct large numbers of site-directed mutants.

Protocol 6: Cloning into Bacteriophage M13 Vectors

This protocol describes three standard methods to construct bacteriophage M13 recombinants: (1) ligating insert DNA to a linearized vector, prepared by cleavage of M13 RF with a single restriction enzyme; (2) using alkaline phosphatase to suppress self-ligation of the linearized vector, and (3) using M13 RF cleaved with two restriction enzymes for directional cloning.

Protocol 7: Analysis of Recombinant Bacteriophage M13 Clones

A rapid method to analyze the size of the single-stranded DNA of M13 recombinants.

Protocol 8: Producing Single-stranded DNA with Phagemid Vectors

This protocol describes methods to superinfect bacteria carrying a recombinant phagemid with a high-titer stock of an appropriate helper virus and to assay the yield of filamentous virus particles that carry single-stranded copies of the phagemid DNA. The key to success in using phagemids is to prepare a stock of helper virus whose titer is accurately known.

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Protocol 1

Plating Bacteriophage M13

Bacteriophage M13 forms turbid plaques on lawns of male strains of *E. coli*.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- IPTG (20% w/v)
- ⚠ X-gal solution (2% w/v)

Media

- LB agar plates containing tetracycline or kanamycin
  These plates are needed only if a tetracycline-resistant strain of E. coli*, such as XL1-Blue, or a kanamycin-resistant strain of* E. coli*, such as XL1-Blue MRF´ Kan, is used to propagate the virus.*
- M9 minimal agar plates, supplemented
  These plates are needed when using E. coli *strains that carry a deletion of the proline biosynthetic operon (Δ[lac-proAB]) in the bacterial chromosome and the complementing proAB genes on the F´ plasmid.*
- Rich M13 medium
- Rich M13 agar medium plates containing 5 mM $MgCl_2$
- Rich M13 top agar or agarose containing 5 mM $MgCl_2$

Vectors and Bacterial Strains

Bacteriophage M13 stock
LB or YT medium from a fully grown liquid culture of bacteria infected with bacteriophage M13 contains between 10^{10} and 10^{12} pfu/ml. A bacteriophage M13 plaque contains between 10^6 and 10^8 pfu.

E. coli F´ strain, prepared as a master culture

METHOD

1. Streak a master culture of a bacterial strain carrying an F´ plasmid onto either a supplemented minimal (M9) agar plate or an LB plate containing tetracycline (XL1-Blue) or kanamycin (XL1-Blue MRF´ Kan). Incubate the plate for 24-36 hours at 37°C.
2. To prepare plating bacteria, inoculate 5 ml of LB or YT medium in a 20-ml sterile culture tube with a single, well-isolated colony picked from the agar plate prepared in Step 1. Agitate the culture for 6-8 hours at 37°C in a rotary shaker. Chill the culture in an ice bath for 20 minutes and then store it at 4°C. These plating bacteria can be stored for periods of up to 1 week at 4°C.
   Do not grow the cells to saturation, as this will increase the risk of losing the pili encoded by the F´ plasmid.
3. Prepare sterile tubes (13 x 100 mm or 17 x 100 mm) containing 3 ml of melted LB or YT medium top agar or agarose, supplemented with 5 mM $MgCl_2$. Allow the tubes to equilibrate to 47°C in a heating block or water bath.
4. Label a series of sterile tubes (13 x 100 mm or 17 x 100 mm) according to the dilution factor and amount of bacteriophage stock to be added (please see Step 5), and deliver 100 µl of plating bacteria from Step 2 into each of these tubes.
5. Prepare tenfold serial dilutions (10^{-6} to 10^{-9}) of the bacteriophage stock in LB or YT medium. Dispense 10 µl or 100 µl of each dilution to be assayed into a sterile tube containing plating bacteria from Step 4. Mix the bacteriophage particles with the bacterial culture by vortexing gently.
6. Add 40 µl of 2% X-gal solution and 4 µl of 20% IPTG solution to each of the tubes containing top agar. Immediately pour the contents of one of these tubes into one of the infected cultures. Mix the culture with the agar/agarose by gently vortexing for 3 seconds, and then pour the mixture onto a labeled plate containing LB or YT agar medium supplemented with 5 mM $MgCl_2$ and equilibrated to room temperature. Swirl the plate gently to ensure an even distribution of bacteria and top agar.
   Work quickly so that the top agar spreads over the entire surface of the agar before it sets.
7. Repeat the addition of top agar with X-gal and IPTG for each tube of infected culture prepared in Step 5.
8. Replace the lids on the plates and allow the top agar/agarose to harden for 5 minutes at room temperature. Wipe excess condensation off the lids with Kimwipes. Invert the plates and incubate them at 37°C.
   Pale blue plaques begin to appear after 4 hours. The color gradually intensifies as the plaques enlarge and is complete after 8-12 hours of incubation.

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Protocol 2

Growing Bacteriophage M13 in Liquid Culture

Most manipulations with M13, including preparations of viral stocks and isolation of single- and double-stranded DNAs, begin with small-scale liquid cultures that are infected with an M13 plaque, picked from an agar plate.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Media

- LB containing tetracycline or kanamycin
  These media are needed only if a tetracycline-resistant strain of E. coli*, such as XL1-Blue, or a kanamycin-resistant strain of* E. coli *is used to propagate the virus.*
- M9 minimal medium, supplemented
  This media is needed when using E. coli *strains that carry a deletion of the proline biosynthetic operon (Δ[lac-proAB]) in the bacterial chromosome and the complementing proAB genes on the F´ plasmid.*
- Rich M13 medium
- 2x YT medium containing 5 mM $MgCl_2$

Vectors and Bacterial Strains

Bacteriophage M13 plaques plated onto an agar or agarose plate
Please see either Chapter 3, Protocol 1 *or* Chapter 3, Protocol 6 *.*

E. coli F´ strain, grown as well-isolated colonies on an agar plate

METHOD

1. Inoculate 5 ml of supplemented M9 medium (or, for antibiotic-resistant strains, LB medium with the appropriate antibiotic) with a single freshly grown colony of *E. coli* carrying an F´ plasmid. Incubate the culture for 12 hours at 37°C with moderate shaking.
2. Transfer 0.1 ml of the *E. coli* culture into 5 ml of 2x YT medium containing 5 mM $MgCl_2$. Incubate the culture for 2 hours at 37°C with vigorous shaking.
3. Dilute the 5-ml culture into 45 ml of 2x YT containing 5 mM $MgCl_2$ and dispense 1-ml aliquots into as many sterile tubes (13 x 100 mm or 17 x 100 mm) as there are plaques to be propagated. Dispense two additional aliquots for use as positive and negative controls for bacteriophage growth. Set these cultures aside for use at Step 7.
4. Dispense 1 ml of YT or LB medium into sterile 13 x 100-mm tubes. Prepare as many tubes as there are plaques. Dispense two additional aliquots for use as positive and negative controls for bacteriophage growth.
5. Prepare a dilute suspension of bacteriophage M13 by touching the surface of a plaque with the end of a sterile inoculating needle and immersing the end of the needle into the YT or LB medium. Pick one blue M13 plaque as a positive control for bacteriophage growth. Also pick an area of the *E. coli* lawn from the plate that does not contain a plaque as a negative control.
6. Allow the suspension to stand for 1-2 hours at room temperature to allow the bacteriophage particles to diffuse from the agar.
7. Use 0.1 ml of the bacteriophage suspension (Step 6) as an inoculum to infect 1-ml cultures of *E. coli* (Step 3) for isolation of viral DNA. Incubate the inoculated tubes for 5 hours at 37°C with moderate shaking.
   Alternatively, transfer a plaque directly into the E. coli *culture.*
   To minimize the possibility of selecting deletion mutants, grow cultures infected with recombinant M13 bacteriophages for the shortest time that will produce a workable amount of single-stranded DNA (usually 5 hours).
8. Transfer the culture to a sterile microfuge tube and centrifuge at maximum speed for 5 minutes at room temperature. Transfer the supernatant to a fresh microfuge tube without disturbing the bacterial pellet.
9. Transfer 0.1 ml of the supernatant to a sterile microfuge tube.
10. Use the remaining 1 ml of the culture supernatant to prepare single-stranded bacteriophage DNA (Chapter 3, Protocol 4). Use the bacterial cell pellet to prepare double-stranded RF DNA (Chapter 3, Protocol 3).

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Protocol 3

Preparation of Double-stranded (Replicative Form) Bacteriophage M13 DNA

The double-stranded replicative form (RF) of bacteriophage M13 is isolated from infected cells using methods similar to those used to purify plasmid DNA. Several micrograms of RF DNA can be isolated from a 1-2-ml culture of infected cells.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Alkaline lysis solution I
- Alkaline lysis solution II
- Alkaline lysis solution III

Ethanol

- ⚠ Phenol:chloroform (1:1, v/v)
- TE (pH 8.0) containing 10 µg/ml RNase A

Enzymes and Buffers

Restriction endonucleases

Vectors and Bacterial Strains

E. coli culture infected with bacteriophage M13

METHOD

1. Centrifuge 1 ml of the M13-infected cell culture at maximum speed for 5 minutes at room temperature in a microfuge to separate the infected cells from the medium. Transfer the supernatant to a fresh microfuge tube and store at 4°C. Keep the infected bacterial cell pellet on ice.
   The supernatant contains M13 bacteriophage housing single-stranded DNA. If desired, prepare M13 DNA from this supernatant at a later stage (Chapter 3, Protocol 4).
2. Centrifuge the bacterial cell pellet for 5 seconds at 4°C and remove residual medium with an automatic pipetting device.
3. Resuspend the cell pellet in 100 µl of ice-cold Alkaline lysis solution I by vigorous vortexing.
   Make sure that the bacterial pellet is completely dispersed in Alkaline lysis solution I.
4. Add 200 µl of freshly prepared Alkaline lysis solution II to the tube. Close the tube tightly and mix by inverting the tube rapidly five times. *Do not vortex.* Store the tube on ice for 2 minutes after mixing.
5. Add 150 µl of ice-cold Alkaline lysis solution III to the tube. Close the tube to disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 3-5 minutes.
6. Centrifuge the bacterial lysate at maximum speed for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh tube.
7. Add an equal volume of phenol:chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the tube at maximum speed for 2-5 minutes. Transfer the aqueous (upper) phase to a fresh tube.
8. Precipitate the double-stranded DNA by adding 2 volumes of ethanol. Mix the contents of the tube by vortexing and then allow the mixture to stand for 2 minutes at room temperature.
9. Recover the DNA by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.
10. Remove the supernatant by gentle aspiration. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Remove any drops of fluid adhering to the walls of the tube.
    An additional ethanol precipitation step here helps to ensure that the double-stranded DNA is efficiently cleaved by restriction enzymes.
    - *Dissolve the pellet of RF DNA in 100 µl of TE (pH 8.0).*
    - *Add 50 µl of 7.5 M ammonium acetate, mix well, and add 300 µl of ice-cold ethanol.*
    - *Store the tube for 15 minutes at room temperature or overnight at -20°C and then collect the precipitated DNA by centrifugation at maximum speed for 5-10 minutes at 4°C in a microfuge. Remove the supernatant by gentle aspiration.*
    - *Rinse the pellet with 250 µl of ice-cold 70% ethanol, centrifuge again for 2-3 minutes, and discard the supernatant.*
    - *Allow the pellet of DNA to dry in the air for 10 minutes and then dissolve the DNA as described in Step 12.*
11. Add 1 ml of 70% ethanol at 4°C and centrifuge again for 2 minutes. Remove the supernatant as described in Step 10, and allow the pellet of nucleic acid to dry in the air for 10 minutes.
12. To remove RNA, resuspend the pellet in 25 µl of TE (pH 8.0) with RNase. Vortex briefly.
13. Analyze the double-stranded RF DNA by digestion with appropriate restriction endonucleases followed by electrophoresis through an agarose gel.

REFERENCES

1. Birnboim H.C. and Doly J. 1979. A rapid extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523.
2. Ish-Horowicz D. and Burke J.F. 1981. Rapid and efficient cosmid cloning. *Nucleic Acids Res.* 9:2989-2998.

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Protocol 4

Preparation of Single-stranded Bacteriophage M13 DNA

Bacteriophage M13 single-stranded DNA is prepared from virus particles secreted by infected cells into the surrounding medium. The filamentous particles are concentrated by precipitation from a high-ionic-strength buffer with polyethylene glycol. Subsequent extraction with phenol releases the single-stranded DNA, which is then collected by precipitation with ethanol. This protocol is generally used to prepare single-stranded DNA from a small number of M13 isolates. Typically, the yield of single-stranded DNA is 5-10 µg/ml infected culture.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

Ethanol

⚠ Phenol

⚠ PEG 8000 (20% w/v) in 2.5 M NaCl

Sodium acetate (3 M, pH 5.2)

Gel-loading buffer IV

TE (pH 8.0)

Nucleic Acids and Oligonucleotides

Single-stranded bacteriophage M13 vector of recombinant DNA

Vectors and Bacterial Strains

E. coli cultures infected with bacteriophage M13

Prepare an infected culture as described in Chapter 3, Protocol 2. These cultures should be infected with both the hoped-for recombinant bacteriophage and a control culture infected with nonrecombinant bacteriophage.

E. coli cultures, uninfected

Prepare a mock-infected culture by picking an area of the E. coli *lawn from the plate that does not contain a plaque as a negative control. Use this culture to monitor the recovery of bacteriophage M13 particles.*

METHOD

1. Transfer 1 ml of the infected and uninfected cultures to separate microfuge tubes and centrifuge the tubes at maximum speed for 5 minutes at room temperature. Transfer each supernatant to a fresh microfuge tube at room temperature.
2. To the supernatant add 200 µl of 20% PEG in 2.5 M NaCl. Mix the solution well by inverting the tube several times, followed by gentle vortexing. Allow the tube to stand for 15 minutes at room temperature.
3. Recover the precipitated bacteriophage particles by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.
4. Carefully remove all of the supernatant using a disposable pipette tip linked to a vacuum line or a drawn-out Pasteur pipette attached to a rubber bulb. Centrifuge the tube again for 30 seconds and remove any residual supernatant.
   A tiny, pinhead-sized, pellet of precipitated bacteriophage particles should be visible at the bottom of the tube. No pellet should be visible in the negative control tube in which a portion of the uninfected E. coli *lawn was inoculated.*
5. Resuspend the pellet of bacteriophage particles in 100 µl of TE (pH 8.0) by vortexing.
   It is important to resuspend the bacteriophage pellet completely to allow efficient extraction of the single-stranded DNA by phenol in the next step.
6. To the resuspended pellet add 100 µl of equilibrated phenol. Mix well by vortexing for 30 seconds. Allow the sample to stand for 1 minute at room temperature, and then vortex for another 30 seconds.
7. Centrifuge the sample at maximum speed for 3-5 minutes at room temperature in a microfuge. Transfer as much as is easily possible of the upper, aqueous phase to a fresh microfuge tube.
   Do not try to transfer all of the aqueous phase. Much cleaner preparations of single-stranded DNA are obtained when approx. 5 µl of the aqueous phase is left at the interface.
8. Recover the M13 DNA by standard precipitation with ethanol in the presence of 0.3 M sodium acetate. Vortex briefly to mix, and incubate the tubes for 15-30 minutes at room temperature or overnight at -20°C.
9. Recover the precipitated single-stranded bacteriophage DNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge.
10. Remove the supernatant by gentle aspiration, being careful not to disturb the DNA pellet (which is often only visible as a haze on the side of the tube). Centrifuge the tube again for 15 seconds and remove any residual supernatant.
11. Add 200 µl of cold 70% ethanol and centrifuge at maximum speed for 5-10 minutes at 4°C. Immediately remove the supernatant by gentle aspiration.
    At this stage, the pellet is not firmly attached to the wall of the tube. It is therefore important to work quickly and carefully to avoid losing the DNA.
12. Invert the open tube on the bench for 10 minutes to allow any residual ethanol to drain and evaporate. Dissolve the pellet in 40 µl of TE (pH 8.0). Warm the solution to 37°C for 5 minutes to speed dissolution of the DNA. Store the DNA solutions at -20°C.
    The yield of single-stranded DNA is usually 5-10 µg/ml of the original infected culture.
13. Estimate the DNA concentration of a few of the samples by mixing 2-µl aliquots of the DNA from Step 12 each with 1 µl of sucrose gel-loading buffer. Load the samples into the wells of a 1.2% agarose gel cast in 0.5x TBE and containing 0.5 µg/ml ethidium bromide. As controls, use varying amounts of M13 DNA preparations of known concentrations. Examine the gel after electrophoresis for 1 hour at 6 V/cm. Estimate the amount of DNA from the intensity of the fluorescence.
    Usually 2-3 µl of a standard bacteriophage M13 DNA preparation is required for each set of four dideoxy cycle sequencing reactions using dye primers.

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1. Messing J. 1983. New M13 vectors for cloning. *Methods Enzymol.* 101:20-78.
2. Sanger F., Coulson A.R., Barrell B.G., Smith A.J., and Roe B.A. 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* 143:161-178.

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Protocol 5

Large-scale Preparation of Single-stranded and Double-stranded Bacteriophage M13 DNA

This protocol, a scaled-up version of Chapter 3, Protocol 3 and Chapter 3, Protocol 4 , is used chiefly to generate large stocks of double-stranded DNA of strains of M13 that are routinely used as cloning vectors. Large amounts of single-stranded DNA of an individual recombinant may occasionally be needed for specific purposes, e.g., to generate many preparations of a particular radiolabeled probe or to construct large numbers of site-directed mutants.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol

NaCl (solid)

⚠ Phenol

⚠ Phenol:chloroform (1:1, v/v)

⚠ PEG 8000 (20% w/v) in H_2O

Sodium acetate (3 M, pH 5.2)

STE

TE (pH 8.0)

Tris-Cl (10 mM, pH 8.0)

Media

Rich M13 medium containing 5 mM $MgCl_2$

Transfer 250 ml of the medium into a 2-liter flask and warm to 37°C before Step 2.

Additional Reagents

Step 5 of this protocol requires the reagents listed in Chapter 1, Protocol 3 , Chapter 1, Protocol 8 , Chapter 1, Protocol 9 , and Chapter 1, Protocol 10 .

Vectors and Bacterial Strains

E. coli F´ plating bacteria

Bacteriophage M13 Stock

METHOD

1. Transfer 2.5 ml of plating bacteria (please see Chapter 3, Protocol 1) to a sterile tube (13 x 100 mm or 17 x 100 mm). Add 0.5 ml of bacteriophage M13 stock (approx. 5 x 10^{11} pfu) and mix by tapping the side of the tube. Incubate the infected cells for 5 minutes at room temperature.
2. Dilute the infected cells into 250 ml of fresh LB or YT medium containing 5 mM $MgCl_2$ prewarmed to 37°C in a 2-liter flask. Incubate for 5 hours at 37°C with constant, vigorous shaking.
3. Harvest the infected cells by centrifugation at 4000*g* (5000 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C. Recover the supernatant, which may be used for large-scale preparations of single-stranded bacteriophage M13 DNA, as described in Steps 7-17 below.
4. Resuspend the bacterial pellet in 100 ml of ice-cold STE. Recover the washed cells by centrifugation at 4000*g* (5000 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C.
5. Isolate the bacteriophage M13 closed circular RF DNA by the alkaline lysis method (please see Chapter 1, Protocol 3). Scale up the volumes of lysis solutions appropriately. Purify the DNA either by precipitation with PEG, by column chromatography, or by equilibrium centrifugation in CsCl-ethidium bromide gradients.
6. Measure the concentration of the DNA spectrophotometrically and confirm its integrity by agarose gel electrophoresis. Store the closed circular DNA in small (1-5 µg) aliquots at -20°C.
7. To isolate single-stranded DNA from the bacteriophage particles in the infected cell medium, transfer the 250-ml supernatant from Step 3 to a 500-ml beaker containing a magnetic stirring bar.
8. Add 10 g of PEG and 7.5 g of NaCl to the supernatant. Stir the solution for 30-60 minutes at room temperature.
9. Collect the precipitate by centrifugation at 10,000*g* (7800 rpm in a Sorvall GSA rotor) for 20 minutes at 4°C. Invert the centrifuge bottle for 2-3 minutes to allow the supernatant to drain, and then use Kimwipes to remove the last traces of supernatant from the walls and neck of the bottle.
   Avoid touching the thin whitish film of precipitated bacteriophage particles on the side and bottom of the centrifuge bottle.
10. Add 10 ml of 10 mM Tris-Cl (pH 8.0) to the bottle. Swirl the solution in the bottle and use a Pasteur pipette to rinse the sides of the bottle thoroughly. When the bacteriophage pellet is dissolved, transfer the solution to a 30-ml Corex centrifuge tube.
11. To the bacteriophage suspension, add an equal volume of equilibrated phenol, seal the tube with a silicon rubber stopper, and mix the contents by vortexing vigorously for 2 minutes.
12. Centrifuge the solution at 3000*g* (5000 rpm in a Sorvall SS-34 rotor) for 5 minutes at room temperature. Transfer the upper aqueous phase to a fresh tube and repeat the extraction with 10 ml of phenol:chloroform.
    If there is a visible interface between the organic and aqueous layers, then extract the aqueous supernatant once more with chloroform.
13. Transfer equal amounts of the aqueous phase to each of two 30-ml Corex tubes. Add 0.5 ml of 3 M sodium acetate (pH 5.2) and 11 ml of ethanol to each tube. Mix the solutions well and then store them for 15 minutes at room temperature.
14. Recover the precipitate of single-stranded DNA by centrifugation at 12,000*g* (10,000 rpm in a Sorvall SS-34 rotor) for 20 minutes at 4°C. Carefully remove all of the supernatant.
    Most of the precipitated DNA will collect in a thin film along the walls of the centrifuge tubes.
15. Add 30 ml of 70% ethanol at 4°C to each tube, and centrifuge at 12,000*g* (10,000 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C. Carefully remove as much of the supernatant as possible, invert the tubes to allow the last traces of supernatant to drain away from the precipitate, and wipe the neck of the tubes with Kimwipes.
16. Allow the residual ethanol to evaporate at room temperature. Dissolve the pellets in 1 ml of TE (pH 8.0). Store the DNA at -20°C.
17. Measure the concentration of the DNA spectrophotometrically and confirm its integrity by agarose gel electrophoresis. Store the closed circular DNA in small (10-50 µg) aliquots at -20°C.

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Protocol 6

Cloning into Bacteriophage M13 Vectors

This protocol describes three standard methods to construct bacteriophage M13 recombinants: (1) ligating insert DNA to a linearized vector, prepared by cleavage of M13 RF with a single restriction enzyme; (2) using alkaline phosphatase to suppress self-ligation of the linearized vector, and (3) using M13 RF cleaved with two restriction enzymes for directional cloning.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)

Ethanol

IPTG (20% w/v)

⚠ Phenol:chloroform (1:1, v/v)

Sodium acetate (3 M, pH 5.2)

TE (pH 7.6 and pH 8.0)

⚠ X-gal solution (2% w/v)

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Restriction endonucleases
The choice of restriction enzymes to be used in Steps 1 and 6 depends on the cloning strategy.

Nucleic Acids and Oligonucleotides

Foreign DNA
Individual fragments of foreign DNA to be cloned in M13 vectors are usually derived from a larger segment of DNA that has already been cloned and characterized in another vector.

Test DNA

Media

Rich M13 agar plates

Rich M13 medium

Rich M13 top agar or agarose

Additional Reagents

Step 4 of this protocol requires the reagents listed in Chapter 1, Protocol 20 .

Vectors and Bacterial Strains

Bacteriophage M13 vector DNA (RF)

E. coli competent cells of an appropriate strain carrying an F´ plasmid
Competent cells may be prepared in the laboratory as described in Chapter 1, Protocol 25 or purchased from commercial suppliers.

E. coli F´ plating bacteria
Plating bacteria may be prepared in the laboratory as described in Chapter 3, Protocol 1 or purchased from commercial suppliers.

METHOD

1. Digest 1-2 µg of the bacteriophage M13 vector RF DNA to completion with a three- to fivefold excess of the appropriate restriction enzyme(s). Set up a control reaction containing M13 RF DNA but no restriction enzyme(s).
2. At the end of the incubation period, remove a small sample of DNA (50 ng) from each of the reactions and analyze the extent of digestion by electrophoresis through an 0.8% agarose gel. If digestion is incomplete (i.e., if any closed circular DNA is visible), add more restriction enzyme(s) and continue the incubation.
3. When digestion is complete, purify the M13 DNA by extraction with phenol:chloroform followed by standard precipitation with ethanol in the presence of 0.3 M sodium acetate (pH 5.2). Dissolve the DNA in TE (pH 8.0) at a concentration of 50 µg/ml.
4. If required, dephosphorylate the linearized vector DNA by treatment with calf alkaline phosphatase or shrimp alkaline phosphatase. At the end of the dephosphorylation reaction, inactivate the alkaline phosphatase by heat and/or by digestion with proteinase K, followed by extraction with phenol:chloroform (for details, please see Chapter 1, Protocol 20).
5. Recover the linearized M13 DNA as outlined in Step 3. Dissolve the dephosphorylated DNA in TE (pH 7.6) at a concentration of 50 µg/ml.
6. Generate individual restriction fragments of foreign DNA by cleavage with the appropriate restriction enzymes and purify them by agarose gel electrophoresis. Dissolve the final preparation of foreign DNA in TE (pH 7.6) at a concentration of 50 µg/ml.
   When ligating DNAs with complementary cohesive termini, please follow Steps 7-9 below. For methods to set up blunt-ended ligation reactions, please see Chapter 1, Protocol 19.

7. In a microfuge tube (Tube A), mix together approx. 50 ng of vector DNA and a one- to fivefold molar excess of the target (foreign) DNA fragment(s). The combined volume of the two DNAs should not exceed 8 µl. If necessary, add TE (pH 7.6) to adjust the volume to 7.5-8.0 µl. As controls, set up three ligation reactions containing:

Tube	DNA
B	the same amount of vector DNA, but no foreign DNA
C	the same amount of vector DNA and a one- to fivefold molar excess of the target DNA fragment(s)
D	the same amount of vector DNA together with an equal amount by weight of a test DNA that has been successfully cloned into bacteriophage M13 on previous occasions

   As a test DNA, we routinely use a standard preparation of bacteriophage λ DNA cleaved with restriction enzymes that recognize tetranucleotide sequences and generate termini that are complementary to the M13 vectors to be used.
8. Add 1 µl of 10x ligation buffer and 1 µl of 10 mM ATP to all four reactions (Tubes A-D).

Omit ATP if using a commercial buffer that contains ATP.

9. Add 0.5 Weiss unit of bacteriophage T4 DNA ligase to Tubes A, B, and D. Mix the components by gently tapping the side of each tube for several seconds. Incubate the ligation reactions for 4-16 hours at 12-16°C.
   At the end of the ligation reaction, analyze 1 µl of each ligation reaction by electrophoresis through an 0.8% agarose gel. Bands of circular recombinant molecules containing vector and fragment(s) of foreign DNA should be visible in the test reaction (Tube A) but not in the control (Tube C).
   After ligation, the reactions may be stored at -20°C until transformation.
10. Prepare and grow an overnight culture of plating bacteria (please see Chapter 3, Protocol 1) in YT or LB medium at 37°C with constant shaking.
11. Remove from the -70°C freezer an aliquot of frozen competent cells of the desired strain carrying an F´ plasmid, allow the cells to thaw at room temperature, and then place them on ice for 10 minutes.
12. Transfer 50-100 µl of the competent F´ bacteria to each of 16 sterile 5-ml culture tubes (Falcon 2054, Becton Dickinson) that have been chilled to 0°C.
13. Immediately add 0.1-, 1.0-, and 5-µl aliquots of the ligation reactions and controls (Tubes A-D) to separate tubes of competent cells. Mix the DNAs with the bacteria by tapping the sides of the tubes gently for a few seconds. Store on ice for 30-40 minutes. Include two transformation controls, one containing 5 pg of bacteriophage M13 RF DNA and the other containing no added DNA.
14. While the ligated DNA is incubating with the competent cells, prepare a set of 16 sterile culture tubes containing 3 ml of melted YT or LB top agar. Store the tubes at 47°C in a heating block or water bath until needed in Step 16.
15. Transfer the tubes containing the competent bacteria and DNA to a water bath equilibrated to 42°C. Incubate the tubes for exactly 90 seconds. Immediately return the tubes to an ice-water bath.
16. Add 40 µl of 2% X-gal, 4 µl of 20% IPTG, and 200 µl of the overnight culture of *E. coli* cells (Step 10) to the tubes containing the melted top agar prepared in Step 14, and mix the contents of the tubes by gentle vortexing for a few seconds. Transfer each sample of the transformed bacteria to the tubes. Cap the tubes and mix the contents by gently inverting the tubes three times. Pour the contents of each tube in turn onto a separate labeled LB agar plate. Swirl the plate to ensure an even distribution of bacteria and top agar.
17. Close the plates and allow the top agar to harden for 5 minutes at room temperature. Use a Kimwipe to remove any condensation from the top of the plate, invert the plates, and incubate at 37°C.
    Plaques will be fully developed after 8-12 hours.

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Protocol 7

Analysis of Recombinant Bacteriophage M13 Clones

A rapid method to analyze the size of the single-stranded DNA of M13 recombinants.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ SDS (2% w/v)

20x SSC

Gel-loading buffer IV

Nucleic Acids and Oligonucleotides

Single-stranded recombinant bacteriophage M13 DNA
Choose previously characterized recombinants that carry foreign sequences of known size to use as positive controls during gel electrophoresis.

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 3, Protocol 2.

Step 7 of this protocol may require the reagents listed in Chapter 2, Protocol 21 and Chapter 2, Protocol 22.

Vectors and Bacterial Strains

Bacteriophage M13 recombinant plaques in top agarose
Prepared as described in Chapter 3, Protocol 6 .

Bacteriophage M13 nonrecombinant vector, grown as well-isolated plaques in top agarose

E. coli F´ strain

METHOD

1. Prepare stocks of putative recombinant bacteriophages from single plaques, grown in an appropriate F´ host, as described in Chapter 3, Protocol 2 .
   As controls, prepare stocks of several nonrecombinant bacteriophages (picked from well-isolated dark blue plaques).
2. Use a micropipettor with a sterile tip to transfer 20 µl of each of the supernatants into a fresh microfuge tube. Store the remainder of the supernatants at 4°C until needed.
3. To each 20-µl aliquot of supernatant, add 1 µl of 2% SDS. Tap the sides of the tubes to mix the contents, and then incubate the tubes for 5 minutes at 65°C.
4. To each tube, add 5 µl of sucrose gel-loading buffer. Again mix the contents of the tubes by tapping and then analyze each sample by electrophoresis through an 0.7% agarose gel. Run the gel at 5 V/cm. As positive controls, use single-stranded DNA preparations of previously characterized M13 recombinants that carry foreign sequences of known size.
5. When the bromophenol blue has traveled the full length of the gel, photograph the DNA under UV illumination.
6. Compare the electrophoretic mobilities of the single-stranded DNAs liberated from the putative recombinants with those of the DNAs liberated from the control nonrecombinant bacteriophages.
   The single-stranded DNAs of recombinants carrying sequences of foreign DNA longer than 200-300 nucleotides migrate slightly more slowly than empty vector through 0.7% agarose gels. Once recombinants of the desired size have been identified, single-stranded DNAs can be prepared from supernatants stored at 4°C (Step 2).
7. If necessary, confirm the presence of foreign DNA sequences by transferring single-stranded DNAs from the gel to a nitrocellulose or nylon membrane (please see Chapter 2, Protocol 21) and hybridizing to an appropriate radiolabeled probe (please see Chapter 2, Protocol 22). Soak the gel in 10 volumes of 20x SSC for 45 minutes, and then transfer the DNA directly to the membrane.

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Protocol 8

Producing Single-stranded DNA with Phagemid Vectors

This protocol describes methods to superinfect bacteria carrying a recombinant phagemid with a high-titer stock of an appropriate helper virus and to assay the yield of filamentous virus particles that carry single-stranded copies of the phagemid DNA. The key to success in using phagemids is to prepare a stock of helper virus whose titer is accurately known.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Kanamycin (10 mg/ml)

⚠ SDS (2% w/v)

Gel-loading buffer IV

Media

M9 minimal agar plates, supplemented
This medium is needed when using E. coli *strains that carry a deletion of the proline biosynthetic operon (Δ[lac-proAB]) in the bacterial chromosome and the complementing proAB genes on the F´ plasmid.*

YT agar plates containing 60 µg/ml ampicillin

2x YT

2x YT containing 60 µg/ml ampicillin

2x YT containing 60 µg/ml kanamycin

Additional Reagents

Steps 2 and 5 of this protocol require the reagents listed in Chapter 3, Protocol 1 .

Step 14 of this protocol requires the reagents listed in Chapter 3, Protocol 4 .

Vectors and Bacterial Strains

Bacteriophage M13K07 (helper)
M13K07 may be obtained commercially (e.g., from Pharmacia or New England Biolabs) and propagated as described in Steps 1-3 below. Store stocks of helper virus at 4°C in growth medium or at -20°C in growth medium containing 50% (v/v) glycerol.

E. coli F´ strain

E. coli strain DH11S
DH11S should be plated on supplemented minimal agar plates.

E. coli strain DH11S, transformed with bacteriophage M13 phagemid vector
Transform E. coli *with the phagemid vector as described in Chapter 3, Protocol 6 . The transformed strain may be propagated as a culture as described in Chapter 3, Protocol 2 .*

E. coli strain DH11S, transformed with bacteriophage M13 recombinant phagemid vector clone carrying foreign DNA
Transform E. coli *with the recombinant phagemid vector as described in Chapter 3, Protocol 6 . The transformed strain may be propagated as a culture as described in Chapter 3, Protocol 2 .*

METHOD

1. In 20 ml of 2x YT medium, establish a culture of *E. coli* strain DH11S from a single colony freshly picked from supplemented minimal agar plates. Incubate the culture at 37°C with moderate agitation until the OD_{600} reaches 0.8.
2. Prepare a series of tenfold dilutions of bacteriophage M13K07 in 2x YT medium, and plate aliquots of the bacteriophage as described in Chapter 3, Protocol 1 to obtain well-isolated plaques on a lawn of DH11S cells.
3. Pick well-separated, single plaques and place each plaque in 2-3 ml of 2x YT medium containing kanamycin (25 µg/ml) in a 15-ml culture tube. Incubate the infected cultures for 12-16 hours at 37°C with moderate agitation (250 cycles/minute).
   *Kanamycin is used in this protocol to ensure that all bacterial cells containing a phagemid genome are infected by the helper M13K07 bacteriophage. During propagation of M13K07 (e.g., Steps 1-3), there is selection for bacteriophage genomes that have lost the p15A origin and the Tn*903 *transposon. For this reason, it is essential to include kanamycin in the medium used to prepare the stock of helper virus in this step.*
   IMPORTANT *Use stocks of M13K07 derived from single freshly picked plaques in the following steps.*
4. Transfer the infected cultures to 1.5-ml sterile microfuge tubes and centrifuge them at maximum speed for 2 minutes at 4°C in a microfuge. Transfer the supernatants to fresh tubes and store them at 4°C.
5. Measure the titer of each of the bacteriophage stocks by plaque formation (Chapter 3, Protocol 1) on a strain of *E. coli* F´ (TG1, DH11S, NM522, or XL1-Blue) that supports the growth of bacteriophage M13.
   The titer of infectious bacteriophage particles in the stocks should be 10^{10} pfu/ml. Discard any stock with a lower titer.
6. Streak DH11S cells transformed by (i) the recombinant phagemid and (ii) the empty (parent) phagemid vector onto two separate YT agar plates containing 60 µg/ml ampicillin. Incubate the plates for 16 hours at 37°C.
7. Pick (i) several colonies transformed by the recombinant phagemid and (ii) one or two colonies transformed by the parent vector into sterile 15-ml culture tubes that contain 2-3 ml of 2x YT medium containing 60 µg/ml ampicillin.
8. To each culture, add M13K07 helper bacteriophage to achieve a final concentration of 2×10^7 pfu/ml. Incubate the cultures for 1.0-1.5 hours at 37°C with strong agitation (300 cycles/ minute).
9. Add kanamycin to the cultures to a final concentration of 25 µg/ml. Continue incubation for a further 14-18 hours at 37°C.
10. Transfer the cell suspensions to microfuge tubes and separate the bacterial cells from the growth medium by centrifugation at maximum speed for 5 minutes at room temperature in a microfuge. Transfer the supernatants to fresh tubes and store them at 4°C.
11. Combine 40 µl of each supernatant with 2 µl of 2% SDS in 0.5-ml microfuge tubes. Mix the contents of the tubes by tapping and then incubate the tubes for 5 minutes at 65°C.
12. Add 5 µl of sucrose gel-loading buffer to each sample of the phagemid DNA, mix the samples, and load them into separate wells of an 0.7% agarose gel.
13. Carry out electrophoresis for several hours at 6 V/cm until the bromophenol blue has migrated approximately half the length of the gel. Examine and photograph the gel by UV light.
    Yields vary depending on the size and nature of foreign DNA in the phagemid, but are generally approx. 1 µg/ml of culture volume.
14. Isolate single-stranded phagemid DNA from the supernatants containing the largest amount of single-stranded DNA. Follow the steps outlined in Chapter 3, Protocol 4 , scaling up the volumes two- to threefold.

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Chapter 4 Working with High-Capacity Vectors

Protocol 1: Construction of Genomic DNA Libraries in Cosmid Vectors

The protocol describes how to construct a library of 35-45-kb fragments of genomic DNA in the double *cos* site cosmid vector, SuperCos-1. The steps include:
• Linearization and dephosphorylation of SuperCos-1 DNA.
• Partial digestion of high-molecular-weight DNA with *Mbo*I.
• Dephosphorylation of high-molecular-weight genomic DNA.
• Ligation of cosmid arms to genomic DNA: Packaging and plating recombinants.
• Isolation and analysis of recombinant cosmids: Validation of the library.

Protocol 2: Screening an Unamplified Cosmid Library by Hybridization: Plating the Library onto Filters

Unamplified cosmid libraries are plated at high density onto 150-mm nitrocellulose or nylon filters and screened by hybridization.

Protocol 3: Amplification and Storage of a Cosmid Library: Amplification in Liquid Culture

Amplification of cosmid libraries may result in distorted representation of cloned genomic sequences and should be avoided wherever possible. If amplification should become necessary, it is best to expand the library by growth in liquid medium, as described here.

Protocol 4: Amplification and Storage of a Cosmid Library: Amplification on Filters

Amplification of cosmid libraries may result in distorted representation of cloned genomic sequences and should be avoided wherever possible. In this method of amplification, distortion of the library is rarely a problem because at no stage are bacteria containing different recombinant cosmids grown in competition with one another. The major problem with this method is its tediousness.

Protocol 5: Working with Bacteriophage P1 and Its Cloning Systems

This protocol describes methods for recovery and purification of recombinant clones of bacteriophage P1 or PAC DNAs from bacteria. Because of their large size, these DNAs are sensitive to shearing forces and must be handled carefully. This protocol generally yields P1 DNA that works well as a substrate or template in enzymatic reactions.

Protocol 6: Transferring Bacteriophage P1 Clones between *E. coli* Hosts

Problems with low yield or poor quality can sometimes be overcome by transferring the P1 or PAC recombinant into a strain of *E. coli* that does not express Cre recombinase. This protocol for transfer of P1 clones by electroporation was supplied by Ray MacDonald (University of Texas Southwestern Medical Center, Dallas).

Protocol 7: Working with Bacterial Artificial Chromosomes

This protocol describes how to transform *E. coli* with BAC DNA by electroporation.

Protocol 8: Isolation of BAC DNA from Small-scale Cultures

BAC DNAs are prepared from 5-ml cultures of BAC-transformed cells by a modification of the standard alkaline lysis method (Chapter 1, Protocol 1). The yield typically varies between 0.1 and 0.4 µg of BAC DNA.

Protocol 9: Isolation of BAC DNA from Large-scale Cultures

The procedure for isolation of BAC DNA is scaled-up to accommodate 500-ml cultures, which, on average, yield 20-25 µg of purified BAC DNA.

Protocol 10: Working with Yeast Artificial Chromosomes

This protocol outlines a procedure to validate and store yeast strains carrying YACs.

Protocol 11: Growth of *S. cerevisiae* and Preparation of DNA

This protocol describes methods for isolation of DNA from a strain of *S. cerevisiae* carrying a recombinant YAC. Because the linear YAC DNAs are sensitive to shearing forces, pipettes with wide-bore tips should be used to transfer DNAs. The method is suitable for preparing DNA that will be used for agarose gel electrophoresis, Southern blotting, subcloning, genomic library construction, PCR, or other methods that do not require intact high-molecular-weight DNA. The expected yield from a 10-ml culture is 2-4 µg of yeast DNA.

Protocol 12: Small-scale Preparations of Yeast DNA

Yeast DNA is prepared by digestion of the cell wall and lysis of the resulting spheroplasts with SDS. This method reproducibly yields several micrograms of yeast DNA that can be efficiently cleaved by restriction enzymes and used as a template in PCR.

Protocol 13: Analyzing Yeast Colonies by PCR

Yeast colonies are suspended in complete PCR buffer and transferred to a thermal cycler for 35 cycles of PCR. The products of the amplification reaction are analyzed by gel electrophoresis.

Protocol 14: Isolating the Ends of Genomic DNA Fragments Cloned in High-capacity Vectors: Vectorette Polymerase Chain Reactions

This protocol describes the use of vectorette PCR and single-site PCR to amplify the terminal sequences of genomic sequences cloned in high-capacity vectors such as PACs and YACs.

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Protocol 1

Construction of Genomic DNA Libraries in Cosmid Vectors

The protocol describes how to construct a library of 35-45-kb fragments of genomic DNA in the double *cos* site cosmid vector, SuperCos-1. The steps include:
• Linearization and dephosphorylation of SuperCos-1 DNA.
• Partial digestion of high-molecular-weight DNA with *Mbo*I.
• Dephosphorylation of high-molecular-weight genomic DNA.
• Ligation of cosmid arms to genomic DNA: Packaging and plating recombinants.
• Isolation and analysis of recombinant cosmids: Validation of the library.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform
10x CIP Dephosphorylation buffer
Ethanol
⚠ Phenol:chloroform (1:1, v/v)
SM
Sodium acetate (3 M, pH 5.2)
TE (pH 8.0)

Enzymes and Buffers

Bacteriophage T4 DNA ligase
Calf intestinal phosphatase (CIP)
Restriction endonucleases: *Bam*HI, *Mbo*I, *Xba*I
Restriction endonucleases that cleave cosmid vector but not the genomic insert DNA

Media

TB agar plates containing 25 µg/ml kanamycin
TB
TB containing 25 µg/ml kanamycin

Nucleic Acids and Oligonucleotides

Control DNA: bacteriophage λ DNA digested with *Hin*dIII
Control DNA: superhelical SuperCos-1 DNA
High-molecular-weight genomic DNA
Linearized plasmid DNA
Marker DNA: linear bacteriophage λ DNA

Additional Reagents

Step 10 of this protocol requires the reagents listed in Chapter 2, Protocol 17 .
Step 23 of this protocol requires the reagents listed in Chapter 1, Protocol 1 .

Vectors and Bacterial Strains

Bacteriophage λ packaging mixtures
Bacteriophage λ stock
E. coli plating bacteria
Use the appropriate strain for titering packaged cosmid (e.g., XL1-Blue, ED8767, NM554, DH5αMCR).
SuperCos-1 DNA

METHOD

1. Combine 20 µg of SuperCos-1 DNA with 50 units of *Xba*I in a volume of 200 µl of 1x *Xba*I digestion buffer and incubate the reaction mixture for 2-3 hours at 37°C.
2. After 2 hours of incubation, transfer an aliquot (approx. 1 µl) of the reaction mixture to a fresh tube. Analyze the aliquot of cosmid DNA by electrophoresis through an 0.8% agarose gel, using as controls (i) 50-100 ng of superhelical SuperCos-1 DNA and (ii) 50-100 ng of a bacteriophage λ DNA digested with *Hin*dIII.
3. Extract the digestion reaction once with phenol:chloroform and once with chloroform.
4. Transfer the aqueous phase to a fresh tube and recover the linearized cosmid DNA by standard precipitation with ethanol and subsequent washing in 70% ethanol. Store the open tube in an inverted position on a bed of paper towels to allow the ethanol to drain and evaporate. Dissolve the damp pellet of DNA in 180 µl of H_2O. Remove a 100-ng aliquot of the DNA for use as a control.
5. Add 20 µl of 10x dephosphorylation buffer to the remainder of the DNA solution. Add 0.1 unit of CIP and incubate the reaction for 30 minutes at 37°C. Add a second aliquot (0.1 unit) of CIP and continue digestion for an additional 30 minutes. Transfer the reaction to a water bath set at 65°C and incubate for 30 minutes to inactivate CIP. Remove two 100-ng aliquots of the DNA for use as controls.
6. Extract the reaction mixture once with phenol:chloroform and once with chloroform. Recover the linearized, dephosphorylated SuperCos-1 DNA by standard precipitation with ethanol and subsequent washing in 70% ethanol. Dissolve the damp pellet of DNA in 180 µl of H_2O.
7. Transfer an aliquot of the dephosphorylated DNA (50-100 ng) to a fresh microfuge tube and store it on ice. Add 20 µl of 10x *Bam*HI restriction buffer to the remainder of the desphosphorylated DNA. Add 40 units of *Bam*HI and incubate the reaction for 2-3 hours at 37°C.
8. After 2 hours of incubation, remove a second aliquot of DNA to a separate microfuge tube. Analyze both aliquots of DNA by agarose gel electrophoresis. After digestion with *Bam*HI, the linear 7.9-kb fragment of dephosphorylated SuperCos-1 DNA should be quantitatively cleaved into two DNA fragments of approx. 1.1 kb and approx. 6.8 kb.
If traces of the 7.9-kb DNA are still visible, add 10 more units of Bam*HI to the digest and continue incubation at 37°C until the reaction has gone to completion.*
9. Extract the digestion reaction once with phenol:chloroform and once with chloroform. Recover the DNA by standard precipitation with ethanol followed by washing with 70% ethanol. Dissolve the damp pellet of DNA in 20 µl of H_2O and store the solution at 4°C until needed.
10. Establish the conditions for partial digestion of a 30-µg sample of high-molecular-weight genomic DNA with *Mbo*I. The aim is to establish conditions that produce the highest yield of DNA fragments with a modal size of 38-52 kb.

11. Using the conditions for partial digestion established in Step 10, set up three large-scale reactions each containing 100 µg of high-molecular-weight genomic DNA and amounts of *Mbo*I that bracket the optimal concentration, as determined in Step 10. At the end of the incubation period, check the size of an aliquot of each partially digested DNA by agarose gel electrophoresis.
12. Pool the two samples of partially digested genomic DNA that contain the highest amounts of DNA in the 38-52-kb range. Extract the pooled DNAs once with phenol:chloroform and once with chloroform. Recover the DNA by standard precipitation with ethanol and subsequent washing in 70% ethanol. Dissolve the damp pellet of DNA in 180 µl of TE (pH 8.0).
Resuspension is best accomplished by allowing the DNA pellet to soak in TE overnight at 4°C. Do not vortex the DNA. Instead, mix the DNA by gently tapping the sides of the tube. If only small amounts of DNA are available, as is the case when constructing cosmid libraries from flow-sorted eukaryotic chromosomes or from purified YACs, then, instead of ethanol precipitation, purify the DNA by drop dialysis against TE (pH 8.0) with floating membranes, as described in Chapter 4, Protocol 5 .
13. To the resuspended partially digested genomic DNA, add 20 µl of 10x dephosphorylation buffer and 2 units of CIP. Immediately withdraw a sample of DNA for use as a control:
    a. Remove an aliquot of the reaction containing 0.1-1.0 µg of DNA to a small (0.5 ml) microfuge tube containing 0.3 µg of a linearized plasmid in 1x dephosphorylation buffer (e.g., pUC cleaved with *Bam*HI).
    b. Set up a second control that contains 0.3 µg of the same linearized plasmid in 10 µl of 1x dephosphorylation buffer.
    Do not add CIP to this control.
    c. Follow the instructions in Steps 14-16.
14. Incubate the large-scale dephosphorylation reaction and the two controls for 30 minutes at 37°C. Then transfer the three reactions to a water bath set at 65°C and incubate them for 30 minutes to inactivate CIP.
15. Cool the reactions to room temperature. Purify the DNAs by extracting once with phenol:chloroform and once with chloroform. Recover the DNA by standard precipitation with ethanol and subsequent washing in 70% ethanol.
16. Dissolve the two control DNAs in 10 µl of 1x ligation buffer. Add 0.1 Weiss unit of bacteriophage T4 DNA ligase to each tube and incubate them for 3 hours at room temperature. Examine the ligated DNAs by agarose gel electrophoresis.
17. Allow the DNA in the large-scale reaction to dissolve overnight at 4°C in a small volume of H_2O. Aim for a final concentration of approx. 500 µg of DNA/ml. Estimate the concentration of DNA by agarose gel electrophoresis, or better, by measuring A_{260}.
18. Set up a series of ligation reactions (final volume 20 µl) containing:

cosmid arms DNA (Step 9)	2 µg
dephosphorylated genomic DNA (Step 17)	0.5, 1, or 2.5 µg
10x ligation buffer	2 µg
bacteriophage T4 DNA ligase	2 Weiss units

    Incubate the ligation reactions for 12-16 hours at 16°C.
19. Package 5 µl of each of the ligation reactions in bacteriophage λ particles (equivalent to 0.5 µg of vector arms) using a commercial packaging kit and following the conditions recommended by the supplier. After packaging, add 500 µl of SM and 20 µl of chloroform to the reactions and then store the diluted reactions at 4°C.
20. Measure the titer of the packaged cosmids in each of the packaging reactions by transduction into an appropriate *E. coli* host. Mix 0.1 ml of a 10^{-2} dilution of an aliquot of each reaction with 0.1 ml of SM and 0.1 ml of fresh plating bacteria. Allow the bacteriophage particles containing the recombinant cosmids to adsorb by incubating the infected bacterial cultures for 20 minutes at 37°C. Add 1 ml of TB medium and continue the incubation for a further 45 minutes at 37°C to allow expression of the kanamycin resistance gene in the SuperCos-1 vector.
Store the remainder of the packaging mixtures at 4°C until Steps 21-24 have been completed (2-3 days).
21. Spread 0.5 ml and 0.1 ml of the bacterial culture onto TB agar plates containing kanamycin (25 µg/ml). After incubating the plates overnight at 37°C, count the number of bacterial colonies.
22. Pick 12 individual colonies and grow small-scale (2.5 ml) cultures in TB containing 25 µg/ml kanamycin for periods of no longer than 6-8 hours. Shake the cultures vigorously during incubation.
23. Isolate cosmid DNA from 1.5 ml of each of the 12 small-scale bacterial cultures using the alkaline lysis method, described in Chapter 1, Protocol 1 .
24. Digest 2-4 µl of each of the DNA preparations with restriction enzymes (e.g., *Not*I and *Sal*I) that cleave the cosmid vector but are unlikely to cleave the cloned insert of genomic DNA. Analyze the sizes of the resulting fragments by electrophoresis through a 0.7% agarose gel.
Use as markers linear bacteriophage λ DNA and Hind*III fragments of bacteriophage λ DNA, which can be prepared easily in the laboratory and are also available commercially.*
25. Calculate the proportion of colonies that carry inserts.
26. Estimate the average size of the inserts by isolating a few dozen clones and measuring the size of inserts by PFGE.
27. Calculate the "depth" of the library, i.e., how many genome equivalents it contains.
If the library is satisfactory in size and quality, proceed to plate and screen the library by hybridization (Chapter 4, Protocol 2) or, alternatively, to amplify and store the library (Chapter 4, Protocol 3 and Chapter 4, Protocol 4).

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Protocol 2

Screening an Unamplified Cosmid Library by Hybridization: Plating the Library onto Filters

Unamplified cosmid libraries are plated at high density onto 150-mm nitrocellulose or nylon filters and screened by hybridization.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Media

- TB agar plates (150 mm) containing 25 µg/ml kanamycin
- TB

Additional Reagents

Step 16 of this protocol requires the reagents listed in Chapter 1, Protocol 31 and Chapter 1, Protocol 32 .

Vectors and Bacterial Strains

Bacteriophage λ packaging reaction
Please see Chapter 4, Protocol 1 , Step 19.

E. coli plating bacteria
Use the appropriate strain (e.g., XL1-Blue, ED8767, NM554, DH5αMCR).

METHOD

1. Calculate the volume of the packaging reaction (Chapter 4, Protocol 1 , Steps 20-21) that will generate 30,000-50,000 transformed bacterial colonies.
2. Set up a series of sterile test tubes containing this volume of packaging reaction and 0.2 ml of plating bacteria.
   The actual number of tubes to set up for plating the packaging reaction depends on the genome size, average size of fragments cloned, and the coverage of the genome required to find the particular clone of interest. In most cases, 15-20 tubes should be sufficient.
3. Incubate the tubes for 20 minutes at 37°C.
4. To each tube, add 0.5 ml of TB. Continue the incubation for a further 45 minutes.
5. Place sterile filters onto a series (equal in number to the series of tubes in Step 2) of 150-mm TB agar plates containing kanamycin (25 µg/ml).
6. Use a sterile spreader to smear the contents of each tube over the surface of a filter on an agar plate. After the inoculum has been absorbed into each filter, transfer the plates to a 37°C incubator for several hours to overnight (12-15 hours).
   Try to avoid spreading the inoculum within 3 mm of the edge of the master filters.
7. Place a sterile, numbered 137-mm filter on a fresh TB agar plate containing kanamycin (25 µg/ml).
8. Place a sterile Whatman No. 1 filter on a thick, sterile glass plate.
9. Use blunt-ended forceps to remove the replica filter from the fresh TB agar plate (Step 7) and place it on the Whatman No. 1 filter.
10. Again use forceps to remove a master filter now carrying transformed colonies from its TB agar plate (Step 6) and place it, colony side down, exactly on top of the numbered replica filter on the Whatman No. 1 filter. Cover the two filters with another sterile Whatman No. 1 filter.
11. Place a second sterile glass plate on top of the stack of filters. Press the plates together.
12. Remove the upper glass plate and the upper Whatman No. 1 filter. Use a 17-gauge hypodermic needle to key the two nitrocellulose or nylon filters to each other by making a series of holes (approx. 5 will do), placed asymmetrically around the edge of the filters.
13. Peel the two nitrocellulose or nylon filters apart, and working quickly, replace them on their TB agar plates containing kanamycin (25 µg/ml).
14. Incubate the master and replica filters for a few hours at 37°C, until the bacterial colonies are 0.5-1.0 mm in diameter.
15. Seal the master plates in Parafilm and store them at 4°C in an inverted position.
16. Lyse the colonies on the replica filter (Chapter 1, Protocol 31), and process the filters for hybridization to radiolabeled probes (Chapter 1, Protocol 32).
    Replica filters can be used to replicate the library again or may be stored frozen at -70°C.

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2. Hanahan D. and Meselson M. 1980. Plasmid screening at high colony density. *Gene* 10:63-67.

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Protocol 3

Amplification and Storage of a Cosmid Library: Amplification in Liquid Culture

Amplification of cosmid libraries may result in distorted representation of cloned genomic sequences and should be avoided wherever possible. If amplification should become necessary, it is best to expand the library by growth in liquid medium, as described here.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Glycerol

Media

- TB agar plates containing 25 µg/ml kanamycin
- TB
- TB containing 25 µg/ml kanamycin

Additional Reagents

Step 9 of this protocol requires reagents listed in Chapter 4, Protocol 2.

Step 9 of this protocol also requires the reagents listed in Chapter 1, Protocol 31 and Chapter 1, Protocol 32 .

Vectors and Bacterial Strains

Bacteriophage λ packaging reaction
Please see Chapter 4, Protocol 1 , Step 19.

E. coli plating bacteria
Use the appropriate strain (e.g., XL1-Blue, ED8767, NM554, DH5αMCR).

METHOD

1. Calculate the volume of the packaging reaction (Chapter 4, Protocol 1 , Steps 20-21) that will generate 30,000-50,000 transformed bacterial colonies.
2. Set up a series of sterile test tubes and into each tube deliver 0.2 ml of plating bacteria followed by the volume of packaging reaction determined in Step 1.
   The actual number of tubes to set up for plating the packaging reaction depends on the genome size, average size of fragments cloned, and the coverage of the genome required to find the particular clone of interest. In most cases, 15-20 tubes should be sufficient.
3. Incubate the tubes for 20 minutes at 37°C.
   *Large amounts of packaging mixture can inhibit attachment of the bacteriophage particles to the plating bacteria. If the concentration of packaged bacteriophages is low (<10^4 transducing units/ml of packaging mixture), use more plating bacteria, e.g., 5 ml/ml of packaging reaction. After incubating the cells for 20 minutes at 37°C, recover the bacteria by centrifugation at 5000*g *(6500 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C. Resuspend the cells in 0.5 ml of TB and proceed to Step 4.*
4. Add 0.5 ml of TB to each tube. Continue incubation for a further 45 minutes.
5. Inoculate 0.25-ml aliquots of each culture of infected cells into 100-ml volumes of TB medium containing 25 µg/ml kanamycin in 250-ml flasks.
6. Incubate the inoculated cultures with vigorous shaking at 37°C until the cells reach an optimal density of 0.5-1.0 OD_{600}.
7. Pool the cultures and recover the cells by centrifugation at 5000*g* (5500 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C. Resuspend the cells in a volume of TB that is equal to 0.1x the volume of the original pooled cultures.
   Cosmid DNA can be isolated (please see Step 23 in Chapter 4, Protocol 1) from aliquots of cells taken before the addition of glycerol. This stock of DNA can be used as a template in PCR to determine whether a particular DNA sequence of interest is present in the library.
8. Add sterile glycerol to the cell suspension to a final concentration of 15% (v/v). Mix the suspension well by inverting the closed tube several times. Dispense aliquots (0.5-1.0 ml) of the bacterial suspension into sterile vials. Store the tightly closed vials at -70°C.
9. To screen the library, thaw an aliquot of frozen cells rapidly at 37°C and plate 30,000-50,000 bacteria onto each of a series of numbered filters as described in Chapter 4, Protocol 2 beginning with Step 5. Proceed with lysing the colonies on the replica filters (Chapter 1, Protocol 31) and processing the filters for hybridization to labeled probes (Chapter 1, Protocol 32).

REFERENCES

1. Longmire L.L., Brown N.C., Meincke L.J., Campbell M.L., Albright K.L., Fawcett J.J., Campbell E.W., Moyzis R.K., Hildebrand C.E., Evans G.A. et al. 1993. Construction and characterization of partial digest DNA libraries made from flow-sorted human chromosome 16. *Genet. Anal. Tech. Appl.* 10:69-76.

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Protocol 4

Amplification and Storage of a Cosmid Library: Amplification on Filters

Amplification of cosmid libraries may result in distorted representation of cloned genomic sequences and should be avoided wherever possible. In this method of amplification, distortion of the library is rarely a problem because at no stage are bacteria containing different recombinant cosmids grown in competition with one another. The major problem with this method is its tediousness.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Media

- TB agar plates (150 mm) containing 25 µg/ml kanamycin
- TB

Additional Reagents

Step 7 of this protocol requires reagents listed in Chapter 4, Protocol 2 .

Step 8 of this protocol requires the reagents listed in Chapter 1, Protocol 31 and Chapter 1, Protocol 32 .

Vectors and Bacterial Strains

Bacteriophage λ packaging reaction
Please see Chapter 4, Protocol 1 , Step 19.

E. coli plating bacteria
Use the appropriate strain (e.g., XL1-Blue, ED8767, NM554, DH5αMCR).

METHOD

1. Calculate the volume of the packaging reaction (Chapter 4, Protocol 1 , Steps 20-21) that will generate 30,000-50,000 transformed bacterial colonies.
2. Set up a series of sterile test tubes and into each tube deliver 0.2 ml of plating bacteria followed by the volume of packaging reaction determined in Step 1.
   The actual number of tubes to set up for plating the packaging reaction depends on the genome size, average size of fragments cloned, and the coverage of the genome required to find the particular clone of interest. In most cases, 15-20 tubes should be sufficient.
3. Incubate the tubes for 20 minutes at 37°C.
   Large amounts of packaging mixture can inhibit attachment of the bacteriophage particles to the plating bacteria. If the concentration of packaged bacteriophages is low (<10^4 transducing units/ml of packaging mixture), use more plating bacteria, e.g., 5 ml/ml of packaging reaction. After incubating the cells for 20 minutes at 37°C, recover the bacteria by centrifugation at 5000g (5500 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C. Resuspend the cells in 0.5 ml of TB and proceed to Step 4.
4. Add 0.5 ml of TB to each tube. Continue incubation for a further 45 minutes.
5. Place sterile, numbered filters onto a series (equal in number to the series of tubes in Step 2) of 150-mm TB agar plates containing kanamycin (25 µg/ml).
6. Use a sterile spreader to smear the contents of each tube over the surface of a filter on an agar plate. After the inoculum has absorbed into each filter, transfer the plates to a 37°C incubator for several hours to overnight (12-15 hours).
7. Make a replica of each of the master filters as described beginning with Step 7 of Chapter 4, Protocol 2 . Store the filters at -70°C.
8. To screen the library, thaw the replica filters and proceed with lysing the colonies on the filters (Chapter 1, Protocol 31) and processing the filters for hybridization to labeled probes (Chapter 1, Protocol 32).

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Protocol 5

Working with Bacteriophage P1 and Its Cloning Systems

This protocol describes methods for recovery and purification of recombinant clones of bacteriophage P1 or PAC DNAs from bacteria. Because of their large size, these DNAs are sensitive to shearing forces and must be handled carefully. This protocol generally yields P1 DNA that works well as a substrate or template in enzymatic reactions.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Alkaline lysis solution I
- Alkaline lysis solution II
- Alkaline lysis solution III
- ⚠ Ammonium acetate (0.5 M)
- Ethanol
- IPTG (1 mM)
  Optional, please see note to Step 1.
- Isopropanol
- $MgCl_2$ (1 mM)
- ⚠ Phenol:chloroform (1:1, v/v)
- ⚠ PEG 8000 (40% w/v)
- Sodium acetate (0.3 M, pH 5.2)
- TE (pH 8.0)
- TE (pH 8.0) containing 20 µg/ml RNase

Enzymes and Buffers

- Restriction endonucleases

Media

- LB containing 25 µg/ml kanamycin
  Optional, use with IPTG in Step 1.
- TB containing 25 µg/ml kanamycin

Vectors and Bacterial Strains

- *E. coli* strain transformed with a nonrecombinant bacteriophage P1 or PAC vector (culture)
- *E. coli* strain transformed with a recombinant bacteriophage P1 or PAC vector (culture)

METHOD

1. Transfer 10 ml of TB containing 25 µg/ml kanamycin into each of two 50-ml Falcon tubes or Erlenmeyer flasks. Inoculate one tube with a single colony of bacteria containing the recombinant P1 or PAC. Inoculate the other tube with a single colony of bacteria transformed by the vector alone. Grow the cultures to saturation, with vigorous shaking for 12-16 hours at 37°C.
   The addition of IPTG (1 mM) to cultures of cells carrying P1 recombinants inactivates the lac *repressor and leads to induction of the P1 lytic replicon, which results in an increase in the copy number of the plasmid DNA from 1 to approx. 20 copies/cell.*
   For induction with IPTG, cultures are grown in LB containing 25 µg/ml kanamycin until the OD_{600} reaches approx. 0.8, at which point IPTG is added to a final concentration of 0.5-1 mM. The cells are incubated for a further 3 hours at 37°C and then harvested.
2. Transfer each culture to a 15-ml centrifuge tube. Harvest the cells by centrifugation at 3500*g* (5400 rpm in a Sorvall SS-34 rotor) for 5 minutes at 4°C. Resuspend each cell pellet in 3 ml of sterile H_2O and repeat the centrifugation step.
3. Resuspend each cell pellet in 2 ml of Alkaline lysis solution I and place on ice.
4. Add 3 ml of Alkaline lysis solution II, and gently invert the tube several times to mix the solutions. Transfer the tube to an ice bath for 10 minutes.
   IMPORTANT *Because recombinant P1 and PAC clones are large enough to be sensitive to shearing, keep vortexing, pipetting, and shaking to a minimum during the isolation of DNA. Wherever possible, transfer by pouring the DNA from one tube to another. When pipetting cannot be avoided, use wide-bore pipette tips.*
5. Add 3 ml of ice-cold Alkaline lysis solution III to each cell suspension, and mix the solution by gently inverting the tube several times. Store the tube on ice for 10 minutes.
6. Pellet the cellular debris by centrifugation at 12,000*g* (10,000 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C.
7. Decant the supernatant (approx. 7 ml) into a 30-ml Corex centrifuge tube and add an equal volume of isopropanol. Mix the solutions by gently inverting the tube several times and collect the precipitated nucleic acids by centrifugation at 12,000*g* (10,000 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C.
8. Remove the supernatant by gentle aspiration. Invert the tube on a Kimwipe tissue until the last drops of fluid have drained away. Use a Pasteur pipette attached to a vacuum line to remove any drops remaining attached to the wall of the tube. Dissolve the pellet of nucleic acids in 0.4 ml of 0.3 M sodium acetate (pH 5.2). Heat the solution to 65°C briefly (for a few minutes) to assist in dissolving the nucleic acids.
9. Transfer the DNA solution to a microfuge tube. Extract the solution once with an equal volume of phenol:chloroform. Separate the aqueous and organic phases by centrifugation at maximum speed for 5 minutes at room temperature in a microfuge. Transfer the upper aqueous phase to a fresh microfuge tube.
10. Add 1 ml of ice-cold ethanol. Mix the solutions by inverting the tube several times. Collect the precipitated DNA by centrifugation at maximum speed for 10 minutes at 4°C. Rinse the DNA pellet with 0.5 ml of 70% ethanol and centrifuge again for 2 minutes.
11. Carefully remove the supernatant. Store the open inverted tube at room temperature until no more traces of ethanol are visible. Add 0.4 ml of TE plus RNase to the pellet and place the closed tube at 37°C. Periodically during the next 15 minutes, shake the tube gently to assist in dissolving the DNA. Continue the incubation for a total of 2 hours.
12. Add 4 µl of 1 M $MgCl_2$ and 200 µl of 40% PEG solution. Mix well and collect the precipitated DNA by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge.
13. Remove the supernatant by aspiration and resuspend the pellet in 0.5 ml of 0.5 M ammonium acetate. Add 1 ml of ethanol, mix the solutions by inverting the tube several times, and collect the precipitate by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge.
14. Decant the supernatant and rinse the pellet twice with 0.5 ml of ice-cold 70% ethanol. Store the open inverted tube at room temperature until no more traces of ethanol are visible. Resuspend the damp pellet in 50 µl of TE (pH 8.0).
15. For restriction enzyme analysis, digest 5-15 µl (approx. 1 µg) of resuspended DNA and analyze the products either by 0.5% agarose gel electrophoresis or by PFGE.

REFERENCES

1. Pierce J.C. and Sternberg N.L. 1992. Using the bacteriophage P1 system to clone high molecular weight genomic DNA. *Methods Enzymol.* 216:549-574.

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Protocol 6

Transferring Bacteriophage P1 Clones between *E. coli* Hosts

Problems with low yield or poor quality can sometimes be overcome by transferring the P1 or PAC recombinant into a strain of *E. coli* that does not express Cre recombinase. This protocol for transfer of P1 clones by electroporation was supplied by Ray MacDonald (University of Texas Southwestern Medical Center, Dallas).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Enzymes and Buffers

Restriction endonucleases

Media

- LB agar plates containing 25 µg/ml kanamycin
- SOC
- TB containing 25 µg/ml kanamycin

Additional Reagents

Step 9 of this protocol requires reagents listed in Chapter 4, Protocol 5.

Vectors and Bacterial Strains

Closed circular recombinant Bacteriophage P1 DNA
Prepared as described in Chapter 4, Protocol 5.

E. coli strain
Prepare frozen electrocompetent cells (e.g., DH10B). Please see Chapter 1, Protocol 26 .

METHOD

1. Dilute 2-3 µg of P1 plasmid DNA to a concentration of 60 ng/µl in sterile H_2O. Set up a control lacking P1 DNA (sterile H_2O only), and carry the control through the electroporation procedure in parallel with the DNA sample.
2. Thaw vials of electrocompetent cells on ice and prechill 0.1-cm electroporation cuvettes.
3. Combine 20 µl of cells and 1 µl of P1 DNA in the cold cuvette.
4. Set the electroporation device to 1.8 kV, 200 ohms, and 25 µF.
5. Shock the cells. The optimum time constant is usually approx. 5 milliseconds.
6. Immediately add 0.5 ml of prewarmed (37°C) SOC medium to the cell suspension. Transfer the suspension to a culture tube and incubate the suspension for 1 hour at 37°C with moderate agitation.
7. Plate 100-µl aliquots of the cell suspensions on LB plates containing 25 µg/ml kanamycin. Incubate the plates overnight at 37°C.
   The control plates (no P1 DNA) should remain sterile; plates from the culture treated with P1 DNA should contain several hundred transformed colonies.
8. Transfer 10-12 colonies into separate 11-ml aliquots of TB containing 25 µg/ml kanamycin. Incubate the cultures overnight at 37°C with vigorous agitation.
9. Prepare P1 DNA as described in Chapter 4, Protocol 5 .
   The remainder of the overnight cultures can be stored at -80°C in TB/kanamycin containing 30% (v/v) glycerol.
10. Perform digestions with several different restriction endonucleases and compare the patterns of the newly isolated DNAs with that of the original recombinant by agarose gel electrophoresis.

REFERENCES

1. MacLaren D.C. and Clarke S. 1996. Rapid mapping of genomic P1 clones: The mouse L-isoaspartyl/D-aspartyl methyltransferase gene. *Genomics* 35:299-307.

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Protocol 7

Working with Bacterial Artificial Chromosomes

This protocol describes how to transform *E. coli* with BAC DNA by electroporation.

MATERIALS

CAUTION: Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

LB freezing buffer

Enzymes and Buffers

Restriction endonucleases
Please see Step 5. Choose restriction endonucleases appropriate for measuring insert size in BACs.

Media

LB agar plates containing 12.5 µg/ml chloramphenicol
LB containing 12.5 µg/ml chloramphenicol

Additional Reagents

Step 4 of this protocol requires reagents listed in Chapter 4, Protocol 8 .

Step 5 of this protocol requires either reagents listed in Chapter 8, Protocol 12 or reagents listed in Chapter 6, Protocol 10 .

Vectors and Bacterial Strains

BAC DNA
A culture of E. coli *strain transformed with BAC isolate may be used.*

E. coli strain
Prepare frozen electrocompetent cells (e.g., DH10B). Required only if the BAC isolate is supplied as purified DNA. Please see Chapter 1, Protocol 26 .

METHOD

1. Prepare fresh BAC transformants.
   If the BAC Is Supplied in the Form of DNA
   a. Transform *E. coli* (strain DH10B) with BAC DNA by electroporation.
   Because the efficiency of transformation by large BACs decreases dramatically as a function of the voltage applied during electroporation, it is best to set up a series of electroporation reactions at voltages ranging from 13 kV/cm to 25 kV/cm.
   b. Plate 2.5, 25, and 250 µl of each batch of electroporated bacteria onto LB agar plates containing 12.5 µg/ml chloramphenicol. Incubate the plates for 16-24 hours at 37°C.
   If the BAC Is Supplied as a Transformed Bacterial Stock
   a. Streak the culture without delay onto LB agar plates containing 12.5 µg/ml chloramphenicol.
   b. Incubate the plates for 12-16 hours at 37°C.
2. Select 12 individual transformants and inoculate these into 5-ml aliquots of LB medium containing chloramphenicol (12.5 µg/ml). Grow the cultures overnight at 37°C with vigorous shaking.
3. Use a loopful of each 5-ml culture to set up cultures of the 12 transformants in LB freezing medium. When these cultures have grown, transfer them to a -20°C freezer for storage.
4. Purify the BAC DNA from 4.5 ml of each 5-ml culture from Step 2, as described in Chapter 4, Protocol 8 .
5. Analyze the BAC DNA.
   a. Confirm by PCR that the BACs contain the chromosomal region of interest (please see Chapter 8, Protocol 12) or Southern hybridization (please see Chapter 6, Protocol 10).
   b. Measure the size of the inserts by digestion with restriction enzymes and PFGE.
6. On the basis of the results, select one or more of the BACs for further analysis.

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Protocol 8

Isolation of BAC DNA from Small-scale Cultures

BAC DNAs are prepared from 5-ml cultures of BAC-transformed cells by a modification of the standard alkaline lysis method (Chapter 1, Protocol 1). The yield typically varies between 0.1 and 0.4 µg of BAC DNA.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Alkaline lysis solution I, ice cold
- Alkaline lysis solution II
- Alkaline lysis solution III, ice cold

Ethanol

Isopropanol

STE, ice cold

- TE (pH 8.0)

Enzymes and Buffers

Restriction endonucleases

Media

- LB containing 12.5 µg/ml chloramphenicol

Nucleic Acids and Oligonucleotides

DNA markers for pulsed-field gel electrophoresis

Vectors and Bacterial Strains

E. coli strain transformed with BAC isolate
Please see Chapter 4, Protocol 7.

METHOD

1. Prepare 5-ml cultures of BAC-transformed *E. coli* in LB medium containing 12.5 µg/ml chloramphenicol, and grow the cultures overnight at 37°C with vigorous shaking.
2. Collect the bacterial cells by centrifugation at 2000*g* (4100 rpm in a Sorvall SS-34 rotor) for 5 minutes at 4°C. Decant the medium carefully and remove any residual drops by aspiration.
3. Add 5 ml of ice-cold STE to each tube, and resuspend the bacterial pellet by pipetting. Recover the cells by centrifugation as in Step 2.
   Cleaner preparations of BAC DNA are obtained if the cells are washed briefly in ice-cold STE at this stage.
4. Resuspend the cells in 200 µl of ice-cold Alkaline lysis solution I. Transfer the cells to an ice-cold microfuge tube. Place the tube on ice.
   The cell suspension may be gently vortexed to break up clumps of cells.
5. Add 400 µl of freshly prepared Alkaline lysis solution II to the tube. Gently invert the closed tube several times. Place the tube on ice.
6. Add 300 µl of ice-cold Alkaline lysis solution III to the tube. Gently invert the closed tube several times. Place the tube on ice for 5 minutes.
7. Remove the precipitated cell debris by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge. Decant the supernatant into a fresh microfuge tube. Add 900 µl of isopropanol at room temperature and mix the contents of the tube by gentle inversion.
8. Immediately collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at room temperature in a microfuge. Discard the supernatant and carefully rinse the pellet with 1 ml of 70% ethanol. Centrifuge the tube for 2 minutes at room temperature and remove the ethanol by aspiration. Allow the pellet of nucleic acid to dry in the air for 5-10 minutes. Dissolve the damp pellet in 50 µl of TE (pH 8.0).
9. Digest the BAC DNA with restriction endonucleases.
10. Analyze the digested BAC DNA by PFGE, using DNA markers of an appropriate size.

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Protocol 9

Isolation of BAC DNA from Large-scale Cultures

The procedure for isolation of BAC DNA is scaled-up to accommodate 500-ml cultures, which, on average, yield 20-25 µg of purified BAC DNA.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Alkaline lysis solution I, ice cold
  For large-scale preparations of BAC DNA, sterile Alklaine lysis solution I should be supplemented just before use with DNase-free RNase at a concentration of 100 µg/ml.
- Alkaline lysis solution II
- Alkaline lysis solution III, ice cold

Ethanol

Isopropanol

- ⚠ Phenol:chloroform (1:1, v/v)
- STE, ice cold
- TE (pH 8.0)

Enzymes and Buffers

- Lysozyme

Restriction endonucleases

Nucleic Acids and Oligonucleotides

DNA markers for pulsed-field gel electrophoresis

Media

- LB containing 12.5 µg/ml chloramphenicol

Vectors and Bacterial Strains

E. coli strain transformed with BAC isolate
Please see Chapter 4, Protocol 7. Prepare a fresh overnight culture; please see Step 1.

METHOD

1. Inoculate 500 ml of LB medium containing 12.5 µg/ml of chloramphenicol with 50 µl of a saturated overnight culture of BAC-transformed cells. Incubate the 500-ml culture for 12-16 hours at 37°C with vigorous agitation (300 cycles/minute) until the cells reach saturation.
2. Harvest the cells from the culture by centrifugation at 2500*g* (3900 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C. Pour off the supernatant, and invert the open centrifuge bottle to allow the last drops of the supernatant to drain away.
3. Resuspend the bacterial pellet in 100 ml of ice-cold STE. Collect the bacterial cells by centrifugation as described in Step 2.
4. Resuspend the bacterial pellet in 24 ml of Alkaline lysis solution I containing DNase-free RNase (100 µg/ml). Add lysozyme to a final concentration of 1 mg/ml.
   Make sure that the cells are completely resuspended and that the suspension is free of clumps.
5. Add 24 ml of freshly prepared Alkaline lysis solution II. Close the top of the centrifuge bottle and mix the contents thoroughly by gently inverting the bottle several times. Incubate the bottle for 5 minutes on ice.
6. Add 24 ml of ice-cold Alkaline lysis solution III. Close the top of the centrifuge bottle and mix the contents gently but thoroughly by swirling the bottle until there are no longer two distinguishable liquid phases. Place the bottle on ice for 5 minutes.
7. Centrifuge the bacterial lysate at 15,000*g* (9600 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C. At the end of the centrifugation step, decant the clear supernatant into a polypropylene centrifuge bottle. Discard the pellet remaining in the centrifuge bottle.
8. Add an equal volume of phenol:chloroform. Mix the aqueous and organic phases by gently inverting the tube several times. Separate the phases by centrifugation at 3000*g* (4300 rpm in a Sorvall GSA rotor) for 15 minutes at room temperature.
9. Use a wide-bore pipette to transfer the aqueous layer to a fresh centrifuge bottle and add an equal volume of isopropanol. Invert the bottle several times to mix well.
10. Mark the tube on one side and place it in a centrifuge rotor with the marked side facing away from the center of the rotor. Marking in this way will aid in the subsequent identification of the nucleic acid pellet. Recover the precipitated nucleic acids by centrifugation at 15,000*g* (9600 rpm in a Sorvall GSA rotor) for 15 minutes at *room temperature*.
11. Decant the supernatant carefully, and invert the bottle on a paper towel to allow the last drops of supernatant to drain away. Rinse the pellet and the walls of the bottle with 20 ml of 70% ethanol at room temperature. Drain off the ethanol, and place the inverted tube on a pad of paper towels for a few minutes at room temperature to allow the ethanol to evaporate.
12. Gently dissolve the pellet of BAC DNA in 0.2 ml of TE (pH 8.0). Assist in the dissolution of the DNA by tapping the sides of the bottle rather than vortexing. Measure the concentration of DNA by absorption spectroscopy.
13. Digest the BAC DNA with restriction endonucleases.
14. Analyze the digested BAC DNA by PFGE, using DNA markers of an appropriate size.

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Protocol 10

Working with Yeast Artificial Chromosomes

This protocol outlines a procedure to validate and store yeast strains carrying YACs.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Glycerol (30% v/v) in YPD medium

Media

- CM
- YPD agar plates
- YPD medium

Additional Reagents

Step 3 of this protocol requires reagents listed in Chapter 6, Protocol 7 .

Step 4 of this protocol requires reagents listed in Chapter 5, Protocol 17 or Chapter 5, Protocol 18 .

Step 5 of this protocol requires either reagents listed in Chapter 6, Protocol 10 , or reagents listed in Chapter 8, Protocol 12 .

Vectors and Yeast Strains

S. cerevisiae carrying recombinant YAC clone

METHOD

1. Immediately upon the arrival of clones in the laboratory, streak the cultures onto selective media and incubate for 48 hours at 30°C to obtain single colonies.
   Yeast colonies may be analyzed directly by PCR (please see Chapter 4, Protocol 13).
2. Transfer each of 6-12 individual colonies into 10 ml of YPD medium. Incubate the cultures with vigorous agitation (300 cycles/minute) at 30°C overnight. The cells should reach saturation (OD_{600}= 2.0-3.0, approx. 3 x 10^7 cells/ml) during this time.
3. Extract yeast DNA from 9 ml of each of the cultures following the steps described in Chapter 6, Protocol 7 .
   Store the unused portions of the cultures at 4°C.
4. Analyze the size of the YAC in each of the DNA preparations by PFGE.
5. Confirm by either Southern hybridization or PCR that the target sequence is present in the YAC DNA.
6. If the results are satisfactory, i.e., if the cultures contain YACs of the same size, if there is no sign of instability or rearrangement, and if the target sequences are present, then choose one or two of the cultures for long-term storage.

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Protocol 11

Growth of *S. cerevisiae* and Preparation of DNA

This protocol describes methods for isolation of DNA from a strain of *S. cerevisiae* carrying a recombinant YAC. Because the linear YAC DNAs are sensitive to shearing forces, pipettes with wide-bore tips should be used to transfer DNAs. The method is suitable for preparing DNA that will be used for agarose gel electrophoresis, Southern blotting, subcloning, genomic library construction, PCR, or other methods that do not require intact high-molecular-weight DNA. The expected yield from a 10-ml culture is 2-4 µg of yeast DNA.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)

Ethanol

⚠ Phenol:chloroform (1:1, v/v)

TE (pH 8.0)

TE (pH 8.0) containing 20 µg/ml RNase

Triton/SDS solution

Media

YPD

Vectors and Yeast Strains

Yeast colony carrying the YAC clone of interest

METHOD

1. Inoculate a yeast colony containing the YAC clone of interest into 10 ml of YPD medium and incubate overnight with shaking at 30°C.
   The cells should reach saturation (OD_{600} = 2.0-3.0, approx. 3 x 10^7 cells/ml) during this time.
   If the DNA to be extracted will be used in PFGE, follow the steps described in Chapter 5, Protocol 14 .
2. Collect the cells by centrifugation at 2000*g* (4100 rpm in a Sorvall SS-34 rotor) for 5 minutes.
3. Remove the medium, replace with 1 ml of sterile H_2O, and resuspend the cells by gentle vortexing.
4. Collect the cells by centrifugation as in Step 2.
5. Remove the wash, resuspend cells in 0.5 ml of sterile H_2O, and transfer to a sterile 1.5-ml microfuge tube.
6. Collect the cells by centrifugation at maximum speed for 5 seconds at room temperature in a microfuge, and remove the supernatant.
7. Add 0.2 ml of Triton/SDS solution to the cells and resuspend the cell pellet by tapping the side of the tube.
8. Add 0.2 ml of phenol:chloroform and 0.3 g of glass beads to the cells, and vortex the cell suspension for 2 minutes at room temperature. Add 0.2 ml of TE (pH 8.0), and mix the solution by vortexing briefly.
9. Separate the organic and aqueous phases by centrifugation at maximum speed for 5 minutes at room temperature in a microfuge. Transfer the aqueous upper layer to a fresh microfuge tube, taking care to avoid carrying over any of the material at the interface.
10. Add 1 ml of ethanol to the aqueous solution, cap the centrifuge tube, and gently mix the contents by inversion.
11. Collect the precipitated DNA by centrifugation at maximum speed for 2-5 minutes at 4°C in a microfuge. Remove the supernatant with a drawn out Pasteur pipette. Centrifuge the tube briefly (2 seconds) and remove the last traces of ethanol from the bottom of the tube.
12. Resuspend the nucleic acid pellet in 0.4 ml of TE (pH 8.0) with RNase and incubate the solution for 5 minutes at 37°C.
13. Add to the solution an equal volume of phenol:chloroform and extract the RNase-digested solution, mixing by inversion rather than vortexing. Separate the aqueous and organic phases by centrifugation at maximum speed for 5 minutes at room temperature in a microfuge and transfer the aqueous layer to a fresh microfuge tube.
14. Add 80 µl of 10 M ammonium acetate and 1 ml of ethanol to the aqueous layer. Mix the solution by gentle inversion and store the tube for 5 minutes at room temperature.
15. Collect the precipitated DNA by centrifugation for 5 minutes in a microfuge. Decant the supernatant and rinse the nucleic acid pellet with 0.5 ml of 70% ethanol. Centrifuge at maximum speed for 2 minutes and remove the ethanol rinse with a drawn out Pasteur pipette. Centrifuge the tube briefly (2 seconds) and remove the last traces of ethanol from the bottom of the tube. Allow the pellet of DNA to dry in the air for 5 minutes and then dissolve the pellet in 50 µl of TE (pH 8.0).
    The preparation should contain 2-4 µg of yeast DNA.

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Protocol 12

Small-scale Preparations of Yeast DNA

Yeast DNA is prepared by digestion of the cell wall and lysis of the resulting spheroplasts with SDS. This method reproducibly yields several micrograms of yeast DNA that can be efficiently cleaved by restriction enzymes and used as a template in PCR.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Isopropanol
Potassium acetate (5 M)
⚠ SDS (10% w/v)
Sodium acetate (3 M, pH 7.0)
Sorbitol buffer
TE (pH 7.4)
TE (pH 8.0) containing 20 µg/ml RNase
Yeast resuspension buffer

Enzymes and Buffers

Zymolyase 100T

Media

YPD

Vectors and Yeast Strains

Yeast strain

METHOD

1. Set up 10-ml cultures of yeast in YPD medium. Incubate the cultures overnight at 30°C with moderate agitation.
2. Transfer 5 ml of the cells to a centrifuge tube. Collect the cells by centrifugation at 2000*g* (4100 rpm in a Sorvall SS-34 rotor) for 5 minutes. Store the unused portion of the culture at 4°C.
3. Resuspend the cells in 0.5 ml of sorbitol buffer. Transfer the suspension to a microfuge tube.
4. Add 20 µl of a solution of Zymolyase 100T (2.5 mg/ml in sorbitol buffer), and incubate the cell suspension for 1 hour at 37°C.
5. Collect the cells by centrifugation in a microfuge for 1 minute. Remove the supernatant by aspiration.
6. Resuspend the cells in 0.5 ml of yeast resuspension buffer.
7. Add 50 µl of 10% SDS. Close the top of the tube and mix the contents by rapidly inverting the tube several times. Incubate the tube for 30 minutes at 65°C.
8. Add 0.2 ml of 5 M potassium acetate and store the tube for 1 hour on ice.
9. Pellet the cell debris by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.
10. Use a wide-bore pipette tip to transfer the supernatant to a fresh microfuge tube at room temperature.
11. Precipitate the nucleic acids by adding an equal volume of room-temperature isopropanol. Mix the contents of the tube and store it for 5 minutes at room temperature.
    Do not allow the precipitation reaction to proceed for >5 minutes.
12. Recover the precipitated nucleic acids by centrifugation at maximum speed for 10 *seconds* in a microfuge. Remove the supernatant by aspiration and allow the pellet to dry in the air for 10 minutes.
13. Dissolve the pellet in 300 µl of TE (pH 8.0) containing 20 µg/ml pancreatic RNase. Incubate the digestion mixture for 30 minutes at 37°C.
14. Add 30 µl of 3 M sodium acetate (pH 7.0). Mix the solution and then add 0.2 ml of isopropanol. Mix once again and recover the precipitated DNA by centrifugation at maximum speed for 20 seconds in a microfuge.
15. Remove the supernatant by aspiration and allow the pellet to dry in the air for 10 minutes. Dissolve the DNA in 150 µl of TE (pH 7.4).

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Protocol 13

Analyzing Yeast Colonies by PCR

Yeast colonies are suspended in complete PCR buffer and transferred to a thermal cycler for 35 cycles of PCR. The products of the amplification reaction are analyzed by gel electrophoresis.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

10x Yeast colony PCR buffer

⚠ dNTPs (10 mM) containing all four dNTPs (pH 8.0; PCR grade)

$MgCl_2$ (25 mM)

Enzymes and Buffers

Taq DNA polymerase

Nucleic Acids and Oligonucleotides

Oligonucleotide primers

The oligonucleotides should be 20-24 nucleotides in length, specific for the target DNA sequences, free of potential secondary structures, and contain no less than 10 and no more than 15 G and C residues.

Marker DNA

Vectors and Yeast Strains

Yeast strain carrying recombinant YAC of interest

METHOD

1. In a sterile 0.5-ml microfuge tube, mix in the following order:

10x colony PCR buffer	2 µl
25 mM $MgCl_2$	1.2 µl
10 mM dNTPs	0.4 µl
oligonucleotide primers	10 pmoles of each primer
Taq polymerase	5 units (0.2 µl)
H_2O	to 20 µl

2. Use a disposable yellow pipette tip to transfer a small amount of a yeast colony (0.10-0.25 µl) to the reaction mixture.
   It is important not to be too greedy when sampling the yeast colony because cell wall components inhibit the PCR.
3. Transfer the PCR tube to the thermocycler, programmed as follows, and start the program.

Cycle Number	Denaturation	Annealing	Polymerization
1	4 min at 95°C		
2-35	1 min at 95°C	1 min at 55°C	1 min at 72°C
Last			10 min at 72°C

   These times are suitable for 50-µl reactions assembled in thin-walled 0.5-ml tubes and incubated in thermal cyclers such as the Perkin-Elmer 9600 or 9700, Master cycler (Eppendorf), and PTC 100 (MJ-Research). Times and temperatures may need to be adapted to suit other types of equipment and reaction volumes.
4. Analyze the products of the PCR by electrophoresis through an agarose or polyacrylamide gel, using markers of suitable size.
   If amplification of the target sequence is weak or erratic, repeat the reactions using a polymerization temperature 2-3°C below the calculated melting temperature of the oligonucleotide primer that is richer in A+T. If the results are still unsatisfactory, convert the yeast cells to spheroplasts by removing the cell walls with Zymolyase 100T before beginning the protocol. This takes only 1 hour and almost always clears up any problems. Alternatively, grow 10-ml liquid (YPD) cultures of the colonies under test and make small-scale preparations of yeast DNA (please see Chapter 4, Protocol 12).

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Protocol 14

Isolating the Ends of Genomic DNA Fragments Cloned in High-capacity Vectors: Vectorette Polymerase Chain Reactions

This protocol describes the use of vectorette PCR and single-site PCR to amplify the terminal sequences of genomic sequences cloned in high-capacity vectors such as PACs and YACs.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- 10x Bacteriophage T4 DNA ligase buffer
- ⚠ dNTPs (1 mM) containing all four dNTPs (pH 8.0; PCR grade)
- Ethanol
- ⚠ Phenol:chloroform (1:1, v/v)
- Sodium acetate (3 M, pH 5.2)

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Restriction endonucleases *Pst*I or *Nsi*I

Thermostable DNA polymerase

Nucleic Acids and Oligonucleotides

Oligonucleotide cassette (5.0 OD_{260}/ml [approx. 8.5 µM]) in TE (pH 7.6)

5´CATGCTCGGTCGGGATAGGCACTGGTCTAGAGGGTTAGGTTCCTGCTACATCTCCAGCCTTGCA3´
This 64-nucleotide single-stranded cassette is designed for ligation to target DNAs carrying termini generated by Pst*I or* Nsi*I. The four 3´-terminal nucleotides (underlined) are complementary to the protruding termini of fragments of DNA generated by cleavage with these enzymes. If another restriction enzyme is used, the nucleotides at the 3´ end of the linker must be changed so as to complement the protruding terminus generated by the enzyme.*
Before use, the oligonucleotide should be purified by C_{18} chromatography or electrophoresis through a 12% polyacrylamide gel. The 5´ terminus of the oligonucleotide should not be phosphorylated.

Oligonucleotide (linker) primer (5.0 OD_{260}/ml [approx. 17 µM]) in TE (pH 7.6)

5´CATGCTCGGTCGGGATAGGCACTGGTCTAGAG3´
This oligonucleotide is identical in sequence to the 32 nucleotides at the 5´ end of the oligonucleotide cassette and is used as an amplimer in the PCR.
There is no need to purify or phosphorylate the deprotected oligonucleotide before use. Dissolve the oligonucleotide in TE (pH 7.6) at a concentration of 5.0 OD_{260}/ml solution (approx. 17 µM).

Sequence-specific oligonucleotide (vector) primer (5.0 OD_{260}/ml [approx. 17 µM]) in TE (pH 7.6)

This primer is complementary to the vector when terminal sequences of a cloned segment of DNA are to be amplified or to cloned DNA sequences when a neighboring segment of genomic DNA is to be recovered. The primer should be 28-32 nucleotides in length and its predicted melting temperature should be approximately equal to that of the 32-nucleotide oligonucleotide primer.
There is no need to purify or phosphorylate the deprotected oligonucleotide before use. Dissolve the oligonucleotide in TE (pH 7.6) at a concentration of 5.0 OD_{260}/ml solution (approx. 17 µM).

Template DNAs
Template may be recombinant BAC, YAC, or cosmid DNA.
YACs can either be embedded in an agarose plug (2 µg in 100-µl plug) or in solution. Unless the yeast strain is carrying more than one YAC, there is no need to purify YAC DNA by PFGE before use in vectorette or single-site PCR.
DNAs should be purified by column chromatography using, e.g., Qiagen resin or GeneClean II (please see Chapter 1, Protocol 9), and resuspended at a concentration of 1 µg/µl in TE (pH 7.6).

METHOD

1. Digest approx. 5 µg of template DNA with *Pst*I or *Nsi*I. Check a small aliquot of the reaction by agarose gel electrophoresis to ensure that all of the DNA has been cleaved.
2. Extract the reaction mixture with phenol:chloroform, and recover the DNA by standard precipitation with ethanol and subsequent washing in 70% ethanol. Store the open tube in an inverted position on a bed of paper towels to allow the last traces of ethanol to evaporate, and then dissolve the damp pellet of DNA in 50 µl of H_2O.
3. In a sterile 0.5-ml microfuge tube, mix in the following order:

10x T4 DNA ligase buffer	10 µl
digested template DNA	20 µl
oligonucleotide cassette, 5.0 OD_{260}/ml	2 µl
T4 bacteriophage DNA ligase, 5 Weiss units/µl	2 µl
H_2O	to 100 µl

   Set up three control reactions as described above but without template DNA in one tube, without linker oligonucleotide in another, and without T4 DNA ligase in the third.
4. Incubate the test ligation reaction and controls for 12-16 hours at 15°C.
5. In a sterile 0.5-ml microfuge tube, mix in the following order:

10x amplification buffer	2 µl
1 mM solution of four dNTPs (pH 7.0)	2 µl
linker primer oligonucleotide, 5.0 OD_{260}/ml	1 µl
vector primer oligonucleotide, 5.0 OD_{260}/ml	1 µl
test DNA ligation reaction, from Step 4	1 µl
thermostable DNA polymerase, 5.0 units/µl	0.5 µl
H_2O	to 20 µl

   Set up three control PCRs that contain 1 µl of the control ligation reactions instead of the test ligation reaction.
6. If the thermocycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil to prevent evaporation of the samples during repeated cycles of heating and cooling. Alternatively, place a bead of wax into the tube if using a hot-start approach.
7. Place the PCR tubes in the thermocycler, programmed as follows, and start the amplification program.

Cycle Number	Denaturation	Annealing	Polymerization
1	2 min at 95°C		
2-35	30 sec at 94°C	30 sec at 60°C	3 min at 72°C
Last			5 min at 72°C

8. Analyze aliquots (25%) of each amplification reaction on an agarose gel.
   A prominent DNA product visible by ethidium bromide staining should be present in the PCR containing the products of the test ligation. This DNA should be absent from the control reactions. The size of the product depends on the distance between the vector primer and the first cleavage site in the cloned insert. For PstI *and* NsiI*, the amplified product is typically between 0.5 kb and 2 kb.*
   The amplified DNA can be sequenced directly, cloned, radiolabeled by random hexamer priming or PCR, and even used as a transcription template if a bacteriophage promoter is added to the linker or is present in the amplified segment of vector DNA.

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Chapter 5 Gel Electrophoresis of DNA and Pulsed-Field Agarose

Markers for pulsed-field gel electrophorsis can be generated by ligation of linear monomers of bacteriophage λ DNA (48.5 kb) into a nested series of concatemers. This procedure yields a series of concatemers that contain up to 20 tandemly arranged copies of bacteriophage DNA.

Protocol 17: Pulsed-field Gel Electrophoresis via Transverse Alternating Field Electrophoresis Gels

In this form of pulsed-field gel electrophoresis, electrodes are positioned on opposite sides of a vertically oriented gel. The DNA moves first toward one electrode and then toward the other, forming a zigzag pattern. The vector of this oscillation is a straight line from the loading well to the base of the gel. Variation in voltage and pulse time allows separation of DNAs ranging in size from 2 kb to >6000 kb. This protocol, supplied by Tommy Hyatt and Helen Hobbs (University of Texas Southwestern Medical Center, Dallas), describes the resolution of genomic DNA by TAFE, followed by blotting and hybridization.

Protocol 18: Pulsed-field Gel Electrophoresis via Contour-clamped Homogeneous Electric Field Gels

In CHEF gels, the electric field is generated from multiple electrodes, arranged in a square of hexagonal contour around the horizontal gel and clamped to predetermined potentials. Using a combination of low field strengths, low concentrations of aragose, long switching intervals, and extended periods of electrophoresis, DNAs up to 5000 kb can be resolved. This protocol, supplied by Elsy Jones (University of Texas Southwestern Medical Center, Dallas), describes the resolution of genomic DNA by TAFE, followed by blotting and hybridization.

Protocol 19: Direct Retrieval of DNA Fragments from Pulsed-field Gels

A gel slice containing a fragment of DNA resolved by pulsed-field gel electrophoresis is treated with agarase. The released DNA can be used as a substrate for ligation or restriction without further purification.

Protocol 20: Retrieval of DNA Fragments from Pulsed-field Gels following DNA Concentration

DNA contained in a slice of low-melting-temperature agarose is first concentrated by electrophoresis into a high-percentage agarose gel, and then isolated by treatment with agarase. The resulting DNA preparation is purified by microdialysis.

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Protocol 1

Agarose Gel Electrophoresis

How to pour, load, and run an agarose gel.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Agarose solutions (please see Step 3)

- DNA staining solution
- Electrophoresis buffer
- 6x Gel-loading buffer

Nucleic Acids and Oligonucleotides

DNA samples

DNA size standards

Samples of DNAs of known size are typically generated by restriction enzyme digestion of a plasmid or bacteriophage DNA of known sequence. Alternatively, they are produced by ligating a monomer DNA fragment of known size into a ladder of polymeric forms.

METHOD

1. Seal the edges of a clean, dry glass plate (or the open ends of the plastic tray supplied with the electrophoresis apparatus) with tape to form a mold. Set the mold on a horizontal section of the bench.
2. Prepare sufficient electrophoresis buffer (usually 1x TAE or 0.5x TBE) to fill the electrophoresis tank and to cast the gel.
   It is important to use the same batch of electrophoresis buffer in both the electrophoresis tank and the gel.
3. Prepare a solution of agarose in electrophoresis buffer at a concentration appropriate for separating the particular size fragments expected in the DNA sample(s): Add the correct amount of powdered agarose (please see table below) to a measured quantity of electrophoresis buffer in an Erlenmeyer flask or a glass bottle.
   Range of Separation in Cells Containing Different Amounts of Standard Low-EEO Agarose

Agarose Concentration in Gel (% [w/v])	Range of Separation of Linear DNA Molecules (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0-2-3
2.0	0.1-2

   Agarose gels are cast by melting the agarose in the presence of the desired buffer until a clear, transparent solution is achieved. The melted solution is then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose.
4. Loosely plug the neck of the Erlenmeyer flask with Kimwipes. If using a glass bottle, make certain the cap is loose. Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves.
   Heat the slurry for the minimum time required to allow all of the grains of agarose to dissolve.
5. Use insulated gloves or tongs to transfer the flask/bottle into a water bath at 55°C. When the molten gel has cooled, add ethidium bromide to a final concentration of 0.5 µg/ml. Mix the gel solution thoroughly by gentle swirling.
   IMPORTANT *SYBR Gold should not be added to the molten gel solution.*
6. While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel. Position the comb 0.5-1.0 mm above the plate so that a complete well is formed when the agarose is added to the mold.
7. Pour the warm agarose solution into the mold.
   The gel should be between 3 mm and 5 mm thick. Check that no air bubbles are under or between the teeth of the comb. Air bubbles present in the molten gel can be removed easily by poking them with the corner of a Kimwipe.
8. Allow the gel to set completely (30-45 minutes at room temperature), then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb. Pour off the electrophoresis buffer and carefully remove the tape. Mount the gel in the electrophoresis tank.
9. Add just enough electrophoresis buffer to cover the gel to a depth of approx. 1 mm.
10. Mix the samples of DNA with 0.20 volume of the desired 6x gel-loading buffer.
    The maximum amount of DNA that can be applied to a slot depends on the number of fragments in the sample and their sizes. The minimum amount of DNA that can be detected by photography of ethidium-bromide-stained gels is approx. 2 ng in a 0.5-cm-wide band (the usual width of a slot). More sensitive dyes such as SYBR Gold can detect as little as 20 pg of DNA in a band.
11. Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipettor, or a drawn-out Pasteur pipette or glass capillary tube. Load size standards into slots on both the right and left sides of the gel.
12. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the positive anode (red lead). Apply a voltage of 1-5 V/cm (measured as the distance between the positive and negative electrodes). If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis), and within a few minutes, the bromophenol blue should migrate from the wells into the body of the gel. Run the gel until the bromophenol blue and xylene cyanol FF have migrated an appropriate distance through the gel.
    The presence of ethidium bromide allows the gel to be examined by UV illumination at any stage during electrophoresis. The gel tray may be removed and placed directly on a transilluminator. Alternatively, the gel may be examined using a hand-held source of UV light. In either case, turn off the power supply before examining the gel!
13. When the DNA samples or dyes have migrated a sufficient distance through the gel, turn off the electric current and remove the leads and lid from the gel tank. If ethidium bromide is present in the gel and electrophoresis buffer, examine the gel by UV light and photograph the gel as described in Chapter 5, Protocol 2 . Otherwise, stain the gel by immersing it in electrophoresis buffer or H_2O containing ethidium bromide (0.5 µg/ml) for 30-45 minutes at room temperature or by soaking in a 1:10,000-fold dilution of SYBR Gold stock solution in electrophoresis buffer.

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Protocol 2

Detection of DNA in Agarose Gels

Nucleic acids that have been subjected to electrophoresis through agarose gels may be detected by staining and visualized by illumination with 300-nm UV light. Methods for staining and visualization of DNA using either ethidium bromide or SYBR Gold are described here. The most convenient and commonly used method to visualize DNA in agarose gels is staining with the fluorescent dye ethidium bromide. Ethidium bromide can be used to detect both single- and double-stranded nucleic acids (both DNA and RNA). However, the affinity of the dye for single-stranded nucleic acid is relatively low and the fluorescent yield is comparatively poor. In fact, most fluorescence associated with staining single-stranded DNA or RNA is attributable to binding of the dye to short intrastrand duplexes in the molecules.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- DNA-staining solution

METHOD

1. Detect DNA banding patterns in agarose gels using either ethidium bromide or SYBR Gold.
   To detect DNA using **Ethidium bromide**, prepare a stock solution of 10 mg/ml in H_2O, and store it at room temperature in dark bottles or bottles wrapped in aluminum foil. The dye is usually incorporated into agarose gels and electrophoresis buffers at a concentration of 0.5 µg/ml. Note that polyacrylamide gels cannot be cast with ethidium bromide because it inhibits polymerization of the acrylamide. Acrylamide gels are therefore stained with the ethidium solution after the gel has been run (please see Chapter 5, Protocol 10). Although the electrophoretic mobility of linear double-stranded DNA is reduced approx. 15% in the presence of the dye, the ability to examine the agarose gels directly under UV illumination during or at the end of the run is a great advantage. However, sharper DNA bands are obtained when electrophoresis is carried out in the absence of ethidium bromide. Staining is accomplished by immersing the gel in electrophoresis buffer or H_2O containing ethidium bromide (0.5 µg/ml) for 30-45 minutes at room temperature. Destaining is not usually required. However, detection of very small amounts (<10 ng) of DNA is made easier if the background fluorescence caused by unbound ethidium bromide is reduced by soaking the stained gel in H_2O or 1 mM $MgSO_4$ for 20 minutes at room temperature.
   To stain DNA in agarose gels using **SYBR Gold**, prepare a 1:10,000-fold dilution of the stock dye solution and, after electrophoretic separation of the DNA fragments, soak the gel in this solution. SYBR Gold should not be added to the molten agarose or to the gel before electrophoresis, because its presence in the hardened gel will cause severe distortions in the electrophoretic properties of DNA and RNA. The greatest sensitivity is obtained when the gel is illuminated with UV light at 300 nm. The dye is sensitive to fluorescent light, and working solutions containing SYBR Gold (1:10,000 dilution of the stock solution supplied by Molecular Probes) should be freshly prepared in electrophoresis buffer on a daily basis and stored at room temperature.
   SYBR Gold is the trade name of a new ultrasensitive dye with high affinity for DNA and a large fluorescence enhancement upon binding to nucleic acid. The quantum yield of the SYBR Gold-DNA complex is greater than that of the equivalent ethidium bromide-DNA complex and the fluorescence enhancement is >1000 times greater. As a result, <20 pg of double-stranded DNA can be detected in an agarose gel (up to 25 times less than the amount visible after ethidium bromide staining). In addition, staining of agarose or polyacrylamide gels with this dye can reveal as little as 100 pg of single-stranded DNA in a band or 300 pg of RNA. SYBR Gold shows maximum excitation at 495 nm and has a secondary excitation peak at 300 nm. Fluorescent emission occurs at 537 nm. Photography is carried out with green or yellow filters as described below in Step 2.
2. Record the banding pattern of DNA resolved through the gel by photography.
   Photographs of ethidium-bromide-stained gels may be made using transmitted or incident UV light. Most commercially available devices (transilluminators) emit UV light at 302 nm. The fluorescent yield of ethidium bromide-DNA complexes is considerably greater at this wavelength than at 366 nm and slightly less than at short-wavelength (254 nm) light. However, the amount of nicking of the DNA is much less at 302 nm than at 254 nm. A further 10-20-fold increase in the sensitivity of conventional photography can be obtained by staining DNA with SYBR Gold (Molecular Probes). Detection of DNAs stained with this dye requires the use of a yellow or green gelatin or cellophane filter (S-7569, available from Molecular Probes or Kodak) with the camera and illumination with 300-nm UV light.

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Protocol 3

Recovery of DNA from Agarose Gels: Electrophoresis onto DEAE-cellulose Membranes

Recovery of bands of DNA from agarose gels by electrophoresis onto a sliver of DEAE-cellulose membrane can be performed simultaneously on many samples and reliably gives high yields of fragments between 500 bp and 5 kb in length.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
DEAE high-salt elution buffer
DEAE low-salt wash buffer
EDTA (10 mM, pH 8.0)
Ethanol
6x Gel-loading buffer
⚠ NaOH (0.5 N)
⚠ Phenol:chloroform (1:1, v/v)
Sodium acetate (3 M, pH 5.2)
TE (pH 8.0)

Enzymes and Buffers

Restriction endonucleases

Nucleic Acids and Oligonucleotides

DNA sample
DNA standards
Carrier RNA

METHOD

1. Digest an amount of DNA that will yield at least 100 ng of the fragment(s) of interest. Separate the fragments by electrophoresis through an agarose gel of the appropriate concentration that contains 0.5 µg/ml ethidium bromide, and locate the band of interest with a hand-held, long-wavelength UV lamp.
2. Use a sharp scalpel or razor blade to make an incision in the gel directly in front of the leading edge of the band of interest and approx. 2 mm wider than the band on each side.
   If DNA is to be eluted from an entire lane of an agarose gel (e.g., a restriction digest of mammalian genomic DNA), make the incision in the gel parallel to the lane of interest and place a single long piece of DEAE-cellulose membrane (prepared as in Step 3) into the incision. Reorient the gel so that the DNA can be transferred electrophoretically from the gel to the membrane. After electrophoresis, remove the membrane and cut it into segments. Elute DNA of the desired size from the appropriate segment(s) of the membrane as described in Steps 7-11.
3. Wearing gloves, cut a piece of DEAE-cellulose membrane that is the same width as the incision and slightly deeper (1 mm) than the gel. Soak the membrane in 10 mM EDTA (pH 8.0) for 5 minutes at room temperature. To activate the membrane, replace the EDTA with 0.5 N NaOH, and soak the membrane for a further 5 minutes. Wash the membrane six times in sterile H_2O.
4. Use blunt-ended forceps or tweezers to hold apart the walls of the incision on the agarose gel and insert the membrane into the slit. Remove the forceps and close the incision, being careful not to trap air bubbles.
   Minimize the chance of contamination with unwanted species of DNA by either
   - *cutting out a segment of gel containing the band of interest and transfering it to a hole of the appropriate size cut in another region of the gel far from any other species of DNA*

     or
   - *inserting a second piece of membrane above the band of interest to trap unwanted species of DNA*
5. Resume electrophoresis (5 V/cm) until the band of DNA has just migrated onto the membrane. Follow the progress of the electrophoresis with a hand-held, long-wavelength (302 nm) UV lamp.
   Electrophoresis should be continued for the minimum time necessary to transfer the DNA from the gel to the membrane. Extended electrophoresis can result in cross-contamination with other DNA fragments (see above) or unnecessary accumulation of contaminants from the agarose.
6. When all of the DNA has left the gel and is trapped on the membrane, turn off the electric current. Use blunt-ended forceps to recover the membrane and rinse it in 5-10 ml of DEAE low-salt wash buffer at room temperature to remove any agarose pieces from the membrane.
   Do not allow the membrane to dry; otherwise, the DNA becomes irreversibly bound.
7. Transfer the membrane to a microfuge tube. Add enough DEAE high-salt elution buffer to cover the membrane completely. The membrane should be crushed or folded gently, but not tightly packed. Close the lid of the tube and incubate it for 30 minutes at 65°C.
8. While the DNA is eluting from the membrane, photograph the gel as described in Chapter 5, Protocol 2 to establish a record of which bands were isolated.
9. Transfer the fluid from Step 7 to a fresh microfuge tube. Add a second aliquot of DEAE high-salt elution buffer to the membrane, and incubate the tube for a further 15 minutes at 65°C. Combine the two aliquots of DEAE high-salt elution buffer.
10. Extract the high-salt eluate once with phenol:chloroform. Transfer the aqueous phase to a fresh microfuge tube, and add 0.2 volume of 10 M ammonium acetate and 2 volumes of ethanol at 4°C. Store the mixture for 10 minutes at room temperature, and recover the DNA by centrifugation at maximum speed for 10 minutes at room temperature in a microfuge. Carefully rinse the pellet with 70% ethanol, store the open tube on the bench for a few minutes to allow the ethanol to evaporate, and then redissolve the DNA in 3-5 µl of TE (pH 8.0).
    The addition of 10 µg of carrier RNA before precipitation may improve the recovery of small amounts of DNA. However, before adding the RNA, make sure that the presence of RNA will not compromise any subsequent enzymatic reactions in which the DNA is used as a substrate or template.
11. If exceptionally pure DNA is required (e.g., for microinjection of fertilized mouse eggs or electroporation of cultured cells), reprecipitate the DNA with ethanol as follows.
    a. Suspend the DNA in 200 µl of TE (pH 8.0), add 25 µl of 3 M sodium acetate (pH 5.2), and precipitate the DNA once more with 2 volumes of ethanol at 4°C.
    b. Recover the DNA by centrifugation at maximum speed for 5-15 minutes at 4°C in a microfuge.
    c. Carefully rinse the pellet with 70% ethanol. Store the open tube on the bench for a few minutes to allow the ethanol to evaporate, and then dissolve the DNA in 3-5 µl of TE (pH 8.0).

12. Check the amount and quality of the DNA by gel electrophoresis. Mix a small aliquot (approx. 10-50 ng) of the final preparation of the fragment with 10 µl of TE (pH 8.0), and add 2 µl of the desired gel-loading buffer. Load and run an agarose gel of the appropriate concentration, using as markers restriction digests of known quantities of the original DNA and the appropriate DNA size standards. The isolated fragment should comigrate with the correct fragment in the restriction digest. Examine the gel carefully for the presence of faint fluorescent bands that signify the presence of contaminating species of DNA.
It is often possible to estimate the amount of DNA in the final preparation from the relative intensities of fluorescence of the fragment and the markers.

REFERENCES

1. Dretzen G., Bellard M., Sassone-Corsi P., and Chambon P. 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. *Anal. Biochem.* 112:295-298.
2. Girvitz S.C., Bacchetti S., Rainbow A.J., and Graham F.L. 1980. A rapid and efficient procedure for the purification of DNA from agarose gels. *Anal. Biochem.* 106:492-496.

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Protocol 4

Recovery of DNA from Agarose and Polyacrylamide Gels: Electroelution into Dialysis Bags

A messy but reliable technique that works well for DNAs ranging in size from 200 bp to >50 kb.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- DNA staining solution
  Ethanol
- ⚠ Phenol:chloroform (1:1, v/v)
- Sodium acetate (3 M, pH 5.2)
- 0.25x TBE electrophoresis buffer
  Other electrophoresis buffers such as TAE or 0.5x TBE can be used for electroelution of DNA fragments from agarose and polyacrylamide gels. Buffers are used at reduced strength (0.25—0.5x) to increase the rate at which the DNA migrates through the gel.
- 0.25x TBE electrophoresis buffer containing 0.5 µg/ml ethidium bromide

Enzymes and Buffers

Restriction endonucleases

Nucleic Acids and Oligonucleotides

DNA sample

Additional Reagents

Step 3 of this protocol requires reagents listed in Chapter 5, Protocol 2.

Step 10 of this protocol may require reagents listed in Chapter 5, Protocol 5.

METHOD

1. Digest an amount of the sample DNA that will yield at least 100 ng of the fragment(s) of interest. Separate the fragments by electrophoresis through an agarose or polyacrylamide gel of the appropriate concentration, stain with 0.5 µg/ml ethidium bromide or SYBR Gold, and locate the band(s) of interest with a hand-held, long-wavelength UV lamp. *Agarose gels may be cast with ethidium bromide or run and subsequently stained either with ethidium bromide or with SYBR Gold (please see Chapter 5, Protocol 2). If the DNA is separated by electrophoresis through acrylamide, the gel is subsequently stained either with ethidium bromide or with SYBR Gold (please see Chapter 5, Protocol 9 and Chapter 5, Protocol 10). Excitation of the ethidium bromide-DNA complex may cause photobleaching of the dye and single-strand breaks. Use of a source that emits at 302 nm instead of 254 nm will minimize both effects.*
2. Use a sharp scalpel or razor blade to cut out a slice of agarose or polyacrylamide containing the band of interest, and place it on a square of Parafilm wetted with 0.25x TBE. Cut the smallest slice of gel possible to reduce the amount of contamination of DNA with inhibitors, to minimize the distance the DNA need migrate to exit the gel, and to ensure an easy fit into the dialysis tubing on hand.
3. After excising the band, photograph the gel as described in Chapter 5, Protocol 2 to establish a record of which band was removed.
4. Wearing gloves, seal one end of a piece of dialysis tubing with a secure knot. Fill the dialysis bag to overflowing with 0.25x TBE. Holding the neck of the bag and slightly squeezing the tubing to open it, use a thin spatula to transfer the gel slice into the buffer-filled bag.
5. Allow the gel slice to sink to the bottom of the bag. Squeeze out most of the buffer, leaving just enough to keep the gel slice in constant contact with the buffer. Place a dialysis clip just above the gel slice to seal the bag. Avoid trapping air bubbles and clipping the gel slice itself. Use a permanent felt-tipped marker to label the dialysis clip with the name of the DNA fragment.
6. Immerse the bag in a shallow layer of 0.25x TBE in a horizontal electrophoresis tank. Use a glass rod or pipette to prevent the dialysis bag from floating and to maintain the gel fragment in an orientation that is parallel to the electrodes. Pass an electric current through the bag (7.5 V/cm) for 45-60 minutes. Use a hand-held, long-wavelength UV lamp to monitor the movement of the DNA fragment out of the gel slice.
7. Reverse the polarity of the current for 20 seconds to release the DNA from the wall of the bag. Turn off the electric current and recover the bag from the electrophoresis chamber. Gently massage the bag to mix the eluted DNA into the buffer.
8. After the reverse electrophoresis, remove the dialysis clip, and transfer the buffer surrounding the gel slice to a plastic tube. Remove the gel slice from the bag and stain it as described in Step 9. Use a Pasteur pipette to wash out the empty bag with a small quantity of 0.25x TBE after the initial transfer, and add the wash to the tube.
9. Stain the gel slice by immersing it in 0.25x TBE containing ethidium bromide (0.5 µg/ml) for 30-45 minutes at room temperature. Examine the stained slice by UV illumination to confirm that all of the DNA has eluted.
10. Purify the DNA either by passage through DEAE-Sephacel (please see Chapter 5, Protocol 5), by chromatography on commercial resins, or by extraction with phenol:chloroform and standard ethanol precipitation.

REFERENCES

1. McDonell M.W., Simon M.N., and Studier F.W. 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *J. Mol. Biol.* 110:119-146.

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Protocol 5

Purification of DNA Recovered from Agarose and Polyacrylamide Gels by Anion-exchange Chromatography

Fragments of DNA recovered from agarose gels are sometimes poor templates or substrates in subsequent enzymatic reactions. This problem can be solved by binding the DNA to a positively charged matrix, such as DEAE-Sephadex or DEAE-Sephacel, in buffers of low ionic strength. After washing the matrix, the DNA is eluted by raising the strength of the buffer.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol

Isopropanol

⚠ Phenol:chloroform (1:1, v/v)

- TE (pH 7.6)
- TE (pH 7.6) containing 0.1 M NaCl
- TE (pH 7.6) containing 0.2 M NaCl
- TE (pH 7.6) containing 0.3 M NaCl
- TE (pH 7.6) containing 0.6 M NaCl

These four buffers should be sterilized by autoclaving or filtration and stored at room temperature.

Nucleic Acids and Oligonucleotides

DNA samples in TE (pH 7.6)

Additional Reagents

Step 10 of this protocol requires reagents listed in Chapter 5, Protocol 12.

METHOD

1. Suspend the DEAE resin in 20 volumes of TE (pH 7.6) containing 0.6 M NaCl. Allow the resin to settle, and then remove the supernatant by aspiration. Add another 20 volumes of TE (pH 7.6) containing 0.6 M NaCl, and gently resuspend the resin. Allow the resin to settle once more, and then remove most of the supernatant by aspiration. Store the equilibrated resin at 4°C.
2. Pack 0.6 ml (sufficient to bind 20 µg of DNA) of the slurry of DEAE resin into a small column or into the barrel of a 2-cc syringe.
3. Wash the column as follows:

TE (pH 7.6) containing 0.6 M NaCl	3 ml
TE (pH 7.6)	3 ml
TE (pH 7.6) containing 0.1 M NaCl	3 ml

4. Mix the DNA (in TE at pH 7.6) with an equal volume of TE (pH 7.6) containing 0.2 M NaCl. Load the mixture directly onto the column. Collect the flow-through and reapply it to the column.
5. Wash the column twice with 1.5 ml of TE (pH 7.6) containing 0.3 M NaCl.
6. Elute the DNA with three 0.5-ml washes of TE (pH 7.6) containing 0.6 M NaCl.
7. Extract the eluate once with phenol:chloroform.
8. Divide the aqueous phase equally between two microfuge tubes, and add an equal volume of isopropanol to each tube. Store the mixtures for 15 minutes at room temperature, and then recover the DNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge.
9. Wash the pellets carefully with 70% ethanol, store the open tube on the bench for a few minutes to allow the ethanol to evaporate, and then redissolve the DNA in a small volume (3-5 µl) of TE (pH 7.6).
10. Check the amount and quality of the fragment by polyacrylamide or high-resolution agarose gel electrophoresis.
    a. Mix a small aliquot (approx. 20 ng) of the final preparation of the fragment with 10 µl of TE (pH 8.0), and add 2 µl of the desired gel-loading buffer.
    b. Load and run a polyacrylamide or high-resolution agarose gel of the appropriate concentration, using as markers restriction digests of known quantities of the original DNA. The isolated fragment should comigrate with the correct fragment in the restriction digest.
    c. Examine the gel carefully for the presence of faint fluorescent bands that signify the presence of contaminating species of DNA. It is often possible to estimate the amount of DNA in the final preparation from the relative intensities of fluorescence of the fragment and the markers.

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Protocol 6

Recovery of DNA from Low-melting-temperature Agarose Gels: Organic Extraction

DNA fragments separated by electrophoresis through gels cast with low-melting-temperature agarose are recovered by melting the agarose and extracting the resulting solution with phenol:chloroform. The protocol works best for DNA fragments ranging in size from 0.5 kb to 5 kb.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
⚠ Chloroform
DNA staining solution
Ethanol
6x Gel-loading buffer
LMT elution buffer
⚠ Phenol:chloroform (1:1, v/v)
⚠ Phenol, equilibrated to pH 8.0
1x TAE electrophoresis buffer
TE (pH 8.0)

Nucleic Acids and Oligonucleotides

DNA sample

METHOD

1. Prepare a gel containing the appropriate concentration of low-melting-temperature agarose in 1x TAE buffer.
2. Cool the gel to room temperature, and then transfer it and its supporting glass plate to a horizontal surface in a gel box.
3. Mix the samples of DNA with gel-loading buffer, load them into the slots of the gel, and carry out electrophoresis at 3-6 V/cm.
4. If needed, stain the agarose gel with ethidium bromide or with SYBR Gold as described in Chapter 5, Protocol 2 , and locate the DNA band of interest using a hand-held, long-wavelength (302 nm) UV lamp.
5. Use a sharp scalpel or razor blade to cut out a slice of agarose containing the band of interest and transfer it to a clean, disposable plastic tube.
6. After cutting out the band, photograph the gel as described in Chapter 5, Protocol 2 to record which band of DNA was removed.
7. Add approx. 5 volumes of LMT elution buffer to the slice of agarose, close the top of the tube, and melt the gel by incubation for 5 minutes at 65°C.
8. Cool the solution to room temperature, and then add an equal volume of equilibrated phenol. Vortex the mixture for 20 seconds, and then recover the aqueous phase by centrifugation at 4000*g* (5800 rpm in a Sorvall SS-34 rotor) for 10 minutes at 20°C.
   The white substance at the interface is agarose.
9. Extract the aqueous phase once with phenol:chloroform and once with chloroform.
10. Transfer the aqueous phase to a fresh centrifuge tube. Add 0.2 volume of 10 M ammonium acetate and 2 volumes of absolute ethanol at 4°C. Store the mixture for 10 minutes at room temperature, and then recover the DNA by centrifugation, for example, at 5000*g* (6500 rpm in a Sorvall SS-34 rotor) for 20 minutes at 4°C.
11. Wash the DNA pellet with 70% ethanol and dissolve in an appropriate volume of TE (pH 8.0).

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1. Parker R.C. and Seed B. 1980. Two-dimensional agarose gel electrophoresis. "SeaPlaque" agarose dimension. *Methods Enzymol.* 65:358-363.
2. Wieslander L. 1979. A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. *Anal. Biochem.* 98:305-309.

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Protocol 7

Recovery of DNA from Low-melting-temperature Agarose Gels: Enzymatic Digestion with Agarase

A fragment of gel containing a band of DNA is excised and digested with agarase, which hydrolyzes the polymer to disaccharide subunits. The released DNA is then purified by phenol extraction and ethanol precipitation. The method works well for DNAs ranging in size from <5 kb to >20 kb.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- DNA staining solution
  Ethanol
- Gel equilibration buffer
- NaCl (5 M)
- ⚠ Phenol, equilibrated to pH 8.0
- TE (pH 8.0)

Enzymes and Buffers

Agarase

Nucleic Acids and Oligonucleotides

DNA sample

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 5, Protocol 6 .

METHOD

1. Follow Steps 1 through 4 of Chapter 5, Protocol 6 to prepare a gel cast with low-melting-temperature agarose, to load the DNA sample, and to perform electrophoresis.
2. Excise a segment of gel containing the DNA of interest and incubate the gel slice for 30 minutes at room temperature in 20 volumes of gel equilibration buffer.
3. After cutting out the band, photograph the gel as described in Chapter 5, Protocol 2 to record which band of DNA was removed.
4. Transfer the segment of gel to a fresh tube containing a volume of gel equilibration buffer approximately equal to that of the gel slice.
5. Melt the gel slice by incubation for 10 minutes at 65°C. Cool the solution to 40°C and add DNase-free agarase, using 1-2 units of agarase per 200-µl gel slice. Incubate the sample for 1 hour at 40°C.
6. Purify and concentrate the DNA:

To purify small DNA fragments (<20 kb)

a. Extract the DNA solution twice with equilibrated phenol.

b. After the second extraction, transfer the aqueous phase to a fresh tube and add 2 volumes of TE (pH 8.0).

c. Add 0.05 volume of 5 M NaCl followed by 2 volumes of ethanol. (Here, 1 volume is equal to the volume of DNA at the end of Step 6b.) Incubate the tube for 15 minutes at 0°C and then collect the precipitate by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge.

d. Carefully remove the ethanol and add 0.5 ml of 70% ethanol at room temperature. Vortex the mixture and then centrifuge as described in Step c.

e. Remove the supernatant and store the open tube on the bench for a few minutes at room temperature to allow the ethanol to evaporate. Dissolve the DNA in an appropriate volume of TE (pH 8.0).

To purify large DNA fragments (>20 kb)

a. Transfer the agarase-digested sample to a dialysis bag, seal, and place the bag in a beaker or flask containing 100 ml of TE (pH 8.0).

b. Dialyze the sample for several hours at 4°C.

REFERENCES

1. Burmeister M. and Lehrach H. 1989. Isolation of large DNA fragments from agarose gels using agarase. *Trends Genet.* 5:41.

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Protocol 8

Alkaline Agarose Gel Electrophoresis

Alkaline agarose gels are run at a pH that is sufficiently high to denature double-stranded DNA. The denatured DNA is maintained in a single-stranded state and migrates through the alkaline gel as a function of its size. Alkaline agarose gels are used chiefly to measure the size of first and second strands of cDNA (Chapter 1, Protocol 11) and to analyze the size of the DNA strand after digestion of DNA-RNA hybrids with nucleases such as S1.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Agarose

- 10x Alkaline agarose gel electrophoresis buffer
- 6x Alkaline gel-loading buffer
- DNA staining solution

Ethanol

- Neutralizing solution for alkaline agarose gels
- Sodium acetate (3 M, pH 5.2)
- 1x TAE electrophoresis buffer

Nucleic Acids and Oligonucleotides

DNA samples (usually radiolabeled)

METHOD

1. Prepare the agarose solution by adding the appropriate amount of powdered agarose (please see Chapter 5, Protocol 1) to a measured quantity of H_2O in an Erlenmeyer flask or a glass bottle.
2. Loosely plug the neck of the Erlenmeyer flask with Kimwipes. When using a glass bottle, make sure that the cap is loose. Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves.
   Heat the slurry for the minimum time required to allow all of the grains of agarose to dissolve. Check that the volume of the solution has not been decreased by evaporation during boiling; replenish with H_2O if necessary.
3. Cool the clear solution to 55°C. Add 0.1 volume of 10x alkaline agarose gel electrophoresis buffer, and immediately pour the gel as described in Chapter 5, Protocol 1 . After the gel is completely set, mount it in the electrophoresis tank and add freshly made 1x alkaline electrophoresis buffer until the gel is just covered.
   Do not add ethidium bromide because the dye will not bind to DNA at high pH.
   The addition of NaOH to a hot agarose solution causes hydrolysis of the polysaccharide. For this reason, the agarose is first melted in H_2O and then made alkaline by the addition of NaOH just before the gel is poured.
4. Collect the DNA samples by standard precipitation with ethanol. Dissolve the damp precipitates of DNA in 10-20 µl of 1x gel buffer. Add 0.2 volume of 6x alkaline gel-loading buffer.
   It is important to chelate all Mg^{2+} with EDTA before adjusting the electrophoresis samples to alkaline conditions. In solutions of high pH, Mg^{2+} forms insoluble $Mg(OH)_2$ precipitates that entrap DNA.
   It is not strictly necessary to denature the DNA with base before electrophoresis. The exposure of the samples to the alkaline conditions in the gel is usually enough to render the DNA single-stranded.
5. Load the DNA samples dissolved in 6x alkaline gel-loading buffer into the wells of the gel as described in Chapter 5, Protocol 1 . Start the electrophoresis at <3.5 V/cm and, when the bromocresol green has migrated into the gel approx. 0.5-1 cm, turn off the power supply, and place a glass plate on top of the gel. Continue electrophoresis until the bromocresol green has migrated approximately two thirds of the length of the gel.
   Alkaline gels draw more current than neutral gels at comparable voltages and heat up during the run. Alkaline agarose electrophoresis should therefore be carried out at <3.5 V/cm. A glass plate placed on top of the gel after the run is started slows the diffusion of the bromocresol green dye out of the gel and prevents the gel from detaching and floating in the buffer.
6. Process the gel according to one of the procedures described below, as appropriate for the goal of the experiment:
   Southern hybridization
   a. Soak the gel in neutralizing solution for 45 minutes at room temperature, and transfer the DNA to an uncharged nitrocellulose or nylon membrane as described in Chapter 6, Protocol 8 .
      Alternatively, transfer the DNA directly (without soaking the gel) from the alkaline agarose gel to a charged nylon membrane (please see Chapter 6, Protocol 8).
   b. Detect the target sequences in the immobilized DNA by hybridization to an appropriate labeled probe (please see Chapter 6, Protocol 10).
   Staining
   a. Soak the gel in neutralizing solution for 45 minutes at room temperature.
   b. Stain the neutralized gel with 0.5 µg/ml ethidium bromide in 1x TAE or with SYBR Gold.
      A band of interest can be sliced from the gel and subsequently eluted by one of the procedures described in Chapter 5, Protocol 3 or Chapter 5, Protocol 4 .

REFERENCES

1. McDonell M.W., Simon M.N., and Studier F.W. 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *J. Mol. Biol.* 110:119-146.

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Protocol 9

Neutral Polyacrylamide Gel Electrophoresis

How to pour and run a neutral polyacrylamide gel.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Acrylamide:bisacrylamide (29:1) (% w/v)

⚠ Ammonium persulfate (10% w/v)
Ammonium persulfate is used as a catalyst for the copolymerization of acrylamide and bisacrylamide gels. The polymerization reaction is driven by free radicals that are generated by an oxido-reduction reaction in which a diamine (e.g., TEMED) is used as the adjunct catalyst.

Ethanol

6x Gel-loading buffer

⚠ KOH/methanol solution

Siliconizing fluid (e.g., Sigmacote or Acrylease) (optional)

5x TBE electrophoresis buffer
Polyacrylamide gels are poured and run in 0.5x or 1x TBE at low voltage (1-8 V/cm) to prevent denaturation of small fragments of DNA by Joulic heating. Other electrophoresis buffers such as 1x TAE (please see Chapter 5, Protocol 1) can be used, but they are not as good as TBE. The gel must be run more slowly in 1x TAE, which does not provide as much buffering capacity as TBE. For electrophoresis runs greater than 8 hours, we recommend that 1x TBE buffer be used to ensure that adequate buffering capacity is available throughout the run.

⚠ TEMED

Nucleic Acids and Oligonucleotides

DNA samples

METHOD

1. If necessary, clean the glass plates and spacers with KOH/methanol.
2. Wash the glass plates and spacers in warm detergent solution and rinse them well, first in tap water and then in deionized H_2O. Hold the plates by the edges or wear gloves, so that oils from the hands do not become deposited on the working surfaces of the plates. Rinse the plates with ethanol and set them aside to dry.
   The glass plates must be free of grease spots to prevent air bubbles from forming in the gel.
3. (*Optional*) Treat one surface of one of the two plates with siliconizing fluid (e.g., Sigmacote or Acrylease): Place the glass on a pad of paper in a chemical fume hood and pour a small quantity of siliconizing fluid onto the surface. Wipe the fluid over the surface of the plate with a pad of Kimwipes, and then rinse the plate in deionized H_2O. Dry the plate with paper towels.
   This treatment prevents the gel from sticking tightly to one plate and reduces the possibility that the gel will tear when the mold is dismantled after electrophoresis.
4. Assemble the glass plates with spacers:
   a. Lay the larger (or unnotched) plate flat on the bench and arrange the spacers at each side parallel to the two edges.
   b. Apply minute dabs of petroleum jelly to keep the spacer bars in position during the next steps.
   c. Lay the inner (notched) plate in position, resting on the spacer bars.
   d. Clamp the plates together with binder or "bulldog" paper clips and bind the entire length of the two sides and the bottom of the plates with gel-sealing tape to make a watertight seal.
      Take particular care with the bottom corners of the plates, as these are the places where leaks often occur. An extra band of tape around the bottom of the plates can help to prevent leaks.
      There are many types of electrophoresis apparatuses available commercially, and the arrangement of the glass plates and spacers differs slightly from manufacturer to manufacturer. Whatever the design, the aim is to form a watertight seal between the plates and the spacers so that the unpolymerized gel solution does not leak out. Several manufacturers also sell precast polyacrylamide gels, which are foolproof but expensive and often can be used only in the manufacturer's gel apparatus.
5. Taking into account the size of the glass plates and the thickness of the spacers, calculate the volume of gel required. Prepare the gel solution with the desired polyacrylamide percentage according to the table below, which gives the amount of each component required to make 100 ml.

Volume of Reagents Used to Cast Polyacrylamide Gels
Volumes of Reagents to Cast Polyacrylamide Gels of Indicated Concentrations in 1x TBE[a]

Polyacrylamide Gel (%)	29% Acrylamide plus 1% N,N´-Methylenebisacrylamide[b] (ml)	H_2O (ml)	5x TBE (ml)	10% Ammonium Persulfate (ml)
3.5	11.6	67.7	20.0	0.7
5.0	16.6	62.7	20.0	0.7
8.0	26.6	52.7	20.0	0.7
12.0	40.0	39.3	20.0	0.7
20.0	66.6	12.7	20.0	0.7

[a]*Some investigators prefer to run acrylamide gels in 0.5x TBE. In this case, adjust the volumes of 5x TBE and H_2O accordingly.*
[b]*Stock solutions other than 29:1 (% w/v) acrylamide:bisacrylamide can be used to cast polyacrylamide gels. However, it is then necessary to recalculate the appropriate amount of stock solution to use. Gels can be cast with acrylamide solutions containing different acrylamide:bisacrylamide (cross-link) ratios, such as 19:1 and 37.5:1, in place of the 29:1 ratio recommended here. The mobility of DNA and dyes in such gels will be different from those given in this protocol.*

6. (*Optional*) Place the required quantity of acrylamide:bis solution in a clean sidearm flask with a magnetic stir bar. De-aerate the solution by applying vacuum, gently at first. Swirl the flask during de-aeration until no more air bubbles are released.
   De-aeration of the acrylamide solution is not essential, but it does reduce the chance that air bubbles will form when thick gels (>1 mm) are poured, as well as reduce the amount of time required for polymerization.
7. Perform the following manipulations over a tray so that any spilled acrylamide:bis solution will not spread over the bench. Wear gloves. Work quickly to complete the gel before the acrylamide polymerizes.

a. Add 35 µl of TEMED for each 100 ml of acrylamide:bis solution, and mix the solution by gentle swirling.
Gels can be cast with as much as 1 µl of TEMED per milliliter of gel solution to increase the rate of polymerization.

b. Draw the solution into the barrel of a 50-cc syringe. Invert the syringe and expel any air that has entered the barrel. Introduce the nozzle of the syringe into the space between the two glass plates. Expel the acrylamide gel solution from the syringe, filling the space almost to the top.
Keep the remaining acrylamide solution at 4°C to reduce the rate of polymerization. If the plates have been well cleaned and well sealed, there should be no trapped air bubbles and no leaks. If air bubbles form, they can sometimes be coaxed to the top of the mold by gentle tapping or may be snagged with a bubble hook made of thin polypropylene tubing. If these methods fail, empty the gel mold, thoroughly clean the glass plates, and pour a new gel.

c. Place the glass plates against a test tube rack at an angle of approx. 10° to the bench top.

8. Immediately insert the appropriate comb into the gel, being careful not to allow air bubbles to become trapped under the teeth. The tops of the teeth should be slightly higher than the top of the glass. Clamp the comb in place with bulldog paper clips. If necessary, use the remaining acrylamide gel solution to fill the gel mold completely. Make sure that no acrylamide solution is leaking from the gel mold.
9. Allow the acrylamide to polymerize for 30-60 minutes at room temperature, adding more acrylamide:bis gel solution if the gel retracts significantly.
10. After polymerization is complete, surround the comb and the top of the gel with paper towels that have been soaked in 1x TBE. Then seal the entire gel in Saran Wrap and store it at 4°C until needed.
11. When ready to proceed with electrophoresis, squirt 1x TBE buffer around and on top of the comb and carefully pull the comb from the polymerized gel. Use a syringe to rinse out the wells with 1x TBE. Remove the gel-sealing tape from the bottom of the gel with a razor blade or scalpel.
12. Attach the gel to the electrophoresis tank, using large bulldog clips on the sides or clamps built into the apparatus. The notched plate should face inward toward the buffer reservoir.
13. Fill the reservoirs of the electrophoresis tank with electrophoresis buffer prepared from the same batch of 5x TBE used to cast the gel. Use a bent Pasteur pipette or syringe needle to remove any air bubbles trapped beneath the bottom of the gel.
It is important to use the same batch of electrophoresis buffer in both of the reservoirs and in the gel. Small differences in ionic strength or pH produce buffer fronts that can greatly distort the migration of DNA.
14. Use a Pasteur pipette or a syringe to flush out the wells once more with 1x TBE. Mix the DNA samples with the appropriate amount of 6x gel-loading buffer. Load the mixture into the wells using a Hamilton syringe or a micropipette equipped with a drawn-out plastic tip.
Usually, approx. 20-100 µl of DNA sample is loaded per well depending on the size of the slot. Do not attempt to expel all of the sample from the loading device, as this almost always produces air bubbles that blow the sample out of the well. In many cases, the same device can be used to load many samples, provided it is thoroughly washed between each loading. However, it is important not to take too long to complete loading the gel; otherwise, the samples will diffuse from the wells.
15. Connect the electrodes to a power pack (positive electrode connected to the bottom reservoir), turn on the power, and begin the electrophoresis run.
Nondenaturing polyacrylamide gels are usually run at voltages between 1 V/cm and 8 V/cm. If electrophoresis is carried out at a higher voltage, differential heating in the center of the gel may cause bowing of the DNA bands or even melting of the strands of small DNA fragments. Therefore, with higher voltages, gel boxes that contain a metal plate or extended buffer chamber should be used to distribute the heat evenly. Many types of gel apparatuses are equipped with thermal sensors that monitor the temperature of the gel during the run. These are particularly useful when striving to minimize variation from one gel run to the next. Alternatively, use a gel-temperature-monitoring strip.
16. Run the gel until the marker dyes have migrated the desired distance. Turn off the electric power, disconnect the leads, and discard the electrophoresis buffer from the reservoirs.
17. Detach the glass plates, and use a scalpel or razor blade to remove the gel-sealing tape. Lay the glass plates on the bench (siliconized plate uppermost). Use a spacer or plastic wedge to lift a corner of the upper glass plate. Check that the gel remains attached to the lower plate. Pull the upper plate smoothly away. Remove the spacers.
18. Use one of the methods described in Chapter 5, Protocol 10 or Chapter 5, Protocol 11 to detect the positions of bands of DNA in the polyacrylamide gel.

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Protocol 10

Detection of DNA in Polyacrylamide Gels by Staining

Staining bands of DNA in polyacrylamide gels with ethidium bromide, SYBR-Gold, and methylene blue.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- DNA staining solution

METHOD

1. Gently submerge the gel and its attached glass plate in the appropriate staining solution. Use just enough staining solution to cover the gel completely, and stain the gel for 30-45 minutes at room temperature.
2. Remove the gel from the staining solution, using the glass plate as a support, rinse the gel with water, and carefully blot excess liquid from the surface of the gel with a pad of Kimwipes.
   IMPORTANT *Do not use absorbent paper; it will stick to the gel.*
3. Cover the gel with a piece of Saran Wrap. Smooth out any air bubbles or folds in the Saran Wrap with the broad end of a slot comb or a crumpled Kimwipe.
4. Place a piece of Saran Wrap on the surface of a UV transilluminator. Invert the gel, and place it on the transilluminator. Remove the glass plate, leaving the gel on the Saran Wrap.
5. Photograph the gel as described in Chapter 5, Protocol 2 .

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Protocol 11

Detection of DNA in Polyacrylamide Gels by Autoradiography

Detection of radioactive DNA in polyacrylamide gels by autoradiography.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffer and Solutions

⚠ Acetic acid (7% v/v)
⚠ Radioactive Ink

METHOD

1. Immerse the gel, together with its attached glass plate, in 7% acetic acid for 5 minutes. Remove the gel from the fixative by carefully lifting the glass plate from the fluid.
2. Rinse the gel briefly in deionized H_2O. Remove excess fluid from the surface of the gel with a pad of Kimwipes.
   IMPORTANT *Do not use absorbent paper; it will stick to the gel.*
3. (*Optional*) Dry the gel onto a piece of Whatman 3MM paper using a commercial gel dryer.
   Drying the gel is generally necessary only when the gel contains DNA labeled with weak β-emitting isotopes such as ^{35}S or such small amounts of ^{32}P-labeled DNA that long exposures (longer than 24 hours) are necessary to obtain an adequate autoradiographic image.
4. Wrap the gel, together with its supporting glass plate, in Saran Wrap. Smooth out any air bubbles or folds in the Saran Wrap with the broad end of a slot comb or a crumpled Kimwipe.
   If the DNA samples separated through the gel have been labeled with ^{35}S, it is better not to use Saran Wrap because the plastic film will block weak β particles. Make sure that the gel is very dry (in Step 3) and proceed to Step 5.
5. To align the gel and the film, attach adhesive dot labels marked with radioactive ink or with chemiluminescent markers to the surface of the Saran Wrap. Cover the radioactive ink labels with cellophane tape to prevent contamination of the film holder or intensifying screen.
6. Invert the gel and expose it to X-ray film (e.g., Kodak XAR-5 or equivalent) as follows:
   a. In a darkroom, tape the sealed gel to a piece of X-ray film cut to the same size as the glass plate.
      The plate serves as a weight to ensure good contact between the Saran Wrap and the X-ray film.
   b. Wrap the gel and film in light-tight aluminum foil.
      Do not use a metal film cassette; it may break the glass plate and crush the gel. If the gel has been dried onto a piece of Whatman 3MM paper (Step 3), a metal film cassette may be used.
   c. Expose the film for the appropriate period of time at room temperature or at -70°C with or without an intensifying screen.
   d. Develop, fix, and dry the X-ray film as recommended by the manufacturer.

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Protocol 12

Isolation of DNA Fragments from Polyacrylamide Gels by the Crush and Soak Method

The "crush and soak" method, which works best for DNAs <1 kb in size, can be used to recover both single- and double-stranded DNAs from polyacrylamide gels. The yield of eluted DNA varies from <30% to >90% depending on the size of the DNA fragment.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Acrylamide gel elution buffer
- ⚠ Chloroform
- Ethanol
- 6x Gel-loading buffer
- ⚠ Phenol:chloroform (1:1, v/v)
- Sodium acetate (3 M, pH 5.2)
- TE (pH 8.0)

Nucleic Acids and Oligonucleotides

DNA markers generated by restriction digests of known quantities of the DNA sample

DNA sample

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 5, Protocol 9 .

Step 1 of this protocol also requires the reagents listed in either Chapter 5, Protocol 10 or Chapter 5, Protocol 11 .

METHOD

1. Carry out polyacrylamide gel electrophoresis of the DNA sample and markers as described in Chapter 5, Protocol 9 . Locate the DNA of interest by autoradiography (Chapter 5, Protocol 11) or by examination of ethidium bromide- or SYBR Gold-stained gels in long-wavelength (302 nm) UV light (Chapter 5, Protocol 10).
2. Use a clean sharp scalpel or razor blade to cut out the segment of the gel containing the band of interest, keeping the size of the polyacrylamide slice as small as possible. This can be achieved by any of the following methods:
   - While the DNA is illuminated with UV light, cut through both the gel and the Saran Wrap, and then peel the small piece of gel containing the DNA from the Saran Wrap.
   - Use a permanent felt-tipped marker (e.g., Sharpie pen) to outline the DNA band on the back of the glass plate while the gel is illuminated from below with UV light. Invert the gel, remove the Saran Wrap, and cut out the band using the marker outline as a guide.
   - In the case of a fragment of DNA identified by autoradiography, place the exposed autoradiographic film on the Saran Wrap and align it with the gel. Use a permanent marker to outline the position of the desired DNA fragment on the back of the glass plate. Remove the exposed film and Saran Wrap and cut out the band.

   Photograph or autoradiograph the gel after the bands of DNA have been excised to produce a permanent record of the experiment.
3. Transfer the gel slice to a microfuge tube or a polypropylene tube. Use a disposable pipette tip or inoculating needle to crush the polyacrylamide gel against the wall of the tube.
4. Calculate the approximate volume of the slice and add 1-2 volumes of acrylamide gel elution buffer to the microfuge tube.
5. Close the tube and incubate it at 37°C on a rotating wheel or rotary platform.
   At this temperature, small fragments of DNA (<500 bp) are eluted in 3-4 hours; larger fragments take 12-16 hours.
6. Centrifuge the sample at maximum speed for 1 minute at 4°C in a microfuge. Transfer the supernatant to a fresh microfuge tube, being extremely careful to avoid transferring fragments of polyacrylamide (a drawn-out Pasteur pipette works well).
7. Add an additional 0.5 volume of acrylamide gel elution buffer to the pellet of polyacrylamide, vortex briefly, and centrifuge again. Combine the supernatants.
8. (*Optional*) Remove any remaining fragments of polyacrylamide by passing the supernatant through a disposable plastic column equipped with a frit (e.g., Isolabs, Inc., Quick-Sep columns) or a syringe barrel plugged with a Whatman GF/C filter or siliconized glass wool.
   The eluted DNA can be extracted with phenol:chloroform and chloroform to remove SDS, which can inhibit subsequent enzymatic manipulation of the DNA. Precipitate the extracted DNA with ethanol as described in Step 9 and continue with the remainder of the protocol.
9. Add 2 volumes of ethanol at 4°C to the flow-through and store the solution on ice for 30 minutes. Recover the DNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge.
   Even small quantities of DNA are efficiently precipitated by ethanol in this method. However, 10 µg of carrier RNA can be added before precipitation, which may improve even further the recovery of small amounts of DNA. Before adding the RNA, make sure that the presence of RNA will not compromise subsequent reactions with the DNA. (For preparation of carrier RNA, please see Chapter 5, Protocol 3 .)
10. Dissolve the DNA in 200 µl of TE (pH 8.0), add 25 µl of 3 M sodium acetate (pH 5.2), and again precipitate the DNA with 2 volumes of ethanol as described in Step 9.
11. Carefully rinse the pellet once with 70% ethanol, and dissolve the DNA in TE (pH 8.0) to a final volume of 10 µl.
12. Check the amount and quality of the fragment by polyacrylamide or high-resolution agarose gel electrophoresis:
    a. Mix a small aliquot (approx. 20 ng) of the final preparation of the fragment with 10 µl of TE (pH 8.0), and add 2 µl of the desired gel-loading buffer.
    b. Load and run a polyacrylamide or high-resolution agarose gel of the appropriate concentration, using as markers restriction digests of known quantities of the original DNA. The isolated fragment should comigrate with the correct fragment in the restriction digest.
    c. Examine the gel carefully for the presence of faint fluorescent bands that signify the presence of contaminating species of DNA. It is often possible to estimate the amount of DNA in the final preparation from the relative intensities of fluorescence of the fragment and the markers.

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1. Maxam A.M. and Gilbert W. 1977. A new method for sequencing DNA. *Proc. Natl. Acad. Sci.* 74:560-564.

29. Maxam A.M. and Gilbert W. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65:499-560.

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Protocol 13

Preparation of DNA for Pulsed-field Gel Electrophoresis: Isolation of DNA from Mammalian Cells and Tissues

Genomic DNAs from mammalian cells are prepared for pulsed-field gel electrophoresis by lysing cells in situ in an agarose plug. Following digestion with an appropriate restriction enzyme, the plug is loaded directly into the well of a pulsed-field gel or it can be melted before loading.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- EDTA (0.5 M, pH 8.0)
- L buffer
- L buffer with proteinase K and Sarkosyl
  Amend the L buffer to a final concentration of 1% (w/v) Sarkosyl. Store L buffer with Sarkosyl at room temperature, and add proteinase K to 0.1 mg/ml just before use.
- PBS
  Use ice-cold PBS for all cell and tissue preparations, except white blood cells. In the latter case, the PBS should be at room temperature.
- Red blood cell lysis buffer
- TE (pH 7.6)
- TE (pH 7.6) containing 40 µg/ml PMSF

Cells and Tissues

Cell or tissue sample
This protocol describes methods for dealing with cultured cell lines, fresh or frozen tissue samples, or white blood cells.

METHOD

1. Prepare cells or tissue samples.
   For cultured cells
   a. Wash cells that have been growing in culture three times in ice-cold PBS.
   b. Harvest the cells by scraping into a small volume of ice-cold PBS-using a sterilized rubber policeman. Collect the cells by low-speed centrifugation.
   c. Resuspend the cells at a concentration of approx. 2 x 10^7 cells/ml in ice-cold L buffer.
   For fresh tissue samples
   a. In a Petri dish, use a clean scalpel to mince freshly excised tissue into small cubes (1-2 mm^3) and then homogenize the cubes in ice-cold PBS in a chilled glass homogenizer with a tight-fitting pestle.
   b. Remove fragments of connective tissue by filtration through two layers of cheesecloth.
   c. Wash the suspended cells three times in ice-cold PBS and resuspend them at a concentration of 2 x 10^7 cells/ml in ice-cold L buffer.
   Use a hemacytometer to count the cells.
   For frozen tissue samples
   a. Grind frozen tissue to a fine powder using a mortar and pestle chilled to -70°C and suspend the powdered tissue in ice-cold PBS.
   b. Remove fragments of connective tissue by filtration through two layers of cheesecloth.
   c. Wash the suspended cells three times in ice-cold PBS and resuspend them at a concentration of 2 x 10^7 cells/ml in ice-cold L buffer.
   For white blood cells
   a. Fractionate 5-10 ml of starting blood by centrifugation in LeucoPrep cell separation tubes to grossly separate white and red blood cells.
   b. To the white blood cell layer (buffy coat) add 4 volumes of red blood cell lysis buffer and gently mix by two to three inversions of the tube.
   c. Incubate the cells in buffer for 5 minutes at room temperature, and then centrifuge the tube at 3000*g* (5000 rpm in a Sorvall SS-34 rotor) for 5 minutes at room temperature.
   d. Resuspend the pellet in 1 ml of PBS at room temperature.
2. Prepare a volume of 1% low-melting-temperature agarose in L buffer that is equal to the volume of the cell preparation in Step 1. Cool the melted agarose to 42°C.
3. When the agarose has cooled to 42°C, warm the cell suspension (Step 1) to the same temperature. Mix the melted agarose with the suspended cells. Stir the mixture with a sealed Pasteur pipette to ensure that the cells are evenly dispersed throughout the agarose.
4. Pipette or pour the molten mixture into preformed Plexiglas molds (50-100 µl), or draw the mixture into an appropriate length of Tygon tubing (1/8-inch or 3.2-mm internal diameter), or a 1-ml plastic syringe barrel. Store the molds for 15 minutes at room temperature, and then transfer them to 4°C for 15-30 minutes.
5. When the agarose has set, gently collect the plugs from the Plexiglas molds or gently blow out the agarose from the Tygon tubing or syringe barrel into a Petri dish. Cut the cylindrical plugs into 1-cm sections.
   Each 1-cm length of agarose (45 µl) should contain approx. 0.5 x 10^6 cells and yield approx. 2-5 µg of DNA. Analysis of larger amounts of DNA in the individual lanes of a pulsed-field gel will distort the calculated molecular weights of the DNA fragments. The migration of large DNA fragments is significantly slowed when electrophoresis is carried out at high DNA concentrations. In turn, this slowing will lead to an overestimate of the size of an individual DNA fragment.
6. Transfer the plugs to 3 volumes of L buffer containing 0.1 mg/ml proteinase K and 1% (w/v) Sarkosyl. Incubate the plugs for 3 hours at 50°C. Replace the original digestion mixture with two volumes of fresh digestion mixture and continue the incubation for 12-16 hours at 50°C.
   Be as careful as possible not to scar the agarose plugs when changing buffer solutions.
   Some DNA isolation protocols include very long incubations (24-48 hours) at 50°C when treating the embedded cells with proteinase K in the presence of Sarkosyl. These extended incubation times are not required and may lead to degradation of the high-molecular-weight DNA.
7. Incubate the plugs at room temperature in 50 volumes of TE (pH 7.6) with three to five changes of buffer over a period of 3 hours.
8. Remove the TE and replace with 2 volumes of TE (pH 7.6) containing 40 µg/ml PMSF. Incubate for 30 minutes at 50°C.
9. Incubate the plugs at room temperature in 50 volumes of TE (pH 7.6) with three to five changes of buffer over a period of 3 hours.

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1. Anand R. 1986. Pulsed field gel electrophoresis: A technique for fractionating large DNA molecules. *Trends Genet.* 2:278-283.
2. Schwartz D.C. and Cantor C.R. 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* 37:67-75.

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Protocol 14

Preparation of DNA for Pulsed-field Gel Electrophoresis: Isolation of Intact DNA from Yeast

Yeast cells are first treated enzymatically to break down the cell walls and then resuspended in low-melting-temperature agarose plugs. The DNA is liberated by infusing the plugs with lysis buffer and proteases. This method is used to prepare both conventional and artificial yeast chromosomes.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- EDTA (0.05 M, pH 8.0)
- L buffer
- L buffer with proteinase K and Sarkosyl
- TE (pH 7.6)
- TE (pH 7.6) containing 40 µg/ml PMSF
- Yeast cell wall digestion enzymes
- Yeast cell wash buffer
- Yeast lysis buffer

Cells and Tissues

Yeast suspension culture

Prepare the yeast culture at a volume and cell density appropriate to yield the required number of plugs of embedded DNA.

METHOD

1. Collect yeast cells growing in suspension by centrifugation at 3000*g* (4300 rpm in a Sorvall GSA rotor) for 5 minutes at 4°C. Wash the cell pellet twice with yeast cell wash buffer.
2. Resuspend the cells at a concentration of 3 x 10^9 cells/ml in 0.05 M EDTA (pH 8.0) at 0°C.
3. Prepare an equal volume of 1% low-melting-temperature agarose in L buffer. Cool the melted agarose to 42°C.
4. Add 75 µl of zymolyase or lyticase solution to the cell suspension of Step 2. Mix well.
5. Warm the cell suspension to 42°C. Mix 5 ml of the melted agarose with 5 ml of the suspended cells. Stir the mixture with a sealed Pasteur pipette to ensure that the cells are evenly dispersed throughout the agarose.
6. Pipette or pour the molten mixture into preformed Plexiglas molds (50-100 µl), or draw the mixture into an appropriate length of Tygon tubing (3/32-inch internal diameter) or a 1-ml plastic syringe barrel. Store the molds for 15 minutes at room temperature, and then transfer them to 4°C for 15-30 minutes.
7. When the agarose has set, collect the plugs from the Plexiglas molds, or blow out the agarose from the Tygon tubing or syringe barrel into a Petri dish. Cut the cylindrical plugs into 1-cm blocks.
   Each block (50 µl) should contain approx. 5 µg of yeast chromosomal DNA.
8. Incubate the blocks in a chemical fume hood for 3 hours at 37°C in 3 volumes of yeast lysis buffer.
9. Add 3 volumes of L buffer containing 0.1 mg/ml proteinase K and 1% (w/v) Sarkosyl into a fresh Petri dish. Transfer the blocks to this buffer, and incubate them for 3 hours at 50°C. Replace the original digestion mixture with an equal volume of fresh digestion mixture and continue incubation for 12-16 hours at 50°C.
   Be as careful as possible not to scar the agarose plugs when changing buffer solutions.
10. Incubate the plugs at 50°C in 50 volumes of TE (pH 7.6) containing 40 µg/ml PMSF. After 1 hour, replace the original rinse buffer (TE containing PMSF) with an equal volume of fresh rinse buffer and continue incubation for another hour at 50°C.
11. Remove the rinse buffer (TE containing PMSF), replace with an equal volume of fresh TE (pH 7.6), and continue incubation for another hour at room temperature.

REFERENCES

1. Schwartz D.C. and Cantor C.R. 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* 37:67-75.

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Protocol 15

Restriction Endonuclease Digestion of DNA in Agarose Plugs

Genomic DNA isolated from mammalian, yeast, or bacterial cells can be digested with restriction endonucleases by incubating agarose plugs containing the DNA in the presence of the desired enzyme. After digestion, the DNA can be fractionated by pulsed-field gel electrophoresis and either isolated from the gel or analyzed by Southern Hybridization.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Restriction enzyme buffer

TE (pH 7.6)

Enzymes and Buffers

Restriction endonucleases

Nucleic Acids and Oligonucleotides

Genomic DNA embedded in low-melting-temperature agarose plugs
Please see Chapter 5, Protocol 13 for the preparation of low-melting-temperature agarose plugs containing mammalian cell DNA or Chapter 5, Protocol 14 for the preparation of plugs containing yeast DNA.

METHOD

1. If plugs have not been stored in TE (e.g., plugs received through the mail or those that have been stored in 0.5 M EDTA [pH 8.0]), incubate them in 50 volumes of TE (pH 7.6) for 30 minutes at room temperature. Transfer the plugs to 50 volumes of fresh TE (pH 7.6) and continue incubation for a further 30 minutes. Otherwise, proceed directly to Step 2.
2. Transfer the plugs to individual microfuge tubes, and add 10 volumes of the appropriate 1x restriction enzyme buffer to each tube. Incubate the tubes for 30 minutes at room temperature.
   Individual buffers should be supplemented with spermidine to enhance the efficiency of restriction digestion. High-salt buffers (containing 100-150 mM salt) should be supplemented to a final concentration of 10 mM spermidine, medium-salt buffers (50-100 mM salt) to 5 mM spermidine, and low-salt buffers (<50 mM salt) to 3 mM spermidine. It is best to supplement the buffers from a 0.1 M spermidine stock (dissolved in H_2O) just before use.
3. Remove the buffer and replace it with 2-3 volumes of fresh 1x restriction enzyme buffer. Add 20-30 units of the appropriate restriction enzyme to each tube, and incubate the tubes at the optimal temperature for the restriction enzyme: 3 hours if YAC DNA is used or 5- 6 hours if mammalian DNA is used.
4. If the DNA is to be digested with only one restriction enzyme, then soak the plugs in 20 volumes of TE (pH 7.6) at 4°C. After 1 hour, proceed with Step 7. If the DNA is to be treated with more than one enzyme, skip the incubation in TE and proceed to Step 5.
5. If the DNA is to be digested with more than one restriction enzyme, reequilibrate the plug buffer before adding the second enzyme. To reequilibrate, use automatic pipetting devices to remove as much as possible of the first restriction enzyme buffer from each tube and replace it with 1 ml of TE (pH 7.6). Remove the TE and replace it with a fresh 1 ml of TE. Store the plug for 30-60 minutes at room temperature. Gently remove the TE buffer.
6. Add 10 volumes of the appropriate second 1x restriction enzyme buffer to each tube. Incubate the tubes for 30 minutes at room temperature. Repeat the restriction enzyme digestion as described in Step 3. Finally, soak each plug in 20 volumes of TE (pH 7.6) for 1 hour at 4°C.
7. Use a disposable pipette tip to push the blocks directly into the slots of a pulsed-field gel, and separate the fragments of DNA by electrophoresis (Chapter 5, Protocol 17 and Chapter 5, Protocol 18).

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Protocol 16

Markers for Pulsed-field Gel Electrophoresis

Markers for pulsed-field gel electrophorsis can be generated by ligation of linear monomers of bacteriophage λ DNA (48.5 kb) into a nested series of concatemers. This procedure yields a series of concatemers that contain up to 20 tandemly arranged copies of bacteriophage DNA.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (0.1 M)
DTT (Dithiothreitol) (1 M)
EDTA (0.5 M, pH 8.0)
1x Ligation buffer with PEG
LMT agarose buffer
$MgCl_2$ (20 mM)
⚠ PEG 8000 (8% w/v)
TE (pH 7.6)

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Nucleic Acids and Oligonucleotides

Purified bacteriophage λ DNA
Please see Chapter 2, Protocol 11 and Chapter 2, Protocol 12.

METHOD

1. Dissolve 0.1 g of low-melting-temperature agarose in 10 ml of LMT buffer by heating it in a boiling water bath or by microwaving. Cool the solution to 37°C.
2. Dissolve 10 µg of purified bacteriophage λ DNA in 172.5 µl of TE (pH 7.6) and heat the solution to 56°C for 5 minutes.
3. Cool the solution to 37°C, and rapidly add the following reagents in the order listed:

8% PEG 8000	62.5 µl
20 mM $MgCl_2$	5.0 µl
0.1 M ATP	5.0 µl
1 M dithiothreitol	5.0 µl
bacteriophage T4 DNA ligase	0.5 Weiss unit
1% LMT agarose solution (Step 1)	250 µl

Polyethylene glycol acts like a crowding agent and increases the efficiency of ligation.

4. Draw the mixture into a short length of Tygon tubing, and chill the tubing on ice until the agarose has completely set.
5. Blow the agarose plug into a sterile, disposable plastic tube containing at least 3 volumes of 1x ligation buffer with polyethylene glycol.
6. Incubate the plug in ligation buffer for 24 hours at room temperature and then transfer it to a tube containing 10 volumes of 20 mM EDTA (pH 8.0).
7. Incubate the plug in EDTA for 1 hour, then transfer the plug to a tube containing 10 volumes of fresh 20 mM EDTA (pH 8.0), and store at 4°C until needed for electrophoresis in Chapter 5, Protocol 17 and Chapter 5, Protocol 18 .

REFERENCES

1. Vollrath D. and Davis R.W. 1987. Resolution of DNA molecules greater than 5 megabases by contour-clamped homogeneous electric fields. *Nucleic Acids Res.* 15:7865-7876.

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Protocol 17

Pulsed-field Gel Electrophoresis via Transverse Alternating Field Electrophoresis Gels

In this form of pulsed-field gel electrophoresis, electrodes are positioned on opposite sides of a vertically oriented gel. The DNA moves first toward one electrode and then toward the other, forming a zigzag pattern. The vector of this oscillation is a straight line from the loading well to the base of the gel. Variation in voltage and pulse time allows separation of DNAs ranging in size from 2 kb to >6000 kb. This protocol, supplied by Tommy Hyatt and Helen Hobbs (University of Texas Southwestern Medical Center, Dallas), describes the resolution of genomic DNA by TAFE, followed by blotting and hybridization.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Denaturation solution
- TAFE gel electrophoresis buffer
- TAFE gel electrophoresis buffer containing 0.5 µg/ml ethidium bromide or an appropriate dilution of SYBR Gold
  A buffer of 0.045 M Tris-borate (pH 8.2) and 0.01 M EDTA has also been used successfully in this protocol. For a discussion of staining agarose gels, please see Chapter 5, Protocol 2 .
- TE (pH 8.0)

Nucleic Acids and Oligonucleotides

DNA size standards
Please see Step 2.

Genomic DNA of interest
Please see Step 2.

Additional Reagents

Step 2 of this protocol requires the reagents listed in Chapter 5, Protocol 13 or Chapter 5, Protocol 14 , Chapter 5, Protocol 15 , and Chapter 5, Protocol 16 .

Steps 9 and 10 of this protocol require the reagents listed in Chapter 6, Protocol 8 .

Step 11 of this protocol requires the reagents listed in Chapter 6, Protocol 10 .

METHOD

1. Cast a 1% agarose gel in 1x TAFE gel buffer without ethidium bromide and allow the gel to set. Pour the gel with the same buffer solution to be used to fill the gel apparatus.
2. Prepare agarose plugs containing the DNA of interest (please see Chapter 5, Protocol 13 for preparation of mammalian DNA or Chapter 5, Protocol 14 for preparation of yeast DNA embedded in plugs) and carry out digestion with restriction enzymes as described in Chapter 5, Protocol 15 . Prepare and embed the appropriate DNA size standards as described in Chapter 5, Protocol 14 and Chapter 5, Protocol 16 .
3. Rinse all of the plugs in 10 volumes of TE (pH 8.0) for 30 minutes with two changes of buffer.
4. Embed the digested and rinsed DNA plugs in individual wells of the gel. Seal the plugs in the wells with molten 1% agarose in 1x TAFE gel buffer.
5. Place the gel in the TAFE apparatus filled with 1x TAFE gel buffer previously cooled to 14°C.
6. Connect the gel apparatus to the appropriate power supply set to deliver 4-second pulses at a constant current of 170-180 mA for 30 minutes. This setting forces the DNA to enter the gel rapidly. After this time period, decrease the current input to 150 mA, change the pulse time to a setting optimum for the size range of DNAs to be resolved (please see the table below), and continue electrophoresis for 12-18 hours.
   TAFE Gel Conditions for Separating DNAs of Various Sizes

Size Range (kb)	Pulse Time (Seconds)	Time (Hours)
5-50	1	10
20-100	3	10
50-250	10	14
100-400	20	14
200-1000	45	18
200-1600	70	20
Larger DNAs	see text	see text

   In a Tris-acetate/EDTA buffer, a pulse time of 15 seconds will separate DNA fragments in the 50-400-kb size range. This same range of fragments can be separated in the Tris-borate/EDTA buffer using a program of 8-second pulses at 350 mA for 12 hours followed by 15-second pulses at 350 mA for an additional 12 hours.
7. Disconnect the power supply, dismantle the gel apparatus, and stain the gel in 1x TAFE buffer containing 0.5 µg/ml ethidium bromide or an appropriate dilution of SYBR Gold. Photograph the gel under UV light.
   The standard technique used to detect DNAs separated by PFGE is staining with ethidium bromide or SYBR Gold (please see Chapter 5, Protocol 2). To facilitate the detection of minor species of DNA stained with ethidium bromide, the gels may be destained in H_2O for up to 1 hour before photography. For details of methods that can be used to maximize the photographic detection of DNA stained with these dyes, please see Chapter 5, Protocol 2.
8. Rinse the stained gel twice with H_2O. Pour off the second H_2O rinse and replace with denaturation solution. Incubate with gentle shaking for 30 minutes. Change the denaturation solution and incubate for an additional 30 minutes.
   In general, standard methods of Southern blotting as described in Chapter 6 (please see Chapter 5, Protocol 8 and Chapter 5, Protocol 9) can be used to detect hybridizing gene sequences in TAFE gels. Some investigators find that the very large DNA fragments typically analyzed by PFGE transfer to nylon membranes more efficiently after partial hydrolysis of the DNA by acid treatment. This treatment causes partial depurination and nicking of larger DNA fragments and in so doing enhances capillary transfer. To include an acid hydrolysis step, after the electrophoresis is complete, rinse the gel twice with H_2O in Step 8, pour off the second H_2O rinse, and replace with 25 mM HCl. Soak the gel with gentle agitation for 3-5 minutes. Rinse the gel with H_2O and continue with the denaturation protocol of Step 8 and onward.
   When acid treatments are used in the protocol, it is important to stain the agarose gel after transfer of the DNA to the nylon membrane. A slight residual smear of DNA should be visible. If no residual DNA is detected, then the acid treatment may have been too harsh (i.e., too long or too strong). Too much depurination and nicking increases the transfer of the DNA but also tends to reduce the intensity of subsequent hybridization signals.
9. Transfer the DNA directly to a nylon membrane by capillary blotting in denaturation solution (for details, please see Chapter 6, Protocol 8).

Some investigators find that transfer of larger DNA fragments is enhanced when 6x SSC buffer is used in capillary blotting rather than the more standard 10x SSC solution.

10. After transfer, affix the DNA to the nylon membrane by baking for 2 hours at 80°C, by UV cross-linking, or by microwaving.
11. Carry out prehybridization and hybridization with labeled probes in a formamide-containing buffer (for details, please see Chapter 6, Protocol 10).

    We typically use ^{32}P-radiolabeled single-stranded bacteriophage M13 probes at a concentration of 5 x 10^6 to 6 x 10^6 cpm/ml of hybridization buffer to detect a single-copy gene in a complex mammalian genome. A membrane hybridized for 12-16 hours in this fashion is washed in 500 ml of 2x SSC containing 1% (w/v) SDS for 15 minutes at room temperature, scrubbed gently with a sponge, then washed in 1 liter of 0.5x SSC containing 1% (w/v) SDS for 2 hours at 68°C, and then subjected to autoradiography.

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1. Gardiner K. and Patterson D. 1989. Transverse alternating field electrophoresis and applications to mammalian genome mapping. *Electrophoresis* 10:296-302.
2. Gardiner K., Laas W., and Patterson D. 1986. Fractionation of large mammalian DNA restriction fragments using vertical pulsed-field gradient gel electrophoresis. *Somat. Cell Mol. Genet.* 12:185-195.

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Protocol 18

Pulsed-field Gel Electrophoresis via Contour-clamped Homogeneous Electric Field Gels

In CHEF gels, the electric field is generated from multiple electrodes, arranged in a square of hexagonal contour around the horizontal gel and clamped to predetermined potentials. Using a combination of low field strengths, low concentrations of aragose, long switching intervals, and extended periods of electrophoresis, DNAs up to 5000 kb can be resolved. This protocol, supplied by Elsy Jones (University of Texas Southwestern Medical Center, Dallas), describes the resolution of genomic DNA by TAFE, followed by blotting and hybridization.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Denaturation solution
- DNA staining solution
  For a discussion of staining agarose gels, please see Chapter 5, Protocol 2 .
- 0.5x TBE gel electrophoresis buffer

Nucleic Acids and Oligonucleotides

DNA size standards
Please see Step 3.

Genomic DNA of interest
Please see Step 3.

Additional Reagents

Step 3 of this protocol requires the reagents listed in Chapter 5, Protocol 13 or Chapter 5, Protocol 14 , Chapter 5, Protocol 15 , and Chapter 5, Protocol 16 .

Steps 10 and 11 of this protocol require the reagents listed in Chapter 6, Protocol 8 .

Step 12 of this protocol requires the reagents listed in Chapter 6, Protocol 10 .

METHOD

1. Cast an agarose gel of the appropriate concentration in 0.5x TBE buffer as described in Chapter 5, Protocol 1 . Use a bubble level to ensure that the casting tray is completely flat on the laboratory bench. Allow the gel to harden for 1 hour at room temperature.
2. Place the agarose gel in the CHEF apparatus, add enough 0.5x TBE to just cover the gel, and cool the remaining buffer to 14°C.
3. Prepare agarose plugs containing the DNA of interest (please see Chapter 5, Protocol 13 for preparation of mammalian DNA or Chapter 5, Protocol 14 for preparation of yeast DNA embedded in plugs) and carry out digestion with restriction enzymes as described in Chapter 5, Protocol 15 . Prepare and embed the appropriate DNA size standards as described in Chapter 5, Protocol 14 or Chapter 5, Protocol 16 .
4. Gently place the plugs in individual microfuge tubes and add 200 µl of 0.5x TBE to each. Incubate the plugs for 15 minutes at room temperature.
5. Embed the digested and rinsed DNA plugs in individual wells of the gel. Seal the plugs in the wells with the same solution of molten agarose used to pour the gel.
6. Allow the sealed plugs to harden in the gel for approx. 5 minutes, and then add additional 0.5x TBE buffer (previously cooled to 14°C in Step 2) to the apparatus to cover the agarose gel completely.
7. Start the buffer circulating and begin the electrophoresis run using power supply settings as described in the table below.
   Conditions for Separating DNAs of Various Sizes in CHEF Gels

% Agarose	Size Range (kb)	Switch Times	V/CM	Time (Hours)
1% Fast Lane	6-500	ramped, 3-80 seconds	6	18
	10-800	ramped, 6-80 seconds	6	20
	100-1000	60 seconds,	6	15
		then 90 seconds	6	9
0.8% Fast Lane	150-2000	ramped, 30-180 seconds	5	24

   All gels are run in 0.5x TBE. When very high resolution is required in the 800-2000-kb range, a lower voltage and a longer run time than those indicated above are used. If the gels are cast with Seakem GTG agarose, add 10% to the electrophoresis time indicated.
8. After electrophoresis, stain the gel in a 1 µg/ml solution of ethidium bromide or an appropriate dilution of SYBR Gold for 30 minutes at room temperature. Destain the gel in H_2O for 30 minutes and photograph the gel under UV light.
9. After photography, incubate the gel in 250 ml of denaturation solution with gentle shaking for 30 minutes. Change the denaturation solution and incubate for an additional 30 minutes.
10. Transfer the DNA directly to a nylon membrane by capillary blotting in denaturation solution (for details, please see Chapter 6, Protocol 8).
    Enhanced transfer of larger DNA fragments has also been noted when 6x SSC buffer is used in capillary blotting rather than the more standard 10x SSC solution.
11. After transfer, affix the DNA to the nylon membrane by baking for 2 hours at 80°C, by UV cross-linking, or by microwaving.
12. Carry out prehybridization and hybridization with labeled probes in a formamide-containing buffer (for details, please see Chapter 6, Protocol 10).

REFERENCES

1. Chu G., Vollrath D., and Davis R.W. 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science* 234:1582-1585.
2. Vollrath D. and Davis R.W. 1987. Resolution of DNA molecules greater than 5 megabases by contour-clamped homogeneous electric fields. *Nucleic Acids Res.* 15:7865-7876.

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Protocol 19

Direct Retrieval of DNA Fragments from Pulsed-field Gels

A gel slice containing a fragment of DNA resolved by pulsed-field gel electrophoresis is treated with agarase. The released DNA can be used as a substrate for ligation or restriction without further purification.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

DNA staining solution
For a discussion of staining agarose gels, please see Chapter 5, Protocol 2 .

⚠ Phenol:chloroform (1:1, v/v) (optional)

Enzymes and Buffers

Agarase digestion buffer
Use the buffer supplied with the enzyme. We recommend that all buffers be supplemented with 100 mM NaCl, 30 µM spermine, 70 µM spermidine to enhance recovery of the DNA.

Nucleic Acids and Oligonucleotides

DNA size standards
For the preparation of two different ranges of sizing standards, please see Chapter 5, Protocol 14 and Chapter 5, Protocol 16 .

Genomic DNA embedded in low-melting-temperature agarose plugs
Please see Chapter 5, Protocol 13 for preparation of mammalian DNA or Chapter 5, Protocol 14 for preparation of yeast DNA embedded in plugs. Carry out digestion with restriction enzymes as described in Chapter 5, Protocol 15 of this chapter.

Additional Reagents

Steps 1 and 2 of this protocol require the reagents listed in Chapter 5, Protocol 17 or Chapter 5, Protocol 18 .

METHOD

1. Prepare a preparative low-melting-point agarose PFGE that will provide optimum resolution of the DNA fragment or size fraction of interest.
2. Load the DNA size standards and single plugs of the digested genomic DNA in lanes located on both sides of the preparative slot. Load the preparative sample into the preparative slot. Carry out electrophoresis as described in Chapter 5, Protocol 17 (for TAFE gel) or Chapter 5, Protocol 18 (for CHEF gel).
3. After electrophoresis, cut the lanes containing the size standards and single genomic DNA plugs from the gel and stain them with ethidium bromide or SYBR Gold for 30 minutes at room temperature. If necessary, destain the gel slices in H_2O for 30 minutes. Do not stain the preparative lane.
4. Examine the stained slices by UV illumination and make notches on the slices to mark the locations of markers flanking the position of DNA of interest.
5. Reassemble the gel with the lanes containing the stained size standards and single plugs and locate the approximate region of the unstained preparative lane containing the DNA of interest. Carefully excise this region with a clean razor blade, and transfer the gel slice to a snap-cap polypropylene tube.
6. Cover the gel fragment with agarase buffer, and incubate for 1 hour at room temperature with occasional agitation. Discard the buffer, and repeat the procedure twice more.
7. After the buffer exchange is complete, melt the agarose slice containing the fractionated DNA at 65-68°C. During the melting step, swirl the tube to ensure complete melting.
8. Add an appropriate amount of agarase to the melted gel and digest the gel at the temperature recommended by the manufacturer.
   Digestions are usually carried out at temperatures between 37°C and 45°C and for times ranging from 1 hour to overnight.
9. After digestion, inactivate the agarase by heating (according to the manufacturer's instructions) or remove by phenol:chloroform extraction.
   Heat inactivation is preferred to avoid possible shearing of the DNA during extraction with organic solvents. Ligation or restriction enzyme digestion of the released DNA can be carried out in the presence of the agarose monomers produced by the agarase. Alternatively, the DNA can be precipitated in the presence of 0.3 M sodium acetate (pH 5.2) and 2 volumes of isopropanol.

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Protocol 20

Retrieval of DNA Fragments from Pulsed-field Gels following DNA Concentration

DNA contained in a slice of low-melting-temperature agarose is first concentrated by electrophoresis into a high-percentage agarose gel, and then isolated by treatment with agarase. The resulting DNA preparation is purified by microdialysis.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- DNA staining solution
  For a discussion of staining agarose, please see Chapter 5, Protocol 2.
- Injection/transfection buffer
- PFGE equilibration buffer

Enzymes and Buffers

Agarase

- Agarase digestion buffer
  Use the buffer supplied with the enzyme. It is critical that all buffers be supplemented with 100 mM NaCl, 30 µM spermine, and 70 µM spermidine to enhance recovery of the DNA.

Nucleic Acids and Oligonucleotides

DNA size standards
Please see Chapter 5, Protocol 14 and Chapter 5, Protocol 16 for the preparation of two different ranges of sizing standards.

Genomic DNA embedded in low-melting-temperature agarose plugs
Please see Chapter 5, Protocol 13 for preparation of mammalian DNA or Chapter 5, Protocol 14 for preparation of yeast DNA embedded in plugs. Carry out digestion with restriction enzymes as described in Chapter 5, Protocol 15 of this chapter.

Additional Reagents

Steps 1 and 2 of this protocol require the reagents listed in Chapter 5, Protocol 17 or Chapter 5, Protocol 18 .

METHOD

1. Prepare a preparative low-melting-point agarose PFGE that will provide optimum resolution of the DNA fragment or size fraction of interest.
2. Load the DNA size standards and single plugs of the digested genomic DNA in lanes located on both sides of the preparative slot. Load the preparative sample into the preparative slot. Carry out electrophoresis as described in Chapter 5, Protocol 17 (TAFE gel) or Chapter 5, Protocol 18 (for CHEF gel).
3. After electrophoresis, cut the lanes containing the size standards and single genomic DNA plugs from the gel and stain them with ethidium bromide or SYBR Gold for 30 minutes at room temperature. If necessary, destain the gel slices in H_2O for 30 minutes. Do not stain the preparative lane.
4. Examine the stained slices by UV illumination and make notches on the slices to mark the locations of markers flanking the position of DNA of interest.
5. Under normal illumination, reassemble the gel with the lanes containing the stained size standards and single plugs and locate the approximate region of the unstained preparative lane containing the DNA of interest. Carefully excise this region with a clean razor blade, and transfer the gel slice to a snap-cap polypropylene tube.
6. Equilibrate the gel slice containing the size-fractionated DNA in 40 ml of PFGE equilibration buffer for 20-30 minutes at room temperature. Agitate the mixture gently throughout this period.
7. Pour off the buffer and melt the gel slice at 65-68°C, gently swirling the tube periodically to ensure complete melting. Record the volume of the melted gel slice.
8. While the 5% NuSieve GTG minigel is still in the taped casting tray, slice off enough of the top of the gel to accommodate the volume of the melted gel slice that contains DNA.
9. Pour the melted gel slice into the casting tray and allow it to harden. Concentrate the size-fractionated DNA in the 5% gel by applying 60 V for 12 minutes per millimeter of low-melting-temperature gel.
10. When electrophoresis is complete, slice a very thin section from the *center* of the gel and stain it with ethidium bromide. Determine how far into the gel the DNA has migrated (usually approx. 2 mm).
    It is important to stain a sliver from the center of the gel as some smiling occurs during electrophoresis.
11. Remove the low-melting portion of the gel and trim as small a portion as possible of the 5% gel containing the concentrated DNA.
12. Equilibrate the gel slice containing the DNA in 12 ml of 1x agarase digestion buffer containing 100 mM NaCl, 30 µM spermine, and 70 µM spermidine. Incubate the gel slice in this buffer for 20 minutes at room temperature with gentle agitation.
13. Drain off the buffer, transfer the gel slice to a microfuge tube, and melt the DNA slice at 65-68°C. Transfer the melted gel to a water bath set at a temperature optimal for the agarase preparation (recommended by the manufacturer).
14. Incubate the melted gel slice for 15 minutes, and then add an appropriate amount of agarase to digest the starting volume of 5% gel.
15. After digestion, centrifuge the tube at maximum speed for 5 minutes in a microfuge to pellet debris, and transfer the supernatant to a fresh microfuge tube.
16. Set up a drop dialysis of the supernatant using membranes with a 0.05-µm pore size:
    a. Spot the supernatant onto the center of the membrane, floating shiny side up on 100 ml of injection/transfection buffer.
    b. Dialyze for 1 hour at room temperature. Replace the original buffer with 100 ml of fresh injection/transfection buffer and dialyze for an additional hour.
    c. Transfer the DNA to a clean microfuge tube.
       Drop dialysis removes the agarase enzyme and the digested carbohydrates released from the agarose.
       After drop dialysis, the concentration of the DNA can be estimated by gel electrophoresis.
       The DNA can be injected directly or combined with a lipofection reagent for transfection into cultured cells.

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Chapter 6 Preparation and Analysis of Eukaryotic Genomic DNA

Protocol 1: Isolation of High-molecular-weight DNA from Mammalian Cells Using Proteinase K and Phenol

This is the method of choice when large amounts of mammalian DNA are required, for example, for Southern blotting (Chapter 6, Protocol 6 , Chapter 6, Protocol 7 , Chapter 6, Protocol 8) or for construction of genomic libraries in bacteriophage λ vectors. Approximately 200 µg of mammalian DNA, 100-150 kb in length, is obtained from 5 x 10^7 cultured aneuploid mammalian cells (e.g., HeLa cells). The usual yield of DNA from 20 ml of normal blood is approx. 250 µg.
The initial stages of the procedure vary, depending on the type of samples used (cells growing in monolayer, cells growing in suspension, freshly drawn blood, or frozen blood). The protocol therefore contains four alternative versions of Step 1.

Protocol 2: Isolation of High-molecular-weight DNA from Mammalian Cells Using Formamide

This procedure, although lengthy, generates preparations of DNA that are large enough (200 kb) to be used for the construction of genomic libraries in high-capacity vectors and for analysis by pulsed-field electrophoresis. However, the concentration of DNA in the final preparation is low (approx. 10 µg/ml), as is the yield (approx. 1 mg of DNA/10^8 cultured aneuploid mammalian cells, such as HeLa cells).

Protocol 3: Isolation of DNA from Mammalian Cells by Spooling

This procedure is used to prepare DNA simultaneously from many different types of samples or tissues. Although the DNA is generally too small (approx. 80 kb) for efficient construction of genomic DNA libraries, it gives excellent results in Southern hybridizations and PCRs. Cultured aneuploid mammalian cells (2 x 10^7, e.g., HeLa cells) yield 100 µg of DNA in a volume of 1 ml.

Protocol 4: Isolation of DNA from Mammalian Cells Grown in 96-well Microtiter Plates

Each well yields sufficient genomic DNA for several standard PCRs or for analysis in a single lane of a Southern hybridization.

Protocol 5: Preparation of Genomic DNA from Mouse Tails and Other Small Samples

This simple protocol is used to extract DNA from small numbers of cultured cells and from fragments of soft or bony tissues. The method is used chiefly to genotype transgenic and knockout mice. Each 6-10-mm snippet of mouse tail yields 50-100 µg of DNA that can be used in dot or slot blotting to detect a transgene of interest, in Southern hybridization to detect DNA fragments that are <20 kb in size, and as a template in PCRs.

Protocol 6: Rapid Isolation of Mammalian DNA

Mammalian DNA prepared from blood or tissues as described in this protocol is 20-50 kb in size and suitable for use as a template in PCRs. The yields of DNA vary between 0.5 and 3.0 µg/mg tissue or 5 and 15 µg per 300 µl of whole blood.

Protocol 7: Rapid Isolation of Yeast DNA

This method is used to isolate genomic yeast DNA or shuttle plasmids that replicate in both *S. cerevisiae* and *E. coli.* The DNA can be used as a template for PCR and for transformation.

Protocol 8: Southern Blotting: Capillary Transfer of DNA to Membranes

This protocol describes the first stages of Southern blotting: digestion of genomic DNA with one or more restriction enzymes, separation of the resulting fragments by electrophoresis through an agarose gel, and transfer of the denatured fragments to a membrane by downward capillary transfer.

Protocol 9: Southern Blotting: Simultaneous Transfer of DNA from a Single Agarose Gel to Two Membranes

DNA can be simultaneously transferred from opposite sides of a single agarose gel to two membranes. Bidirectional transfer occurs rapidly at first, but soon slows down as the gel becomes dehydrated. Because the efficiency of transfer is low, the method works best when the target sequences are present in high concentration, for example, when analyzing restriction digests of cloned DNAs or less complex genomes.

Protocol 10: Southern Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Membranes

This protocol describes how to carry out Southern hybridizations at high stringency in phosphate-SDS buffers. Although a wide variety of formats are available, most Southern hybridizations are carried out in heat-sealable bags, roller bottles, or plastic boxes, as described here.

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Protocol 1

Isolation of High-molecular-weight DNA from Mammalian Cells Using Proteinase K and Phenol

This is the method of choice when large amounts of mammalian DNA are required, for example, for Southern blotting (Chapter 6, Protocol 6 , Chapter 6, Protocol 7 , Chapter 6, Protocol 8) or for construction of genomic libraries in bacteriophage λ vectors. Approximately 200 µg of mammalian DNA, 100-150 kb in length, is obtained from 5 x 10^7 cultured aneuploid mammalian cells (e.g., HeLa cells). The usual yield of DNA from 20 ml of normal blood is approx. 250 µg.
The initial stages of the procedure vary, depending on the type of samples used (cells growing in monolayer, cells growing in suspension, freshly drawn blood, or frozen blood). The protocol therefore contains four alternative versions of Step 1.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- ACD (Step 1D)
- ⚠ Ammonium acetate (10 M) (used as an alternative to dialysis, Step 9)
- Dialysis buffer 6-1 (used as an alternative to ethanol precipitation, Step 9)
- EDTA (Step 1D)
  EDTA-may be used as an alternative to ACD when preparing DNA from whole blood; note, however, that ACD is superior for preserving high-molecular-weight DNA.
  Ethanol (used as an alternative to dialysis, Step 9)
- Mammalian cell lysis buffer
- ⚠ Phenol, equilibrated with 0.5 M Tris-Cl (pH 8.0)
  IMPORTANT *The pH of the phenol must be approx. 8.0 to prevent DNA from becoming trapped at the interface between the organic and aqueous phases.*
- PBS
- TE (pH 8.0)
- TBS (Tris-buffered saline)

Enzymes and Buffers

- Proteinase K (20 mg/ml)
  Use of a genomic grade proteinase K that has been shown to be free of DNase and RNase activity.

Nucleic Acids and Oligonucleotides

Bacteriophage λ DNA, intact
Purify λ DNA as described in Chapter 2, Protocol 11 or Chapter 2, Protocol 12 . The DNA is used as a size standard during gel electrophoresis (please see Step 11).

Cells and Tissues

Monolayers or suspensions of mammalian cells, or fresh tissue, or blood samples

METHOD

1. 1A. Lyse cells growing in monolayer cultures.
   Work with batches of 10-12 culture dishes at a time. Store the remaining culture dishes in the incubator until they are required.

a. Take one batch of culture dishes, containing cells grown to confluency, from the incubator and immediately remove the medium by aspiration. Working quickly, wash the monolayers of cells twice with ice-cold TBS. This is most easily accomplished by gently pipetting approx. 10 ml of TBS onto the first monolayer. Swirl the dish gently for a few seconds and then tip the fluid into a 2-liter beaker. Add another 10 ml of ice-cold TBS and store the dish on a bed of ice. Repeat the procedure until the entire batch of monolayers has been processed.

b. Tip the fluid from the first monolayer into the 2-liter beaker. Remove the last traces of TBS from the culture dish by aspiration. Add 1 ml of fresh ice-cold TBS and store the dish on a bed of ice. Repeat the procedure until the entire batch of monolayers has been processed.

c. Use a rubber policeman to scrape the cells from the first culture dish into the 1 ml of TBS. Use a Pasteur pipette to transfer the cell suspension to a centrifuge tube on ice. Immediately wash the culture dish with 0.5 ml of ice-cold TBS, and combine the washings with the cell suspension in the centrifuge tube. Process the remaining monolayers in the same way.

d. Recover the cells by centrifugation at 1500*g* (2700 rpm in a Sorvall H1000B rotor and swinging buckets) for 10 minutes at 4°C.

e. Resuspend the cells in 5-10 volumes of ice-cold TBS and repeat the centrifugation.

f. Resuspend the cells in TE (pH 8.0) at a concentration of 5 x 10^7 cells/ml. Transfer the solution to an Erlenmeyer flask.
For 1 ml of cell suspension, use a 50-ml flask; for 2 ml, use a 100-ml flask, and so on.

g. Add 10 ml of lysis buffer for each milliliter of cell suspension. Incubate the suspension for 1 hour at 37°C, and then proceed immediately to Step 2.
Make sure that the cells are well dispersed over the inner surface of the Erlenmeyer flask when the lysis buffer is added.

1B. Lyse cells growing in suspension cultures.

a. Transfer the cells to a centifuge tube or bottle and recover them by centrifugation at 1500*g* (2700 rpm in a Sorvall H100B rotor and swinging buckets) for 10 minutes at 4°C. Remove the supernatant medium by aspiration.

b. Wash the cells by resuspending them in a volume of ice-cold TBS equal to the volume of the original culture. Repeat the centrifugation. Remove the supernatant by aspiration and then gently resuspend the cells once more in ice-cold TBS. Recover the cells by centrifugation.

c. Remove the supernatant by aspiration and gently suspend the cells in TE (pH 8.0) at a concentration of 5 x 10^7 cells/ml. Transfer the suspension to an Erlenmeyer flask.
For 1 ml of cell suspension, use a 50-ml flask; for 2 ml, use a 100-ml flask, and so on.

d. Add 10 ml of lysis buffer for each milliliter of cell suspension. Incubate the solution for 1 hour at 37°C and then proceed immediately to Step 2.
Make sure that the cells are well dispersed over the inner surface of the Erlenmeyer flask when the lysis buffer is added.

1C. Pulverize and lyse tissue samples.

a. Drop approx. 1 g of freshly excised tissue into liquid nitrogen in the stainless-steel container of a Waring blender. Blend at top speed until the tissue is ground to a powder.
Alternatively, smaller quantities of tissue can be snap-frozen in liquid nitrogen and then pulverized to a powder using a mortar and pestle precooled with liquid nitrogen.

b. Allow the liquid nitrogen to evaporate, and add the powdered tissue little by little to approx. 10 volumes (w/v) of lysis buffer in a beaker. Allow the powder to spread over the surface of the lysis buffer, and then shake the beaker to submerge the material.

c. When all of the material is in solution, transfer the suspension to a 50-ml centrifuge tube. Incubate the tube for 1 hour at 37°C, and then proceed to Step 2 (p. 6.9).

1D. Collect blood cells from freshly drawn or frozen samples. Human blood must be collected by a trained phlebotomist under sterile conditions.

To collect cells from freshly drawn blood

a. Collect approx. 20 ml of fresh blood in tubes containing 3.5 ml of either ACD (acid citrate dextrose solution B) or EDTA.
The blood may be stored for several days at 0°C or indefinitely at -70°C before the DNA is prepared. Blood should not be collected into heparin, which is an inhibitor of the polymerase chain reaction.

b. Transfer the blood to a centrifuge tube and centrifuge at 1300*g* (2500 rpm in a Sorvall H1000B rotor and 50-ml swinging buckets) for 15 minutes at 4°C.

c. Remove the supernatant fluid by aspiration. Use a Pasteur pipette to transfer the buffy coat carefully to a fresh tube and repeat the centrifugation. Discard the pellet of red cells.
The buffy coat is a broad band of white blood cells of heterogeneous density.

d. Remove residual supernatant from the buffy coat by aspiration. Resuspend the buffy coat in 15 ml of lysis buffer. Incubate the solution for 1 hour at 37°C, and proceed to Step 2.

To collect cells from frozen blood samples

a. Collect approx. 20 ml of fresh blood in tubes containing 3.5 ml of either ACD (acid citrate dextrose solution B) or EDTA.
The blood may be stored for several days at 0°C or indefinitely at -70°C before the DNA is prepared.

b. Thaw the blood in a water bath at room temperature and then transfer it to a centrifuge tube. Add an equal volume of phosphate-buffered saline at room temperature.

c. Centrifuge the blood at 3500*g* (5400 rpm in a Sorvall SS-34 rotor) for 15 minutes at room temperature.

d. Remove the supernatant, which contains lysed red cells, by aspiration. Resuspend the pellet in 15 ml of lysis buffer. Incubate the solution for 1 hour at 37°C, and then proceed to Step 2.

2. Transfer the lysate to one or more centrifuge tubes that fit into a Sorvall SS-34 rotor, or equivalent. The tubes should not be more than one-third full.
3. Add proteinase K (20 mg/ml) to a final concentration of 100 µg/ml. Use a glass rod to mix the enzyme solution gently into the viscous lysate of cells.
4. Incubate the lysate in a water bath for 3 hours at 50°C. Swirl the viscous solution from time to time.
5. Cool the solution to room temperature and add an equal volume of phenol equilibrated with 0.1 M Tris-Cl (pH 8.0). Gently mix the two phases by slowly turning the tube end-over-end for 10 minutes on a tube mixer or roller apparatus. If the two phases have not formed an emulsion at this stage, place the tube on a roller apparatus for 1 hour.
6. Separate the two phases by centrifugation at 5000*g* (6500 rpm in a Sorvall SS-34 rotor) for 15 minutes at room temperature.
7. Use a wide-bore pipette (0.3-cm diameter orifice) to transfer the viscous aqueous phase to a fresh centrifuge tube.
When transferring the aqueous (upper) phase, it is essential to draw the DNA into the pipette very slowly to avoid disturbing the material at the interface and to minimize hydrodynamic shearing forces. If the DNA solution is so viscous that it cannot easily be drawn into a wide-bore pipette, use a long pipette attached to an aspirator to remove the organic (lower) phase.
8. Repeat the extraction with phenol twice more and pool the aqueous phases.
9. Isolate DNA by one of the following two methods.

To isolate DNA in the size range of 150-200 kb

a. Transfer the pooled aqueous phases to a dialysis bag. Close the top of the bag with a dialysis tubing clip, allowing room in the bag for the sample volume to increase 1.5-2-fold during dialysis.

b. Dialyze the solution at 4°C against 4 liters of dialysis buffer 6-1. Change the buffer three times at intervals of ≥6 hours.
Because of the high viscosity of the DNA solution, dialysis generally takes ≥24 hours to complete.

To isolate DNA that has an average size of 100-150 kb

a. After the third extraction with phenol, transfer the pooled aqueous phases to a fresh centrifuge tube and add 0.2 volume of 10 M ammonium acetate. Add 2 volumes of ethanol at room temperature and swirl the tube until the solution is thoroughly mixed.

b. The DNA immediately forms a precipitate. Remove the precipitate in one piece from the ethanolic solution with a Shepherd's crook (a Pasteur pipette whose end has been sealed and shaped into a U; please see Steps 5-7 of Chapter 3, Protocol 6). Contaminating oligonucleotides remain in the ethanolic phase.

c. If the DNA precipitate becomes fragmented, abandon the Shepherd's crook and collect the precipitate by centrifugation at 5000*g* (6500 rpm in a Sorvall SS-34 rotor) for 5 minutes at room temperature.

d. Wash the DNA precipitate twice with 70% ethanol, and collect the DNA by centrifugation as described in Step c.

e. Remove as much of the 70% ethanol as possible, using an aspirator. Store the pellet of DNA in an open tube at room temperature until the last visible traces of ethanol have evaporated.
Do not allow the pellet of DNA to dry completely; desiccated DNA is very difficult to dissolve.

f. Add 1 ml of TE (pH 8.0) for each 0.1 ml of cells (Step 1). Place the tube on a rocking platform and gently rock the solution for 12-24 hours at 4°C until the DNA has completely dissolved. Store the DNA solution at 4°C.

10. Measure the concentration of the DNA by absorbance at 260 nm or by fluorometry.
11. Analyze the quality of the preparation of high-molecular-weight DNA by pulsed-field gel electrophoresis (Chapter 5, Protocol 17 or Chapter 5, Protocol 18) or by electrophoresis through a conventional 0.6% agarose gel (Chapter 5, Protocol 1). Use unit-length and/or linear concatemers of λ DNA as markers. A method to generate linear concatemers of λ DNA is described in Chapter 5, Protocol 16 .
Do not be concerned if some of the DNA remains in the well, since DNA molecules >250 kb have difficulty entering the gel. This problem can usually be solved by embedding the DNA in a small amount of melted agarose (at 55°C) and transferring the molten solution to the well of a preformed agarose gel.

REFERENCES

1. Blin N. and Stafford D.W. 1976. A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res.* 3:2303-2308.

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Protocol 2

Isolation of High-molecular-weight DNA from Mammalian Cells Using Formamide

This procedure, although lengthy, generates preparations of DNA that are large enough (200 kb) to be used for the construction of genomic libraries in high-capacity vectors and for analysis by pulsed-field electrophoresis. However, the concentration of DNA in the final preparation is low (approx. 10 µg/ml), as is the yield (approx. 1 mg of DNA/10^8 cultured aneuploid mammalian cells, such as HeLa cells).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Dialysis buffer 6-2-1
- Dialysis buffer 6-2-2
- Formamide denaturation buffer
- TE (pH 8.0)

Nucleic Acids and Oligonucleotides

Linear monomers and concatemers of bacteriophage λ DNA (please see Chapter 5, Protocol 16)

Cells and Tissues

Monolayers or suspensions of mammalian cells, or fresh tissue, or blood samples

METHOD

1. Prepare lysates of cell suspensions (or frozen cell powders) as described in Steps 1-4 of Chapter 6, Protocol 1 .
2. Cool the solution containing lysed cells and lysis buffer to 15°C. For every 1 ml of cell lysate, add 1.25 ml of formamide denaturation buffer, and mix the solution gently using a glass rod. Store the solution for 12 hours at 15°C.
3. Pour the viscous solution into one or more collodion bags. Secure the open end of the bag with a dialysis clip and dialyze the solution for 45 minutes at 4°C in 2 liters of Dialysis buffer 6-2-1. Replace the buffer with fresh Dialysis buffer 6-2-1 and continue the dialysis for at least 4 hours, followed by a further 4 hours in a third 2-liter aliquot of Dialysis buffer 6-2-1. Then dialyze the DNA against 2 liters of fresh Dialysis buffer 6-2-2, three times, for at least 4 hours each.
   Dialysis intervals should be 45 minutes for the first buffer change and 4 hours for all subsequent changes.
   A total dialysis time of 24 hours is required to remove proteins from the DNA effectively.
   For a description of a convenient and inexpensive device for holding the collodion bags, please see Kupiec et al. (1987).
4. Measure the concentration of the DNA by absorbance at 260 nm or by fluorometry.
5. Analyze the quality of the preparation of high-molecular-weight DNA by pulsed-field gel electrophoresis (Chapter 5, Protocol 17 or Chapter 5, Protocol 18) or by electrophoresis through a conventional 0.6% agarose gel (Chapter 5, Protocol 1). Use unit-length and linear concatemers of λ DNA as markers (please see Chapter 5, Protocol 16). The genomic DNA should be more than 200 kb in size.
   Do not be concerned if some of the DNA remains in the well, since DNA molecules >250 kb have difficulty in entering the gel. This problem can usually be solved by embedding the DNA in a small amount of melted agarose (at 55°C) and transferring the molten solution to the well of a preformed agarose gel.

REFERENCES

1. Kupiec J.J., Giron M.L., Vilette D., Jeltsch J.M., and Emanoil-Ravier R. 1987. Isolation of high-molecular-weight DNA from eukaryotic cells by formamide treatment and dialysis. *Anal. Biochem.* 164:53-59.

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Protocol 3

Isolation of DNA from Mammalian Cells by Spooling

This procedure is used to prepare DNA simultaneously from many different types of samples or tissues. Although the DNA is generally too small (approx. 80 kb) for efficient construction of genomic DNA libraries, it gives excellent results in Southern hybridizations and PCRs. Cultured aneuploid mammalian cells (2×10^7, e.g., HeLa cells) yield 100 µg of DNA in a volume of 1 ml.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol (room temperature)

- Mammalian cell lysis solution
- TE (pH 8.0)

Nucleic Acids and Oligonucleotides

Linear monomers and concatemers of bacteriophage λ DNA (please see Chapter 5, Protocol 16)

Prepare λ DNA as described in Chapter 2, Protocol 11 or Chapter 2, Protocol 12 . The DNA is used as a size standard during gel electrophoresis.

Cells and Tissues

Monolayers or suspensions of mammalian cells, fresh tissue, or blood samples

METHOD

1. Prepare cell suspensions (or frozen cell powders) as described in Step 1 of Chapter 6, Protocol 1 .
2. Lyse the cells by one of the following two methods.

   For lysis of cells from suspensions

   a. Transfer the cell suspensions to disposable 50-ml polypropylene centrifuge tubes.

   b. Add 7.5 volumes of cell lysis solution.

   For lysis of cells from tissues

   a. Add the frozen cell powders little by little to approx. 7.5 volumes of cell lysis solution in beakers. Allow the powders to spread over the surface of the lysis solution, and then shake the beakers to submerge the material.

   b. When all of the material is in solution, transfer the solutions to centrifuge tubes.
3. Close the tubes and incubate them for 1 hour at room temperature on a rocking platform.
4. Dispense 18 ml of ethanol at room temperature into each of a series of disposable 50-ml polypropylene centrifuge tubes. Use wide-bore pipettes to layer the cell suspensions carefully *under* the ethanol.
5. Recover the DNA from each tube by slowly stirring the interface between the cell lysate and the ethanol with a Shepherd's crook. The DNA will adhere to the crook, forming a gelatinous mass. Continue stirring until the ethanol and the aqueous phase are thoroughly mixed.
6. Transfer each Shepherd's crook, with its attached DNA, to a separate polypropylene tube containing 5 ml of ethanol at room temperature. Leave the DNA submerged in the ethanol until all of the samples have been processed.
7. Remove each crook, with its attached DNA, and allow as much ethanol as possible to drain away. By this stage, the DNA should have shrunk into a tightly packed, dehydrated mass; it is then possible to remove most of the free ethanol by capillary action by touching the U-shaped end of the crook to a stack of Kimwipes. Before all of the ethanol has evaporated from the DNA, transfer the crook into a fresh polypropylene tube containing 5 ml of ethanol at room temperature.
8. When all of the samples have been processed, again remove as much ethanol as possible (see Step 7).Do not allow the DNA pellets to dry completely or they will be very difficult to dissolve.
9. Transfer each pipette to a fresh polypropylene tube containing 1 ml of TE (pH 8.0). Allow the DNAs to rehydrate by storing the tubes overnight at 4°C.
10. During rehydration, the DNAs become highly gelatinous but remain attached to their pipettes. Use fresh Shepherd's crooks as scrapers to free the pellets of DNA gently from their pipettes. Discard the pipettes, leaving the DNA pellets floating in the TE. Close the tubes and incubate them at 4°C on a rocking platform until the pellets are completely dissolved (approx. 24-48 hours).

    The level of contamination by RNA is kept within acceptable limits if 1.5 ml or more of lysis solution is used per 10^7 cells. However, the amount of RNA contaminating the DNA sample can be further reduced by adding RNase (final concentration of 1 µg/ml) to the solution DNA.
11. Analyze an aliquot by pulsed-field gel electrophoresis (Chapter 5, Protocol 17 or Chapter 5, Protocol 18) or by electrophoresis through a 0.6% agarose gel (Chapter 5, Protocol 1). Store the DNA at 4°C.

    The DNA should be approx. 80 kb in size and should migrate more slowly than monomers of bacteriophage λ DNA. Because DNA made by this procedure is always contaminated with a small amount of RNA, it is necessary to estimate the concentration of DNA in the final preparation either by fluorometry or by gel electrophoresis and staining with ethidium bromide (Chapter 5, Protocol 2).

REFERENCES

1. Bowtell D.D. 1987. Rapid isolation of eukaryotic DNA. *Anal. Biochem.* 162:463-465.

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Protocol 4

Isolation of DNA from Mammalian Cells Grown in 96-well Microtiter Plates

Each well yields sufficient genomic DNA for several standard PCRs or for analysis in a single lane of a Southern hybridization.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol

- Gel-loading buffer IV
- Microtiter cell lysis buffer
- NaCl/ethanol solution
- PBS
- TE (pH 8.0)

Enzymes and Buffers

DNase-free RNase

Restriction enzymes

Cells and Tissues

Mammalian cells growing in 96-cell plates

Cell cultures in individual flat-bottomed wells of 96-well tissue culture plates should be grown to confluence, or close to it. These plates are available from most suppliers of materials used for tissue culture. Label both the tops and the bottoms of the 96-well plates when working with more than one plate.

METHOD

1. Remove the medium from confluent cultures of cells growing in individual wells of 96-well plates by aspiration through a blue pipette tip or a Pasteur pipette.
2. Rinse the monolayers of cells in the individual wells twice with 100 µl of phosphate-buffered saline.
3. Use a multichannel pipettor to add 50 µl of cell lysis buffer to each well of the microtiter plate. Place several wet paper towels in a polypropylene box (e.g., a Tupperware box) and then stack the microtiter plates containing the lysis buffer and cells on top of the towels. Seal the box tightly with the lid.
4. Incubate the sealed box for 12-16 hours in a 60°C oven.
5. Remove the box from the oven, place the plates on a flat bench top, and allow them to cool for a few minutes before adding 100 µl of NaCl/ethanol solution per well. Store the plates for 30 minutes at room temperature without mixing. A stringy precipitate of nucleic acid should be visible at the end of the incubation.
6. Slowly invert each plate over a sink to decant the ethanolic solution. The precipitated nucleic acid should remain attached to the base of the wells. Place each plate in an upside down position on a bed of dry paper towels and allow the remaining ethanol to drain from the plate.
7. Add 150 µl of 70% ethanol to each well, being careful not to dislodge the precipitate of nucleic acid. Discard the 70% ethanol by inverting the plate as in Step 6. Blot the excess liquid on a bed of paper towels. Rinse the precipitates of DNA twice more with 70% ethanol.
8. Allow the plates to dry at room temperature until the last traces of ethanol have evaporated. If the genomic DNA is to be analyzed by PCR, then proceed to Step 9. If the DNA is to be analyzed by Southern hybridization, proceed to Steps 10, 11, and 12.
9. Add 30-50 µl of TE (pH 8.0) to each well and allow the DNA to dissolve during gentle rocking for 12-16 hours at room temperature.
   Dissolution of the DNA can be accelerated by placing the microtiter dishes on the heating block of a thermal cycler that is programmed to cycle 10 times between 80°C and 50°C (1 minute at each temperature).
   The DNA may now be used as template for standard PCR (please see Chapter 8, Protocol 1).
10. If the DNA is to be analyzed by Southern blotting, make up the following restriction enzyme mixture; 40 µl of the mixture will be required for each well.

H_2O	0.8 volume
10x restriction enzyme buffer	0.1 volume
DNase-free RNase	10 µg/ml

    Just before use, add 10 units of restriction enzyme for each 40 µl of mixture.
11. Use a multichannel pipettor to add 40 µl of the restriction enzyme mixture to each well. Mix the contents of the wells by pipetting up and down several times, taking care to avoid air bubbles. Incubate the reactions at the appropriate digestion temperature for 12-16 hours in a humidified sealed Tupperware box as described in Step 3.
12. Stop the reactions by adding 5-10 µl of sucrose gel-loading buffer and analyze the digested DNA by Southern blotting and hybridization as described in Chapter 6, Protocol 8 , Chapter 6, Protocol 9 , and Chapter 6, Protocol 10 .

REFERENCES

1. Ramirez-Solis R., Rivera-Perez J., Wallace J.D., Wiss M., Zheng H., and Bradley A. 1992. Genomic DNA microextraction: A method to screen numerous samples. *Anal. Biochem.* 201:331-335.

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Protocol 5

Preparation of Genomic DNA from Mouse Tails and Other Small Samples

This simple protocol is used to extract DNA from small numbers of cultured cells and from fragments of soft or bony tissues. The method is used chiefly to genotype transgenic and knockout mice. Each 6-10-mm snippet of mouse tail yields 50-100 µg of DNA that can be used in dot or slot blotting to detect a transgene of interest, in Southern hybridization to detect DNA fragments that are <20 kb in size, and as a template in PCRs.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol

Isopropanol

- PBS
- ⚠ Phenol:chloroform:isoamyl alcohol (25:24:1 v/v)
- SNET
- TE (pH 8.0)

Enzymes and Buffers

- Proteinase K (20 mg/ml)

Cells and Tissues

Cultured mammalian cells

Monolayer cultures, grown to confluence or semiconfluence in 100-mm dishes, should be washed twice with ice-cold phosphate-buffered saline and then immediately lysed by addition of 1 ml of SNET containing 400 µg/ml proteinase K, as described in Step 1. Cells growing in suspension should be recovered by centrifugation, washed twice in ice-cold phosphate-buffered saline, and then resuspended in TE (pH 8.0) at a concentration of 5 x 10^7/ml. Aliquots of the suspension (0.2 ml) are then transferred to a series of 17 x 100-mm Falcon polypropylene tubes and the cells are immediately lysed with SNET containing 400 µg/ml proteinase K, as described in Step 1.

Mouse tails

Samples of mouse tails are generally cut from 10-day-old suckling animals or at the time of weaning (approx. 3 weeks of age). In the former case, the distal one third of the tail is removed and transferred into a microfuge. In the latter case, 6-10 mm of the tail is removed under anesthesia and transferred to a 17 x 100-mm Falcon polypropylene tube. Under rare circumstances, where obtaining a result rapidly is of paramount importance, the entire tail can be removed from newborn animals and transferred to a microfuge tube.

Mouse tissue

To isolate DNA from mouse tissue (other than tail snippets), transfer approx. 100 mg of the freshly dissected tissue to a 17 x 100-mm Falcon polypropylene tube.

All experiments carried out on laboratory mice, including removing sections of tail, require prior authorization from the appropriate institutional ethics committee.

METHOD

1. Prepare the appropriate amount of lysis buffer (see table below) by adding proteinase K to a final concentration of 400 µg/ml in SNET. Add lysis buffer to the mouse tails or other tissues.
   This procedure also can be used to isolate DNA from monolayers of cultured mammalian cells. In this case, 1 ml of SNET containing 400 µg/ml proteinase K is added directly to 100-mm monolayers that have been rinsed twice in phosphate-buffered saline. The viscous cell slurry is scraped from the dish with a rubber policeman, and transferred to a 17 x 100-mm polypropylene Falcon tube.
   Cells growing in suspension that have been washed twice in phosphate-buffered saline are resuspended in TE and lysed with SNET containing 400 µg of proteinase K (1 ml per 10^9 cells).

SNET Lysis Buffer Volumes

Age of Mouse	Amount of Tissue	Type of Tube	Volume of SNET Lysis Buffer (ml)
Newborn	entire tail (1 cm)	microfuge	0.5
10 days old	distal one third	microfuge	0.5
Weanling (3-4 weeks)	6-10 mm	17 x 100-mm polypropylene	4.0
Any age	100 mg fresh tissue	17 x 100-mm polypropylene	4.0

2. Incubate the tube overnight at 55°C in a horizontal position on a rocking platform or with agitation in a shaking incubator.
   It is important that the sample be mixed adequately during digestion. After overnight incubation, the tissue/tails should no longer be visible and the buffer should be a milky-gray.
3. Add an equal volume of phenol:chloroform:isoamyl alcohol, seal the top of the tube, and place it on a rocking platform for 30 minutes at room temperature.
4. Separate the organic and aqueous phases by centrifugation. Centrifuge the samples in 17 x 100-mm polypropylene tubes at 666*g* (1800 rpm in a Sorvall H1000B rotor with swinging buckets or 1600 rpm in a Sorvall SH-3000 swinging bucket rotor) for 5 minutes at room temperature. Alternatively, for smaller sample volumes, centrifuge the samples in microfuge tubes at maximum speed for 5 minutes at room temperature in a microfuge. Transfer the upper aqueous phase to a fresh Falcon or microfuge tube.
5. Precipitate the DNA by adding an equal volume of isopropanol. Collect the precipitated DNA by centrifugation at 13,250*g* (8000 rpm in a Sorvall SH-3000 swinging bucket rotor or maximum speed in a microfuge) for 15 minutes at 4°C.
6. Carefully remove the isopropanol. Rinse the pellet of DNA with 1 ml of 70% ethanol. If the pellets are loose, centrifuge the samples again for 5 minutes. Remove the 70% ethanol, and allow the pellets to dry in air for 15-20 minutes at room temperature.
   Do not allow the DNA pellets to dry completely or they will be very difficult to dissolve.
7. Dissolve the nucleic acid pellet by rocking it gently overnight in 0.5 ml of TE (pH 8.0) at 4°C.
8. Transfer the solution to a microfuge tube and store it at room temperature.
   Between 100 µg and 250 µg of genomic DNA is typically isolated from 1 cm (approx. 100 mg) of mouse tail.
   The addition of bovine serum albumin at a concentration of 100 µg/ml to restriction enzyme digests of genomic DNA prepared by this method will absorb residual SDS and reduce the possibility of incomplete digestions. If problems persist, re-extract the samples once more with phenol:chloroform and precipitate the DNA with 2 volumes of ethanol.

REFERENCES

1. Palmiter R.D., Chen H.Y., Messing A., and Brinster R.L. 1985. SV40 enhancer and large-T antigen are instrumental in development of choroid plexus tumours in transgenic mice. *Nature* 316:457-460.

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Protocol 6

Rapid Isolation of Mammalian DNA

Mammalian DNA prepared from blood or tissues as described in this protocol is 20-50 kb in size and suitable for use as a template in PCRs. The yields of DNA vary between 0.5 and 3.0 µg/mg tissue or 5 and 15 µg per 300 µl of whole blood.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol

Isopropanol

- Potassium acetate (5 M)
- Rapid mammalian cell lysis buffer
- TE (pH 7.6)
- Tris-Cl (20 mM, pH 7.6)

Enzymes and Buffers

DNase-free RNase (4 mg/ml)

- Proteinase K (20 mg/ml)

Cells and Tissues

Mammalian tissue

Whole blood of interest

METHOD

1. Prepare tissue or whole blood for genomic DNA isolation.

   For tissue

   a. Dissect 10-20 mg of tissue.

   b. Either mince the tissue finely with a razor blade/scalpel or freeze the tissue in liquid nitrogen and then grind it to a powder in a mortar prechilled with liquid nitrogen, as described in Chapter 6, Protocol 1.

   For blood

   a. Transfer 300-µl aliquots of whole blood to each of two microfuge tubes. Add 900 µl of 20 mM Tris-Cl (pH 7.6) to each tube and invert the capped tubes to mix the contents. Incubate the solution at room temperature for 10 minutes, occasionally inverting the tubes.

   b. Centrifuge the tubes at maximum speed for 20 seconds at room temperature in a microfuge.

   c. Discard all but 20 µl of each supernatant.

   d. Resuspend the pellets of white cells in the small amount of supernatant left in each tube. Combine the resuspended cell pellets in a single tube.

2. Transfer the minced tissue or the resuspended white blood cell pellets to a microfuge tube containing 600 µl of ice-cold cell lysis buffer. Homogenize the suspension quickly with 30-50 strokes of a microfuge pestle.
   The SDS will precipitate from the ice-cold cell lysis buffer producing a cloudy solution. This precipitation will not affect isolation of DNA.
3. (*Optional*) Add 3 µl of proteinase K solution to the lysate to increase the yield of genomic DNA. Incubate the digest for at least 3 hours but no more than 16 hours at 55°C.
4. Allow the digest to cool to room temperature and then add 3 µl of 4 mg/ml DNase-free RNase. Incubate the digest for 15-60 minutes at 37°C.
5. Allow the sample to cool to room temperature. Add 200 µl of potassium acetate solution and mix the contents of the tube by vortexing vigorously for 20 seconds.
6. Pellet the precipitated protein/SDS complex by centrifugation at maximum speed for 3 minutes at 4°C in a microfuge.
   A pellet of protein should be visible at the bottom of the microfuge tube after centrifugation. If not, incubate the lysate for 5 minutes on ice and repeat the centrifugation step.
7. Transfer the supernatant to a fresh microfuge tube containing 600 µl of isopropanol. Mix the solution well and then recover the precipitate of DNA by centrifuging the tube at maximum speed for 1 minute at room temperature in a microfuge.
8. Remove the supernatant by aspiration and add 600 µl of 70% ethanol to the DNA pellet. Invert the tube several times and centrifuge the tube at maximum speed for 1 minute at room temperature in a microfuge.
9. Carefully remove the supernatant by aspiration and allow the DNA pellet to dry in air for 15 minutes.
10. Redissolve the pellet of DNA in 100 µl of TE (pH 7.6).
    The solubilization of the genomic DNA pellet can be faciltated by incubation for 16 hours at room temperature or for 1 hour at 65°C.

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Protocol 7

Rapid Isolation of Yeast DNA

This method is used to isolate genomic yeast DNA or shuttle plasmids that replicate in both *S. cerevisiae* and *E. coli.* The DNA can be used as a template for PCR and for transformation.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol

⚠ Phenol:chloroform (1:1, v/v)

Sodium acetate (3 M, pH 5.2)

STES buffer

TE (pH 7.6)

Cells and Tissues

Yeast cells, freshly grown either as colonies on an agar plate or as an overnight culture

METHOD

1. Prepare the yeast cells for lysis.

   For yeast colonies on plates
   Use a sterile inoculating loop to transfer one or more large, freshly grown colonies to a microfuge tube containing 50 µl of STES buffer.

   For yeast grown in liquid culture
   a. Transfer 1.5 ml from an overnight culture of yeast cells to a microfuge tube.
   b. Pellet the cells by centrifuging at maximum speed for 1 minute at room temperature in a microfuge.
   c. Remove the culture medium by aspiration and resuspend the pellet in 50 µl of STES buffer.
2. Add approx. 50 µl of acid-washed glass beads to each tube containing the resuspended yeast. Add 20 µl of TE (pH 7.6) to each tube.
3. Add 60 µl of phenol:chloroform, cap the tubes, and mix the organic and aqueous phases by vortexing for 1 minute.
4. Centrifuge the tubes at maximum speed for 5 minutes at room temperature in a microfuge.
5. Transfer the upper aqueous phase to a fresh microfuge tube. Collect the DNA by standard precipitation with ethanol for 15 minutes at 0°C.
6. Recover the precipitate of nucleic acids by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge.
7. Remove the supernatant by aspiration and rinse the pellet with 100 µl of 70% ethanol in H_2O. Centrifuge the tubes at maximum speed for 1 minute at room temperature in a microfuge.
8. Remove the supernatant by aspiration and allow the pellet to dry in the air for 15 minutes. Redissolve the pellet in 40 µl of TE (pH 7.6).
   Use 1-10 µl of the solution of DNA as template in PCRs. Shuttle plasmids can be recovered by transforming preparations of competent E. coli *with 1 µl of the DNA.*

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Protocol 8

Southern Blotting: Capillary Transfer of DNA to Membranes

This protocol describes the first stages of Southern blotting: digestion of genomic DNA with one or more restriction enzymes, separation of the resulting fragments by electrophoresis through an agarose gel, and transfer of the denatured fragments to a membrane by downward capillary transfer.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Alkaline transfer buffer
- Denaturation solution
- DNA-staining solution
- 6x Gel-loading buffer IV
- ⚠ HCl (0.2 N), for depurination of DNA
  Optional, please see note to Step 6.
- Neutral transfer buffer
- Neutralization buffer I
- Neutralization buffer II
- 6x SSC
- TE (pH 8.0)

Enzymes and Buffers

Restriction enzymes

Nucleic Acids and Oligonucleotides

Marker DNA

Genomic DNA

METHOD

1. Digest an appropriate amount of genomic DNA with one or more restriction enzymes.
2. If necessary, concentrate the DNA fragments at the end of the digestion by ethanol precipitation. Dissolve the DNAs in approx. 25 µl of TE (pH 8.0).
   Make sure that the ethanol is removed from the DNA solution before it is loaded on the gel. If significant quantities of ethanol remain, the DNA "crawls" out of the slot of the gel. Heating the solution of dissolved DNA to 70°C in an open tube for 10 minutes is usually sufficient to drive off most of the ethanol. This treatment also disrupts base pairing between cohesive termini of restriction fragments.
3. Measure the concentrations of the digested DNAs by fluorometry or by the ethidium bromide or SYBR Gold spot test. Transfer the appropriate amount of each digest to a fresh microfuge tube. Add 0.15 volume of 6x sucrose gel-loading buffer and separate the fragments of DNA by electrophoresis through an agarose gel (for most genomic DNAs, a 0.7% gel cast in 0.5x TBE or 1x TAE may be used; please see Chapter 5, Protocol 1). Maintain a low voltage through the gel (about <1 V/cm) to allow the DNA to migrate slowly.
   If the digested DNAs have been stored at 4°C, they should be heated to 56°C for 2-3 minutes before they are applied to the gel. This heating disrupts any base pairing that may have occurred between protruding cohesive termini.
4. After electrophoresis is complete, stain the gel with ethidium bromide or SYBR Gold and photograph the gel as described in Chapter 5, Protocol 2 . Place a transparent ruler alongside the gel so that the distance that any band of DNA has migrated can be read directly from the photographic image.
5. Denature the DNA and transfer it from the agarose gel to a nitrocellulose or a neutral or charged-nylon membrane using one of the methods described below.
6. After fractionating the DNA by gel electrophoresis, transfer the gel to a glass baking dish. Use a razor blade to trim away unused areas of the gel, including the section of gel above the wells. Be sure to leave enough of the wells attached to the gel so that the positions of the lanes can be marked on the membrane after transfer of DNA. Cut off a small triangular piece from the bottom left-hand corner of the gel to simplify orientation during the succeeding operations.
   Cut off the lanes containing the molecular-weight markers.
   If the fragments of interest are larger than approx. 15 kb, then transfer may be improved by nicking the DNA by brief depurination before denaturation. After Step 6, soak the gel in several volumes of 0.2 N HCl until the bromophenol blue turns yellow and the xylene cyanol turns yellow/green. Immediately place the 0.2 N HCl in a hazardous-waste container and then rinse the gel several times with deionized H_2O.
   Depurination is best avoided when the size of the target fragments is <20 kb. However, for Southern analysis of higher-molecular-weight DNA separated by conventional or pulsed-field electrophoresis, depurination/nicking is advisable, if not essential.
7. Denature the DNA by soaking in a denaturing (alkaline) solution as follows:

   For transfer to uncharged membranes

   a. Soak the gel for 45 minutes at room temperature in 10 gel volumes of denaturation solution with constant *gentle* agitation (e.g., on a rotary platform).
   b. Rinse the gel briefly in deionized H_2O, and then neutralize it by soaking for 30 minutes at room temperature in 10 gel volumes of Neutralization buffer I with constant gentle agitation. Change the neutralization buffer and continue soaking the gel for a further 15 minutes.

   For transfer to charged nylon membranes

   a. Soak the gel for 15 minutes at room temperature in several volumes of alkaline transfer buffer with constant *gentle* agitation (e.g., on a rotary platform).
   b. Change the solution and continue to soak the gel for a further 20 minutes with gentle agitation.
      If the gel floats to the surface of the liquid, weigh it down with several Pasteur pipettes.
8. Use a fresh scalpel or a paper cutter to cut a piece of nylon or nitrocellulose membrane approx. 1 mm larger than the gel in each dimension. Also cut two sheets of thick blotting paper to the same size as the membrane.
   IMPORTANT *Use appropriate gloves and blunt-ended forceps (e.g., Millipore forceps) to handle the membrane. A membrane that has been touched by oily hands will not wet!*
9. Float the membrane on the surface of a dish of deionized H_2O until it wets completely from beneath, and then immerse the membrane in the appropriate transfer buffer for at least 5 minutes. Use a clean scalpel blade to cut a corner from the membrane to match the corner cut from the gel.
10. While the DNA is denaturing, place a piece of thick blotting paper on a sheet of Plexiglas or a glass plate to form a support that is longer and wider than the gel. The ends of the blotting paper should drape over the edges of the plate.

Place the support inside a large baking dish. The support can be placed on top of four neoprene stoppers to elevate it from the bottom of the dish.

11. Fill the dish with the appropriate transfer buffer until the level of the liquid reaches almost to the top of the support. When the blotting paper on the top of the support is thoroughly wet, smooth out all air bubbles with a glass rod or pipette.
    Neutral transfer buffer (10x SSC or 10x SSPE) is used to transfer DNA to uncharged membranes. Alkaline transfer buffer (0.4 N NaOH with 1 M NaCl) is used to transfer DNA to charged nylon membranes.
12. Remove the gel from the solution in Step 7 and invert it so that its underside is now uppermost. Place the inverted gel on the support so that it is centered on the wet blotting paper.
    Make sure that there are no air bubbles between the blotting paper and the gel.
13. Surround, but do not cover, the gel with Saran Wrap or Parafilm.
14. Wet the top of the gel with the appropriate transfer buffer. Place the wet membrane on top of the gel so that the cut corners are aligned. To avoid bubbles, touch one corner of the membrane to the gel and gently lower the membrane onto the gel. One edge of the membrane should extend just over the edge of the line of slots at the top of the gel.
    IMPORTANT *Do not move the membrane once it has been applied to the surface of the gel. Make sure that there are no air bubbles between the membrane and the gel.*
15. Wet the two pieces of thick blotting paper in the appropriate transfer buffer and place them on top of the wet membrane. Roll a pipette across the surface of the membrane to smooth away any air bubbles.
16. Cut or fold a stack of paper towels (5-8 cm high) just smaller than the blotting papers. Place the towels on the blotting papers. Put a glass plate on top of the stack and weigh it down with a 400-g weight.
17. Allow the transfer of DNA to proceed for 8-24 hours. Replace the paper towels as they become wet. Try to prevent the entire stack of towels from becoming wet with buffer.
18. Remove the paper towels and the blotting papers above the gel. Turn the gel and the attached membrane over and lay them, gel side up, on a dry sheet of blotting paper. Mark the positions of the gel slots on the membrane with a very soft lead pencil or a ballpoint pen.
19. Peel the gel from the membrane and discard the gel.
    Instead of discarding the gel, it can be stained (45 minutes) in a 0.5 µg/ml solution of ethidium bromide in H_2O and visualized on a UV transilluminator to gauge the success of the DNA transfer.
20. Soak the membrane in *one* of the following solutions as appropriate:
    The sequence of steps from immobilization of DNA to the membrane to subsequent hybridization depends on the type of membrane, the method of transfer, and the method of fixation. Because alkaline transfer results in covalent attachment of DNA to positively charged nylon membranes, there is no need to fix the DNA to the membrane before hybridization. DNA transferred to uncharged nylon membranes in neutral transfer buffer should be fixed to the membrane by baking under vacuum or heating in a microwave oven, or cross-linked to the membrane by UV irradiation.

Fixing DNA to the Membrane for Hybridization

Type of Membrane	Type of Transfer	Method of Fixation	Sequence of Steps
Positively charged nylon	alkaline transfer	alkaline transfer	1. Soak membrane in Neutralization buffer II. 2. Proceed to prehybridization.
Uncharged nylon or positively charged nylon	neutral transfer	UV irradiation (please see Step 21 for details)	1. Soak membrane in 6x SSC. 2. Fix the DNA by UV irradiation. 3. Proceed to prehybridization.
Uncharged nylon or positively charged nylon	neutral transfer	baking in vacuum oven or microwave oven (please see Step 21 for details)	1. Soak membrane in 6x SSC. 2. Bake the membrane. 3. Proceed to prehybridization.

For neutral transfer: 6x SSC for 5 minutes at room temperature.
For alkaline transfer: Neutralization buffer II (0.5 M Tris-Cl [pH 7.2] with 1 M NaCl) for 15 minutes at room temperature.
This rinse removes any pieces of agarose sticking to the membrane and, in the latter case, also neutralizes the membrane.

21. Immobilize the DNA that has been transferred to uncharged membranes. Because alkaline transfer results in covalent attachment of DNA to positively charged nylon membranes, there is no need for additional steps to fix the DNA to the membrane.

To fix by baking in a vacuum oven

a. Remove the membrane from the 6x SSC and allow excess fluid to drain away. Place the membrane flat on a paper towel to dry for at least 30 minutes at room temperature.
b. Sandwich the membrane between two sheets of dry blotting paper. Bake for 30 minutes to 2 hours at 80°C in a vacuum oven.
   Overbaking can cause nitrocellulose membranes to become brittle. If the gel was not completely neutralized before the DNA was transferred, nitrocellulose membranes will turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.

To fix by baking in a microwave oven

a. Place the damp membrane on a dry piece of blotting paper.
b. Heat the membrane for 2-3 minutes at full power in a microwave oven (750-900 W).
   Proceed directly to hybridization (Chapter 6, Protocol 10) or dry the membrane and store it between sheets of blotting paper until it is needed.

To cross-link by UV irradiation

a. Place the damp membrane on a dry piece of blotting paper.
b. Irradiate at 254 nm to cross-link the DNA to the membrane.
   Make sure that the side of the membrane carrying the DNA faces the UV light source. Most manufacturers advise that damp membranes be exposed to a total of 1.5 J/cm^2 and that dry membranes be exposed to 0.15 J/cm^2. However, we recommend carrying out a series of preliminary experiments to determine empirically the amount of irradiation required to produce the maximum hybridization signal.

22. Proceed directly to hybridization of immobilized DNA to a probe (Chapter 6, Protocol 10).
    Any membranes not used immediately in hybridization reactions should be thoroughly dried, wrapped loosely in aluminum foil or blotting paper, and stored at room temperature, preferably under vacuum.

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1. Reed K.C. and Mann D.A. 1985. Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res.* 13:7207-7221.
2. Southern E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.

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Protocol 9

Southern Blotting: Simultaneous Transfer of DNA from a Single Agarose Gel to Two Membranes

DNA can be simultaneously transferred from opposite sides of a single agarose gel to two membranes. Bidirectional transfer occurs rapidly at first, but soon slows down as the gel becomes dehydrated. Because the efficiency of transfer is low, the method works best when the target sequences are present in high concentration, for example, when analyzing restriction digests of cloned DNAs or less complex genomes.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Denaturation solution
- DNA-staining solution
- 6x Gel-loading buffer IV
- ⚠ HCl (0.2 N), for depurination of DNA
  Optional, please see note to Step 6, Chapter 6, Protocol 8 .
- Neutral transfer buffer
- Neutralization buffer
- 6x SSC

Enzymes and Buffers

Restriction enzymes

Nucleic Acids and Oligonucleotides

Marker DNA

Target DNA

METHOD

1. Digest the DNA and fractionate it by gel electrophoresis according to Steps 1-3 of Chapter 6, Protocol 8 .
2. After fractionating the DNA by gel electrophoresis, stain the gel with ethidium bromide or SYBR Gold and photograph as described in Chapter 5, Protocol 2 . Place a transparent ruler alongside the gel so that the distance that any band has migrated can be read directly from the photographic image. Prepare the gel for transfer under neutral conditions (Chapter 6, Protocol 8 , Steps 6-7).
3. Use a fresh scalpel or a paper cutter to cut two pieces of nylon or nitrocellulose membrane approx. 1-2 mm larger than the gel in each dimension. Cut a corner from the membranes to match the corner cut from the gel. Also cut four sheets of thick blotting paper to the same size as the membranes.
   IMPORTANT *Use appropriate gloves and blunt-ended forceps (e.g., Millipore forceps) to handle the membranes. A membrane that has been touched by oily hands will not wet!*
   To retain small fragments of DNA (<300 nucleotides), use nitrocellulose membranes with a small pore size (0.2 µm) or nylon membranes.
4. Float the membranes on the surface of a dish of deionized H_2O until they wet completely from beneath, and then immerse the membranes in 10x SSC for at least 5 minutes.
5. Roll a moistened pipette over each layer as it is assembled to ensure that no air bubbles are trapped, especially between the membranes and the gel sides. Place one of the membranes on two pieces of dampened blotting paper. Lay the gel on top of the membrane, aligning the cut corner of the gel with the cut corner of the membrane. Without delay, place the second membrane on the other side of the gel, followed by two sheets of dampened blotting paper.
6. Transfer the entire sandwich of blotting papers, membranes, and gel onto a 2-4-inch stack of paper towels. Cover the sandwich with a second stack of paper towels. Put a glass plate on top of the entire stack and weigh it down with a 400-g weight.
7. After 2-4 hours, remove the paper towels and blotting papers. Transfer the gel and membrane sandwich to a dry sheet of blotting paper, and mark the approximate positions of the gel slots with a very soft lead pencil or a ballpoint pen.
8. Immobilize the DNA onto the membranes by completing Steps 19-21 of Chapter 6, Protocol 8.
9. Proceed directly to hybridization of immobilized DNA to a probe (Chapter 6, Protocol 10).

REFERENCES

1. Reed K.C. and Mann D.A. 1985. Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res.* 13:7207-7221.
2. Southern E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.

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Protocol 10

Southern Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Membranes

This protocol describes how to carry out Southern hybridizations at high stringency in phosphate-SDS buffers. Although a wide variety of formats are available, most Southern hybridizations are carried out in heat-sealable bags, roller bottles, or plastic boxes, as described here.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Phosphate-SDS washing solution 1
- Phosphate-SDS washing solution 2
- ⚠ Prehybridization/hybridization solutions
- Sodium phosphate (1 M, pH 7.2)
- 0.1x SSC
- 0.1x SSC with 0.1% (w/v) SDS
- 2x SSC with 0.1% (w/v) SDS
- 2x SSC with 0.5% (w/v) SDS
- 6x SSC or
- 6x SSPE

Nucleic Acids and Oligonucleotides

DNA immobilized on membrane

Poly(A) RNA (10 mg/ml) in sterile H_2O

Optional, for hybridization buffers. Prepare solution by dissolving poly(A) RNA in sterile H_2O and store in 100-µl aliquots.

⚠ Probe

For Southern analysis of mammalian genomic DNA, where each lane of the gel contains 10 µg of DNA, use 10-20 ng/ml radiolabeled probe DNA or RNA (sp. act. $^3 10^9$ cpm/µg). For Southern analysis of cloned DNA fragments, where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. When analyzing cloned DNA, hybridization is carried out for 6-8 hours using 1-2 ng/ml radiolabeled probe (sp. act. 10^6 to 10^9 cpm/µg). Labeling should be carried out according to the methods described in Chapter 9 or 10.

- Salmon sperm DNA (approx. 10 mg/ml)

METHOD

1. Float the membrane containing the target DNA on the surface of a tray of 6x SSC (or 6x SSPE) until the membrane becomes thoroughly wetted from beneath. Submerge the membrane for 2 minutes.
2. Prehybridize the membrane by one of the following methods.

For hybridization in a heat-sealable bag

a. Slip the wet membrane into a heat-sealable bag (e.g., Sears Seal-A-Meal or equivalent), and add 0.2 ml of prehybridization solution for each square centimeter of membrane. Squeeze as much air as possible from the bag.

b. Seal the open end of the bag with a heat sealer and then make a second seal. Test the strength and integrity of the seal by gently squeezing the bag. Incubate the bag for 1-2 hours submerged in a water bath set to the appropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide; 65°C for phosphate-SDS solvents).

For hybridization in a roller bottle

a. Gently roll the wetted membrane into the shape of a cylinder and place it inside a hybridization roller bottle together with the plastic mesh provided by the manufacturer. Add 0.1 ml of prehybridization solution for each square centimeter of membrane. Close the bottle tightly.

b. Place the hybridization tube inside a prewarmed hybridization oven at the appropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide; 65°C for phosphate-SDS solvents).

For hybridization in a plastic container

a. Place the wetted membrane in a plastic (e.g., Tupperware) container, and add 0.2 ml of prehybridization solution for each square centimeter of membrane.

b. Seal the box with the lid and place the box on a rocking platform in an air incubator set at the appropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide; 65°C for phosphate-SDS solvents).

3. If the radiolabeled probe is double-stranded DNA, denature it by heating for 5 minutes at 100°C. Chill the probe rapidly in ice water.
   Alternatively, denature DNA probes by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, chill the probe to 0°C in an ice-water bath, and add 0.05 volume of 1 M Tris-Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.
   Single-stranded DNA and RNA probes need not be denatured.
4. To hybridize the probe to a blot containing genomic DNA, carry out one of the following methods.

For hybridization in a heat-sealable bag

a. Working quickly, remove the bag containing the membrane from the water bath. Open the bag by cutting off one corner with scissors and pour off the prehybridization solution.

b. Add the denatured probe to an appropriate amount of fresh prehybridization solution and deliver the solution into the bag. Squeeze as much air as possible from the bag.

c. Reseal the bag with the heat sealer; make sure that as few bubbles as possible are trapped in the bag. To avoid radioactive contamination of the water bath, seal the resealed bag inside a second, noncontaminated bag. Incubate the bag submerged in a water bath set at the appropriate temperature for the required period of hybridization.

For hybridization in a roller bottle

a. Pour off the prehybridization solution from the hybridization bottle and replace with fresh hybridization solution containing probe.

b. Seal bottle and replace in hybridization oven. Incubate for the required period of hybridization.

For hybridization in a plastic container

a. Transfer the membrane from the container to a sealable bag or a hybridization bottle.

b. Immediately treat as described above.

5. After hybridization, wash the membrane.

For hybridization in a heat-sealable bag

a. Wearing gloves, remove the bag from the water bath, remove the outer bag, and immediately cut off one corner of the inner bag. Pour out the hybridization solution into a container suitable for disposal of radioactivity, and then cut the bag along the length of three sides.

b. Remove the membrane and immediately submerge it in a tray containing several hundred milliliters of 2x SSC and 0.5% SDS (i.e., approx. 1 ml/cm^2 membrane) at room temperature. Agitate the tray gently on a slowly rotating platform.

For hybridization in a roller bottle

a. Remove the membrane from the hybridization bottle, and briefly drain excess hybridization solution from the membrane by holding the corner of the membrane to the lip of the bottle or container.

b. Place the membrane in a tray containing several hundred milliliters of 2x SSC and 0.5% SDS (i.e., approx. 1 ml/cm^2 membrane) at room temperature. Agitate the tray gently on a slowly rotating platform.
When hybridizing in phosphate-SDS solution, remove the membrane from the hybridization chamber as described in Step 5 and place it in several hundred milliliters (i.e., approx. 1 ml/cm^2 membrane) of Phosphate-SDS washing solution 1 at 65°C. Agitate the tray. Repeat this rinse once.
IMPORTANT *Do not allow the membrane to dry out at any stage during the washing procedure.*

6. After 5 minutes, pour off the first rinse solution into a radioactivity disposal container and add several hundred milliliters of 2x SSC and 0.1% SDS to the tray. Incubate for 15 minutes at room temperature with occasional gentle agitation.
If hybridization was carried out in a phosphate-SDS buffer, rinse the membrane a total of eight times for 5 minutes each in several hundred milliliters of Phosphate-SDS washing solution 2 at 65°C. Skip to Step 9 after the eighth rinse.
7. Replace the rinse solution with several hundred milliliters of fresh 0.1x SSC with 0.1% SDS. Incubate the membrane for 30 minutes to 4 hours at 65°C with gentle agitation.
During the washing step, periodically monitor the amount of radioactivity on the membrane using a hand-held minimonitor. The parts of the membrane that do not contain DNA should not emit a detectable signal. Do not expect to pick up a signal on the minimonitor from membranes containing mammalian DNA that has been hybridized to single-copy probes.
8. Briefly wash the membrane with 0.1x SSC at room temperature.
9. Remove most of the liquid from the membrane by placing it on a pad of paper towels. Place the damp membrane on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink (or phosphorescent dots) to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the membrane. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.
Alternatively, dry the membrane in the air and glue it to a piece of 3MM paper using a water-soluble glue.
10. Cover the membrane with a sheet of Saran Wrap, and expose the membrane to X-ray film for 16-24 hours at -70°C with an intensifying screen to obtain an autoradiographic image.
Alternatively, cover the hybridized and rinsed membrane with Sarap Wrap, and expose it to a phosphorimager plate. An exposure time of 1-4 hours is usually long enough to detect single-copy gene sequences in a Southern blot of mammalian genomic DNA.

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1. Meinkoth J. and Wahl G. 1984. Hybridization of nucleic acids immobilized on solid supports. *Anal. Biochem.* 138:267-284.
2. Southern E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
3. Wahl G.M., Stern M., and Stark G.R. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyl-oxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci.* 76:3683-3687.

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Chapter 7 Extraction, Purification, and Analysis of mRNA from Eukaryotic Cells

Protocol 1: Purification of RNA from Cells and Tissues by Acid Phenol-Guanidinium Thiocyanate-Chloroform Extraction

In this single-step technique, cells are homogenized in guanidnium thiocyanate and the RNA is purified from the lysate by extraction with phenol:chloroform at reduced pH. Many samples can be processed simultaneously and speedily. The yield of total RNA depends on the tissue or cell source and is generally in the range of 4-7 µg/ml starting tissue or 5-10 µg/10^6 cells. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 2: A Single-step Method for the Simultaneous Preparation of DNA, RNA, and Protein from Cells and Tissues

This protocol, a variation of the method described in Chapter 7, Protocol 1 , involves lysis of cells in a monophasic solution of guanidine isothiocyanate and phenol. Addition of chloroform generates a second (organic) phase into which DNA and proteins are extracted, leaving RNA in the aqueous supernatant. The yield of total RNA depends on the tissue or cell source, but it is generally in the range of 4-7 µg/mg starting tissue or 5-10 µg/10^6 cells. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 3: Selection of Poly(A)$^+$ RNA by Oligo(dT)-Cellulose Chromatography

Chromatography on oligo(dT) columns is the preferred method for large-scale purification (>25 µg) of poly(A)$^+$ RNA extracted from mammalian cells. Typically, between 1% and 10% of the RNA applied to the oligo(dT) column is recovered as poly(A)$^+$ RNA. Because the method can be frustratingly slow, it is not recommended for purification of poly(A)$^+$ RNA from multiple samples. For this purpose, batch elution (Chapter 7, Protocol 4) is the better choice. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 4: Selection of Poly(A)$^+$ RNA by Batch Chromatography

When many RNA samples are to be processed or when working with small amounts (<50 µg) of total mammalian RNA, the technique of choice is batch chromatography on oligo(dT)-cellulose. The method described in this protocol uses a combination of temperature and ionic strength to maximize binding and recovery of polyadenylated RNA. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 5: Separation of RNA According to Size: Electrophoresis of Glyoxylated RNA through Agarose Gels

Separation of RNAs according to size is the first stage in northern blotting and hybridization. The method described in this protocol uses glyoxal to denature the RNA, ethidium bromide to stain it, and agarose gel electrophoresis to separate the resulting glyoxal-RNA-ethidium adducts. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 6: Separation of RNA According to Size: Electrophoresis of RNA through Agarose Gels Containing Formaldehyde

Separation of RNAs according to size is the first stage in northern blotting and hybridization. The method described in this protocol uses formaldehyde to denature the RNA, ethidium bromide to stain it, and electrophoresis through agarose gels containing 2.2 M formamide to separate the resulting formaldehyde-RNA-ethidium adducts. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 7: Transfer and Fixation of Denatured RNA to Membranes

This protocol describes the transfer of RNA from agarose gels to neutral or positively charged nylon membranes, using upward capillary flow of neutral or alkaline buffers. RNA becomes covalently fixed to positively charged nylon membranes during transfer in alkaline buffers. However, treatment by UV irradiation or heating is required to fix RNA to neutral membranes. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 8: Northern Hybridization

This protocol describes how to carry out northern hybridization at high stringency in phosphate-SDS-buffers. Although a wide variety of formats are available, hybridization is usually performed in heat-sealable bags, roller bottles, or plastic boxes, as described here. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 9: Dot and Slot Hybridization of Purified RNA

Dot blotting of RNA is best carried out using purified preparations of RNA that are denatured with glyoxal or formaldehyde immediately before loading onto a nylon membrane through a vacuum manifold. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 10: Mapping RNA with Nuclease S1

Preparations of RNA containing an mRNA of interest are hybridized to a complementary single-stranded DNA probe. At the end of the reaction, nuclease S1 is used to degrade unhybridized regions of the probe, and the surviving DNA-RNA hybrids are then separated by gel electrophoresis and visualized by either autoradiography or Southern hybridization. The method can be used to quantitate RNAs, to map the positions of introns, and to identify the locations of 5´ and 3´ ends of mRNAs on cloned DNA templates. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 11: Ribonuclease Protection: Mapping RNA with Ribonuclease and Radiolabeled RNA Probes

Preparations of RNA containing an mRNA of interest are hybridized to a radiolabeled single-stranded RNA probe. At the end of the reaction, a mixture of RNase A and RNase T1 is used to degrade unhybridized regions of the probe, and the surviving molecules are then separated by denaturing gel electrophoresis and visualized by autoradiography. The method can be used to quantitate RNAs, to map the positions of introns, and to identify the locations of 5´ and 3´ ends of mRNAs on cloned DNA templates. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 12: Analysis of RNA by Primer Extension

Primer extension is used chiefly to map the 5´ termini of mRNAs. A preparation of polyadenylated mRNA is first hybridized with an excess of a single-stranded oligodeoxynucleotide primer, which is complementary to the target RNA and radiolabeled at its 5´ terminus. Reverse transcriptase is then used to extend the 3´ end of the primer. The size of the resulting cDNA, measured by denaturing polyacrylamide gel electrophoresis, is equal to the distance between the 5´ end of the priming oligonucleotide and the 5´ terminus of the target mRNA.

IMPORTANT: Prepare all reagents used in this protocol with DEPC-treated H_2O.

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Protocol 1

Purification of RNA from Cells and Tissues by Acid Phenol-Guanidinium Thiocyanate-Chloroform Extraction

In this single-step technique, cells are homogenized in guanidnium thiocyanate and the RNA is purified from the lysate by extraction with phenol:chloroform at reduced pH. Many samples can be processed simultaneously and speedily. The yield of total RNA depends on the tissue or cell source and is generally in the range of 4-7 µg/ml starting tissue or 5-10 µg/10^6 cells. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
○ **RECIPE:** Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform:isoamyl alcohol (49:1, v/v)
Ethanol
⚠ ○ Formamide (Optional)
Deionized formamide is used for the storage of RNA.
Isopropanol
⚠ Liquid nitrogen
⚠ ○ Phenol
○ PBS
Required for cells grown in suspension and monolayers only.
○ Sodium acetate (2 M, pH 4.0)
○ Solution D (denaturing solution)

Cells and Tissues

Mammalian cells
Mammalian tissue samples

METHOD

1. Prepare cells or tissue samples for isolation of RNA as appropriate for the material under study. The table below describes the amounts of Solution D required for each type of sample.

Amount of Solution D Required to Extract RNA from Cells and Tissues

Amount of Tissue or Cells	Amount of Solution D
100 mg of tissue	3 ml
T-75 flask of cells	3 ml
60-mm plate of cells	1 ml
90-mm plate of cells	2 ml

For tissues

a. Isolate the desired tissues by dissection and place them immediately in liquid nitrogen.
b. Transfer approx. 100 mg of the frozen tissue to a mortar containing liquid nitrogen and pulverize the tissue using a pestle. The tissue can be kept frozen during pulverization by the addition of liquid nitrogen.
c. Transfer the powdered tissue to a polypropylene snap-cap tube containing 3 ml of Solution D.
d. Homogenize the tissue for 15-30 seconds at room temperature with a polytron homogenizer.

For mammalian cells grown in suspension

a. Harvest the cells by centrifugation at 200-1900*g* (1000-3000 rpm in a Sorvall RT600 using the H1000 rotor) for 5-10 minutes at room temperature in a benchtop centrifuge.
b. Remove the medium by aspiration and resuspend the cell pellets in 1-2 ml of sterile ice-cold PBS.
c. Harvest the cells by centrifugation, remove the PBS completely by aspiration, and add 2 ml of Solution D per 10^6 cells.
d. Homogenize the cells with a polytron homogenizer for 15-30 seconds at room temperature.

For mammalian cells grown in monolayers

a. Remove the medium and rinse the cells once with 5-10 ml of sterile ice-cold PBS.
b. Remove PBS and lyse the cells in 2 ml of Solution D per 90-mm culture dish (1 ml per 60 mm dish).
c. Transfer the cell lysates to a polypropylene snap-cap tube.
d. Homogenize the lysates with a polytron homogenizer for 15-30 seconds at room temperature.

2. Transfer the homogenate to a fresh polypropylene tube and sequentially add 0.1 ml of 2 M sodium acetate (pH 4.0), 1 ml of phenol, and 0.2 ml of chloroform-isoamyl alcohol per milliliter of Solution D. After addition of each reagent, cap the tube and mix the contents thoroughly by inversion.
3. Vortex the homogenate vigorously for 10 seconds. Incubate the tube for 15 minutes on ice to permit complete dissociation of nucleoprotein complexes.
4. Centrifuge the tube at 10,000*g* (9000 rpm in a Sorvall SS-34 rotor) for 20 minutes at 4°C, and then transfer the upper aqueous phase containing the extracted RNA to a fresh tube.
5. Add an equal volume of isopropanol to the extracted RNA. Mix the solution well and allow the RNA to precipitate for 1 hour or more at -20°C.
6. Collect the precipitated RNA by centrifugation at 10,000*g* (9000 rpm in a Sorvall SS-34 rotor) for 30 minutes at 4°C.
7. Carefully decant the isopropanol and dissolve the RNA pellet in 0.3 ml of Solution D for every 1 ml of this solution used in Step 1.
IMPORTANT *Pellets are easily lost. Decant the supernatant into a fresh tube. Do not discard it until the pellet has been checked.*
8. Transfer the solution to a microfuge tube, vortex it well, and precipitate the RNA with 1 volume of isopropanol for 1 hour or more at -20°C.
9. Collect the precipitated RNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge. Wash the pellet twice with 75% ethanol, centrifuge again, and remove any remaining ethanol with a disposable pipette tip. Store the open tube on the bench for a few minutes to allow the ethanol to evaporate. Do not allow the pellet to dry completely.
10. Add 50-100 µl of DEPC-treated H_2O. Store the RNA solution at -70°C.
Addition of SDS to 0.5% followed by heating to 65°C may assist dissolution of the pellet.
11. Estimate the concentration of the RNA by measuring the absorbance at 260 nm of an aliquot of the final preparation.

REFERENCES

1. Chomczynski P. and Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.

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Protocol 2

A Single-step Method for the Simultaneous Preparation of DNA, RNA, and Protein from Cells and Tissues

This protocol, a variation of the method described in Chapter 7, Protocol 1 , involves lysis of cells in a monophasic solution of guanidine isothiocyanate and phenol. Addition of chloroform generates a second (organic) phase into which DNA and proteins are extracted, leaving RNA in the aqueous supernatant. The yield of total RNA depends on the tissue or cell source, but it is generally in the range of 4-7 µg/mg starting tissue or 5-10 µg/10^6 cells. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform
Ethanol
Isopropanol
⚠ Liquid nitrogen
Monophasic lysis reagent
PBS, ice-cold
Required for cells grown in suspension and monolayers only.
RNA precipitation solution
Sodium acetate (3 M, pH 5.2)

Cells and Tissues

Mammalian cells
Mammalian tissue samples

METHOD

1. Prepare cells or tissue samples for isolation of RNA.

   For tissues

   a. Isolate the desired tissues by dissection and place them immediately in liquid nitrogen.
   b. Transfer approx. 100 mg of the frozen tissue to a mortar containing liquid nitrogen and pulverize the tissue using a pestle. The tissue can be kept frozen during pulverization by the addition of liquid nitrogen.
   c. Transfer the powdered tissue to a polypropylene snap-cap tube containing 1 ml of ice-cold monophasic lysis reagent.
   d. Homogenize the tissue with a polytron homogenizer for 15-30 seconds at room temperature.

   For mammalian cells grown in suspension

   a. Harvest the cells by centrifugation at 200-1900*g* (1000-3000 rpm in a Sorvall H1000 rotor) for 5-10 minutes at room temperature in a benchtop centrifuge.
   b. Remove the medium by aspiration and resuspend the cell pellets in 1-2 ml of sterile ice-cold PBS.
   c. Harvest the cells by centrifugation, remove the PBS completely by aspiration, and add 1 ml of monophasic lysis reagent per 10^6 cells.
   d. Homogenize the cells with a polytron homogenizer for 15-30 seconds at room temperature.

   For mammalian cells grown in monolayers

   a. Remove the medium and rinse the cells once with 5-10 ml of sterile ice-cold PBS.
   b. Remove PBS and lyse the cells in 1 ml of monophasic lysis reagent per 90-mm culture dish (0.7 ml per 60-mm dish).
   c. Transfer the cell lysates to a polypropylene snap-cap tube.
   d. Homogenize the lysates with a polytron homogenizer for 15-30 seconds at room temperature.
2. Incubate the homogenates for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes.
3. Add 0.2 ml of chloroform per milliliter of monophasic lysis reagent. Mix the samples by vigorous shaking or vortexing. The composition of the monophasic lysis reagent used for the simultaneous isolation of RNA, DNA, and proteins has not been published. However, a large number of commercial reagents, with a variety of names, are available (please see table below). These reagents are all monophasic solutions containing phenol, guanidine, or ammonium thiocyanate and solubilizing agents.

   Monophasic Lysis Reagents

Reagent	Commercial Supplier
Trizol Reagent	Life Technologies www.lifetechnol.com
TRI Reagent	Molecular Research Center www.mrcgene.com
Isogen	Nippon Gene, Toyama, Japan www.Nippon-Gene-6Ltd.html
RNA-Stat-60	Tel-Tes www.isotex-daig.com

   When using commercial reagents for the simultaneous isolation of RNA, DNA, and protein, we recommend following the manufacturer's instructions. In most cases, these differ little from the generic instructions given below.
4. Separate the mixture into two phases by centrifuging at 12,000 rpm (10,000*g* in a Sorvall SS-34 rotor) for 15 minutes at 4°C. Transfer the upper aqueous phase to a fresh tube.
5. Precipitate the RNA from the aqueous phase: For each initial milliliter of monophasic lysis reagent, add 0.25 volume of isopropanol and 0.25 volume of RNA precipitation solution. After thorough mixing, store the final solution for 10 minutes at room temperature.
6. Collect the precipitated RNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge. Wash the pellet twice with 75% ethanol, and centrifuge again. Remove any remaining ethanol with a disposable pipette tip. Store the open tube on the bench for a few minutes to allow the ethanol to evaporate. Do not allow pellet to dry completely.
7. Add 50-100 µl of DEPC-treated H_2O. Store the RNA solution at -70°C.
   Addition of SDS to 0.5% followed by heating to 65°C may assist dissolution of the pellet.
8. Estimate the concentration of the RNA by measuring the absorbance at 260 nm of an aliquot of the final preparation.

REFERENCES

1. Chomczynski P. 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *BioTechniques* 15:532-534.

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Protocol 3

Selection of Poly(A)$^+$ RNA by Oligo(dT)-Cellulose Chromatography

Chromatography on oligo(dT) columns is the preferred method for large-scale purification (>25 µg) of poly(A)$^+$ RNA extracted from mammalian cells. Typically, between 1% and 10% of the RNA applied to the oligo(dT) column is recovered as poly(A)$^+$ RNA. Because the method can be frustratingly slow, it is not recommended for purification of poly(A)$^+$ RNA from multiple samples. For this purpose, batch elution (Chapter 7, Protocol 4) is the better choice.
IMPORTANT: Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol
NaCl (5 M), RNase-free
⚠ NaOH (10 N)
Dilute working solution from 10 N stock with sterile DEPC-treated H_2O.
2x Oligo(dT)-cellulose column-loading buffer
Oligo(dT)-cellulose elution buffer
IMPORTANT *Do not attempt to sterilize elution buffer by autoclaving as it froths excessively.*
Sodium acetate (3 M, pH 5.2)

Nucleic Acids and Oligonucleotides

RNA, total
Prepared as described in Chapter 7, Protocol 1 or Chapter 7, Protocol 2 .

METHOD

1. Suspend 0.5-1.0 g of oligo(dT)-cellulose in 0.1 N NaOH.
2. Pour a column of oligo(dT)-cellulose (0.5-1.0-ml packed volume) in a DEPC-treated Dispocolumn (or a Pasteur pipette, plugged with sterile glass wool and sterilized by baking for 4 hours at 300°C). Wash the column with 3 column volumes of sterile DEPC-treated H_2O.
3. Wash the column with sterile 1x column-loading buffer (dilute from 2x stock using sterile DEPC-treated H_2O) until the pH of the effluent is <8.0. Use pH paper for this measurement.
4. Dissolve the RNA in double-distilled, autoclaved H_2O, and heat the solution to 65°C for 5 minutes. Cool the solution to room temperature quickly, and add 1 volume of 2x column-loading buffer.
5. Apply the solution of RNA to the column, and immediately begin to collect in a sterile tube the material flowing through the column. When all of the RNA solution has entered the column, wash the column with 1 column volume of 1x column-loading buffer while continuing to collect the flow-through.
6. When all the liquid has emerged from the column, heat the collected flow-through to 65°C for 5 minutes and reapply it to the top of the column. Again collect the material flowing through the column.
7. Wash the column with 5-10 column volumes of 1x column-loading buffer, collecting 1-ml fractions into sterile plastic tubes (e.g., microfuge tubes).
8. Use quartz or disposable methacrylate cuvettes to measure the absorbance at 260 nm of a 1:20 dilution of each fraction collected from the column using 1x column-loading buffer as a blank.
9. Precipitate the fractions containing a majority of the OD_{260} material by the addition of 2.5 volumes of ethanol.
10. Elute the poly(A)$^+$ RNA from the oligo(dT)-cellulose with 2-3 column volumes of sterile, RNase-free elution buffer. Collect fractions equivalent in size to 1/3 to 1/2 of the column volume.
11. Use quartz or disposable methacrylate cuvettes to measure the absorbance at 260 nm of each fraction collected from the column. Pool the fractions containing the eluted RNA.
12. To purify poly(A)$^+$ RNA further, heat the preparation of RNA to 65°C for 3 minutes and then cool it quickly to room temperature. Adjust the concentration of NaCl in the eluted RNA to 0.5 M using 5 M NaCl and carry out a second round of chromatography on the same column of oligo(dT)-cellulose (i.e., repeat Steps 3 and 5-11).
The material obtained after a single round of chromatography on oligo(dT)-cellulose usually contains approximately equal amounts of polyadenylated and nonpolyadenylated species of RNA. Polyadenylated RNA may be further purified as described.
13. To the poly(A)$^+$ RNA eluted from the second round of oligo(dT)-cellulose chromatography, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M. Mix well. Add 2.5 volumes of ice-cold ethanol, mix, and store the solution for at least 30 minutes on ice.
14. Recover the poly(A)$^+$ RNA by centrifugation at 10,000*g* (9000 rpm in a Sorvall SS-34 rotor) for 15 minutes at 4°C. Carefully discard the supernatant, and wash the pellet (which is often invisible) with 70% ethanol. Recentrifuge briefly, remove the supernatant by aspiration, and store the open tube in an inverted position for a few minutes to allow most of the residual ethanol to evaporate. Do not allow the pellet to dry.
15. Redissolve the damp pellet of RNA in a small volume of sterile, DEPC-treated H_2O. Use quartz or disposable methacrylate cuvettes to measure the absorbance at 260 nm of each fraction collected from the column. Pool the fractions that contain RNA.
16. Store the preparation of poly(A)$^+$ RNA.

REFERENCES

1. Aviv H. and Leder P. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. *Proc. Natl. Acad. Sci.* 69:1408-1412.
2. Edmonds M., Vaughan Jr., M.H., and Nakazato H. 1971. Polyadenylic acid sequences in the heterogeneous nuclear RNA and rapidly-labeled polyribosomal RNA of HeLa cells: Possible evidence for a precursor relationship. *Proc. Natl. Acad. Sci.* 68:1336-1340.

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Protocol 4

Selection of Poly(A)$^+$ RNA by Batch Chromatography

When many RNA samples are to be processed or when working with small amounts (<50 µg) of total mammalian RNA, the technique of choice is batch chromatography on oligo(dT)-cellulose. The method described in this protocol uses a combination of temperature and ionic strength to maximize binding and recovery of polyadenylated RNA. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)

Ethanol

Ice-cold water

NaCl (5 M)

RNA absorption/washing buffer
This buffer is TES containing 0.5 M NaCl.

TES

Nucleic Acids and Oligonucleotides

RNA, total
Prepared as described in Chapter 7, Protocol 1 or Chapter 7, Protocol 2 .

METHOD

1. In a series of sterile microfuge tubes, adjust the volume of each sample of total RNA (up to 1 mg) to 600 µl with TES. Heat the sealed tubes to 65°C for 10 minutes and then cool them quickly in ice to 0°C. Add 75 µl (0.1 volume) of 5 M NaCl to each sample.
2. Add 50 mg (500 µl) of equilibrated oligo(dT)-cellulose to each tube and incubate the closed tubes on a rotating wheel for 15 minutes at room temperature.
3. Centrifuge the tubes at 600-800*g* (approx. 1500-2500 rpm) for 2 minutes at room temperature in a microfuge.
4. Transfer the supernatants to a series of fresh microfuge tubes. Store the tubes on ice.
5. To the pellets of oligo(dT) remaining in the first set of tubes, add 1 ml of ice-cold absorption/washing buffer. Disperse the pellets of oligo(dT) by gentle vortexing. Incubate the closed tubes on a rotating wheel for 2 minutes at room temperature.
6. Centrifuge the tubes at 600-800*g* (approx. 1500-2500 rpm) for 2 minutes at room temperature in a microfuge. Discard the supernatants and then repeat Steps 5 and 6 twice.
7. Resuspend the pellets of oligo(dT) in 0.4 ml of *ice-cold*, double-distilled, autoclaved H_2O by gentle vortexing. Immediately centrifuge the tubes for 2 minutes at 4°C in a microfuge.
8. Remove the supernatants by careful aspiration.
9. Recover the bound poly(A)$^+$ RNA by resuspending the pellets of oligo(dT)-cellulose in 400 µl of double-distilled, autoclaved H_2O. Incubate the suspensions for 5 minutes at 55°C and then centrifuge the tubes for 2 minutes at 4°C in a microfuge.
10. Transfer the supernatants to a series of fresh tubes and repeat Step 9 twice, pooling the recovered supernatants.
11. Add 0.2 volume of 10 M ammonium acetate and 2.5 volumes of ethanol to the supernatants. Store the tubes for 30 minutes at -20°C.
12. Recover the precipitated poly(A)$^+$ RNAs by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge. Carefully discard the supernatants, and wash the pellets (which are often invisible) with 70% ethanol. Centrifuge briefly, remove the supernatants by aspiration, and store the open tubes in an inverted position for a few minutes to allow most of the residual ethanol to evaporate.
13. Dissolve the RNA in a small volume of sterile DEPC-treated H_2O.
14. Estimate the concentration of the RNA by absorbance or by fluorometry.
15. Store the preparations.

REFERENCES

1. Celano P., Vertino P.M., and Casero Jr., R.A. 1993. Isolation of polyadenylated RNA from cultured cells and intact tissues. *BioTechniques* 15:26-28.

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Protocol 5

Separation of RNA According to Size: Electrophoresis of Glyoxylated RNA through Agarose Gels

Separation of RNAs according to size is the first stage in northern blotting and hybridization. The method described in this protocol uses glyoxal to denature the RNA, ethidium bromide to stain it, and agarose gel electrophoresis to separate the resulting glyoxal-RNA-ethidium adducts. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x BPTE electrophoresis buffer
- ⚠ DMSO
  Purchase a high grade of DMSO (HPLC grade or better).
- Glyoxal
  Commercial stock solutions of glyoxal (40% or 6 M) contain various hydrated forms of glyoxal, as well as oxidation products such as glyoxylic acid, formic acid, and other compounds that can degrade RNA, and therefore must be removed.
- Glyoxal reaction mixture
- RNA gel-loading buffer

Nucleic Acids and Oligonucleotides

RNA samples

Samples of total or poly(A)$^+$ RNA should consist of up to 10 µg of RNA in a volume of 1-2 µl. Equivalent amounts of the RNA samples to be analyzed are removed from storage. Precipitate the RNA with ethanol and dissolve the pellet in an appropriate volume of sterile, DEPC-treated H_2O.

The presence of salts or SDS in the samples, or loading of >10 µg of RNA per lane, can cause smearing of the RNA during electrophoresis.

RNA size markers

Glyoxylated RNAs and DNAs of the same size migrate through agarose gels at the same rate. However, we recommend using RNA ladders (e.g., from Life Technologies) that contain RNAs of 9.49, 7.46, 4.40, 2.37, 1.35, and 0.24 kb in length. This allows the markers to be used as sentinels to detect RNase contamination or other problems that may occur during glyoxylation or electrophoresis.

METHOD

1. Set up the glyoxal denaturation reaction. In sterile microfuge tubes mix:

RNA (up to 10 µg)	1-2 µl
glyoxal reaction mixture	10 µl

   Up to 10 µg of RNA may be analyzed in each lane of the gel. Abundant mRNAs (0.1% or more of the mRNA population) can usually be detected by northern analysis of 10 µg of total cellular RNA. Detection of rare RNAs requires at least 1.0 µg of poly(A)$^+$ RNA. Samples containing RNA size markers should be prepared in glyoxal reaction mixture in the same way as the RNA samples under test.
2. Close the tops of the microfuge tubes, and incubate the RNA solutions for 60 minutes at 55°C. Chill the samples for 10 minutes in ice water, and then centrifuge them for 5 seconds to deposit all of the fluid in the bottom of the microfuge tubes.
3. While the samples are incubating, install the agarose gel in a horizontal electrophoresis box. Add sufficient 1x BPTE electrophoresis buffer to cover the gel to a depth of approx. 1 mm.
4. Add 1-2 µl of RNA gel-loading buffer to the glyoxylated RNA samples, and without delay, load the glyoxylated RNA samples into the wells of the gel, leaving the two outermost lanes on each side of the gel empty. Load the RNA size markers in the outside lanes of the gel.
5. Carry out electrophoresis at 5 V/cm until the bromophenol blue has migrated approx. 8 cm.
6. Visualize the RNAs by placing the gel on a piece of Saran Wrap on a UV transilluminator. Align a transparent ruler with the stained gel and photograph the gel under UV illumination.
7. Use the photograph to measure the distance from the loading well to each of the bands of RNA. Plot the $\log_{10}$ of the size of the fragments of RNA against the distance migrated. Use the resulting curve to calculate the sizes of the RNA species detected by blot hybridization.
8. Proceed with immobilization of RNA onto a solid support by upward or downward capillary transfer (please see Chapter 7, Protocol 7) .

REFERENCES

1. Burnett W.V. 1997. Northern blotting of RNA denatured in glyoxal without buffer recirculation. *BioTechniques* 22:668-671.
2. McMaster G. and Carmichael G.G. 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci.* 74:4835-4838.
3. Thomas P.S. 1983. Hybridization of denatured RNA transferred or dotted nitrocellulose paper. *Methods Enzymol.* 100:255-266.

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Protocol 6

Separation of RNA According to Size: Electrophoresis of RNA through Agarose Gels Containing Formaldehyde

Separation of RNAs according to size is the first stage in northern blotting and hybridization. The method described in this protocol uses formaldehyde to denature the RNA, ethidium bromide to stain it, and electrophoresis through agarose gels containing 2.2 M formamide to separate the resulting formaldehyde-RNA-ethidium adducts. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ethidium bromide (200 µg/ml)

⚠ Formaldehyde

⚠ Formamide
Purchase or prepare a distilled-deionized preparation of this reagent and store in small aliquots under nitrogen at -20°C.

10x Formaldehyde gel-loading buffer

10x MOPS electrophoresis buffer

Nucleic Acids and Oligonucleotides

RNA samples
Samples of total or poly(A)$^+$ RNA should consist of up to 20 µg of RNA in a volume of 1-2 µl. Equivalent amounts of the RNA samples to be analyzed are removed from storage. Precipitate the RNA with ethanol and dissolve it in an appropriate volume of sterile, DEPC-treated H_2O.

The presence of salts or SDS in the samples or loading of >20 µg of RNA per lane can cause smearing of the RNA during electrophoresis.

RNA-size markers
DNA and RNA migrate at different rates through agarose gels containing formaldehyde, with RNA migrating faster than DNA of equivalent size. Although DNA markers are preferable because they run as sharp bands, they cannot readily be used to measure the absolute size of unknown RNAs. We therefore recommend using RNA ladders (e.g., from Life Technologies) that contain RNAs of 9.49, 7.46, 4.40, 2.37, 1.35, and 0.24 kb in length. This allows the markers to be used as sentinels to detect RNase contamination or other problems that may occur during electrophoresis.

METHOD

1. Set up the denaturation reaction. In sterile microfuge tubes mix:

RNA (up to 20 µg)	2.0 µl
10x MOPS electrophoresis buffer	2.0 µl
formaldehyde	4.0 µl
formamide	10.0 µl
ethidium bromide (200 µg/ml)	1.0 µl

As much as 20 µg of RNA may be analyzed in each lane of the gel. Abundant mRNAs (0.1% or more of the mRNA population) can usually be detected by northern analysis of 10 µg of total cellular RNA. For detection of rare RNAs, at least 1.0 µg of poly(A)$^+$ RNA should be applied to each lane of the gel. Samples containing RNA size markers should be prepared in the same way as the RNA samples under test.

2. Close the tops of the microfuge tubes, and incubate the RNA solutions for 60 minutes at 55°C. Chill the samples for 10 minutes in ice water, and then centrifuge them for 5 seconds to deposit all of the fluid in the bottom of the microfuge tubes.
   Many investigators prefer to incubate the RNA solutions for 10 minutes at 85°C.
3. Add 2 µl of 10x formaldehyde gel-loading buffer to each sample and return the tubes to an ice bucket.
4. Install the agarose/formaldehyde gel in a horizontal electrophoresis box. Add sufficient 1x MOPS electrophoresis buffer to cover the gel to a depth of approx. 1 mm. Run the gel for 5 minutes at 5 V/cm, and then load the RNA samples into the wells of the gel, leaving the two outermost lanes on each side of the gel empty. Load the RNA size standards in the outside lanes of the gel.
5. Run the gel submerged in 1x MOPS electrophoresis buffer at 4-5 V/cm until the bromophenol blue has migrated approx. 8 cm (4-5 hours).
6. Visualize the RNAs by placing the gel on a piece of Saran Wrap on a UV transilluminator. Align a transparent ruler with the stained gel and photograph under UV illumination.
7. Use the photograph to measure the distance from the loading well to each of the bands of RNA. Plot the $\log_{10}$ of the size of the fragments of RNA against the distance migrated. Use the resulting curve to calculate the sizes of the RNA species detected by blot hybridization.
8. Proceed with immobilization of RNA onto a solid support by upward or downward capillary transfer (please see Chapter 7, Protocol 7).

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1. Goldberg D.A. 1980. Isolation and partial characterization of the *Drosophila* alcohol dehydrogenase gene. *Proc. Natl. Acad. Sci.* 77:5794-5798.
2. Lehrach H., Diamond D., Wozney J.M., and Boedtker H. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions: A critical reexamination. *Biochemistry* 16:4743-4751.
3. Rosen K.M., Lamperti E.D., and Villa-Komaroff L. 1990. Optimizing the northern blot procedure. *BioTechniques* 8:398-403.
4. Seed B. 1982. Attachment of nucleic acids to nitrocellulose and diazonium-substituted supports. *Genet. Eng.* 4:91-102.

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Protocol 7

Transfer and Fixation of Denatured RNA to Membranes

This protocol describes the transfer of RNA from agarose gels to neutral or positively charged nylon membranes, using upward capillary flow of neutral or alkaline buffers. RNA becomes covalently fixed to positively charged nylon membranes during transfer in alkaline buffers. However, treatment by UV irradiation or heating is required to fix RNA to neutral membranes. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (0.1 M) with 0.5 µg/ml ethidium bromide
Optional, please see Step 13.

Methylene blue solution

RNA Transfer buffer
For alkaline transfers to charged membranes, use 0.01 N NaOH with 3 M NaCl; for neutral transfers to uncharged membranes, use 20x SSC.

Soaking solution
For charged membranes, use 0.01 N NaOH combined with 3 M NaCl; for uncharged membranes, use 0.05 N NaOH.

0.2x SSC with 1% (w/v) SDS

20x SSC

Nucleic Acids and Oligonucleotides

RNA sample, fractionated through an agarose gel
Prepared as described in Chapter 7, Protocol 5 or Chapter 7, Protocol 6.

METHOD

1. (*Optional*) Partially hydrolyze the RNA sample, fractionated through agarose, by soaking the gel in the appropriate soaking solution as described below.

   For transfer to uncharged nylon membranes

   a. Rinse the gel with DEPC-treated H_2O.
   b. Soak the gel for 20 minutes in 5 gel volumes of 0.05 N NaOH.
   c. Transfer the gel into 10 gel volumes of 20x SSC for 40 minutes.
   d. Without delay, proceed directly with Step 2 to transfer the partially hydrolyzed RNA to an uncharged nylon membrane by capillary action.

   For transfer to charged nylon membranes

   a. Rinse the gel with DEPC-treated H_2O.
   b. Soak the gel for 20 minutes in 5 gel volumes of 0.01 N NaOH/3 M NaCl.
   c. Without delay, proceed directly with Step 2 to transfer the partially hydrolyzed RNA to a positively charged nylon membrane by capillary action.
2. Move the gel containing fractionated RNA to a glass baking dish, and use a sharp scalpel to trim away unused areas of the gel. Cut along the slot line to allow the top of the trimmed gel to be aligned with the top of the membrane during transfer. Cut off a small triangular piece from the bottom left-hand corner of the gel to simplify orientation during the succeeding operations.
3. Place a piece of thick blotting paper on a sheet of Plexiglas or a glass plate to form a support that is longer and wider than the trimmed gel. Make sure that the ends of the blotting paper drape over the edges of the plate. Place the support inside a large baking dish.
4. Fill the dish with the appropriate RNA transfer buffer (0.01 N NaOH/3 M NaCl for positively charged membranes, and 20x SSC for uncharged membranes) until the level of the liquid reaches almost to the top of the support. When the blotting paper on the top of the support is thoroughly wet, smooth out all air bubbles with a glass rod or pipette.
   Alkaline transfer buffer (0.01 N NaOH, 3 M NaCl) is used to transfer RNA to positively charged nylon membranes. Neutral transfer buffer (20x SSC) is used to transfer RNA to uncharged nylon membranes.
5. Use a fresh scalpel or a paper cutter to cut a piece of the appropriate nylon membrane approx. 1 mm larger than the gel in both dimensions.
6. Float the nylon membrane on the surface of a dish of deionized H_2O until it wets completely from beneath, and then immerse the membrane in 10x SSC for at least 5 minutes. Use a clean scalpel blade to cut a corner from the membrane to match the corner cut from the gel.
7. Carefully place the gel on the support in an inverted position so that it is centered on the wet blotting paper.
8. Surround, but do not cover, the gel with Saran Wrap or Parafilm.
9. Wet the top of the gel with the appropriate transfer buffer (please see Step 4). Place the wet nylon membrane on top of the gel so that the cut corners are aligned. One edge of the membrane should extend just beyond the edge of the line of slots at the top of the gel.
   IMPORTANT *Do not move the membrane once it has been applied to the surface of the gel. Make sure that there are no air bubbles between the membrane and the gel.*
10. Wet two pieces of thick blotting paper (cut to exactly the same size as the gel) in the appropriate transfer buffer and place them on top of the wet nylon membrane. Smooth out any air bubbles with a glass rod.
11. Cut or fold a stack of paper towels (5-8 cm high) just smaller than the blotting papers. Place the towels on the blotting papers. Put a glass plate on top of the stack and weigh it down with a 400-g weight.
12. Allow upward transfer of RNA to occur for no more than 4 hours in neutral transfer buffer and approx. 1 hour in alkaline transfer buffer.
13. Dismantle the capillary transfer system. Mark the positions of the slots on the membrane with a ballpoint pen through the gel. Transfer the membrane to a glass tray containing approx. 300 ml of 6x SSC at 23°C. Place the tray on a platform shaker and agitate the membrane very slowly for 5 minutes.
14. Remove the membrane from the 6x SSC and allow excess fluid to drain away. Lay the membrane, RNA side upward, on a dry sheet of blotting paper for a few minutes.
15. If the RNA is to be fixed by UV irradiation, proceed first to Step 16; otherwise, stain the membrane.

a. Transfer the damp membrane to a glass tray containing methylene blue solution. Stain the membrane for just enough time to visualize the rRNAs (approx. 3-5 minutes).
The order of steps during staining and fixation depends on the type of transfer, the type of membrane, and the method of fixation. Because alkaline transfer results in covalent attachment of RNA to positively charged nylon membranes, there is no need to fix the RNA to the membrane before staining. RNA transferred to uncharged nylon membranes in neutral transfer buffer should be stained and then fixed to the membrane by baking under vacuum or heating in a microwave oven. If the RNA is to be cross-linked to the membrane by UV irradiation, then the staining step should follow fixation (please see table below).

Sequence of Staining RNA and Fixing to the Membrane

Type of Membrane	Method of Fixation	Order of Steps
Positively charged nylon	alkaline transfer	1. Stain with methylene blue. 2. Proceed to prehybridization.
Uncharged nylon or positively charged nylon (nonalkaline transfer)	UV irradiation (please see Step 16 for details)	1. Fix the RNA by UV irradiation. 2. Stain with methylene blue. 3. Proceed to prehybridization.
Uncharged nylon or positively charged nylon (nonalkaline transfer)	baking in vacuum oven or microwave oven (please see Step 16 for details)	1. Stain with methylene blue. 2. Bake the membrane. 3. Proceed to prehybridization.

b. Photograph the stained membrane under visible light with a yellow filter.

c. After photography, destain the membrane by washing in 0.2x SSC and 1% (w/v) SDS for 15 minutes at room temperature.
For RNA transferred to positively charged nylon using alkaline transfer, proceed directly to hybridization (Chapter 7, Protocol 8). Any membranes not used immediately in hybridization reactions should be thoroughly dry, wrapped loosely in aluminum foil or blotting paper, and stored at room temperature, preferably under vacuum.

16. Fix the RNA to the uncharged nylon membrane or to positively charged nylon (nonalkaline transfer).

To fix by baking

- Allow the membrane to dry in air and then bake for 2 hours between two pieces of blotting paper under vacuum at 80°C in a vacuum oven.

or

- Place the damp membrane on a dry piece of blotting paper and heat for 2-3 minutes at full power in a microwave oven (750-900 W).
Proceed directly to hybridization (Chapter 7, Protocol 8). Any membranes not used immediately in hybridization reactions should be thoroughly dry, wrapped loosely in aluminum foil or blotting paper, and stored at room temperature, preferably under vacuum.

To cross-link by UV irradiation

a. Place the damp, unstained membrane on a piece of dry blotting paper and irradiate at 254 nm for 1 minute 45 seconds at 1.5 J/cm^2.

b. After irradiation, stain the membrane with methylene blue as described in Step 15.
Proceed directly to hybridization (Chapter 7, Protocol 8). Any membranes not used immediately in hybridization reactions should be thoroughly dry, wrapped loosely in aluminum foil or blotting paper, and stored at room temperature, preferably under vacuum.

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Protocol 8

Northern Hybridization

This protocol describes how to carry out northern hybridization at high stringency in phosphate-SDS-buffers. Although a wide variety of formats are available, hybridization is usually performed in heat-sealable bags, roller bottles, or plastic boxes, as described here.
IMPORTANT: Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Prehybridization solution for northern hybridization
- SSC (0.5x, 1x, and 2x) with 0.1% (w/v) SDS
- SSC (0.1x and 0.5x) with 0.1% (w/v) SDS
  Optional, please see Step 4.

Nucleic Acids and Oligonucleotides

- ⚠ Probe (>2 x 10^8 cpm/µg)
  Prepare and radiolabel the DNA or RNA probes in vitro to high specific activity with ^{32}P, as described in Chapter 9. High-specific-activity (>2 x 10^8 cpm/µg) strand-specific probes (either DNA or RNA) can detect mRNAs that are present at low to medium abundance. The highest sensitivity in northern blotting is obtained from single-stranded probes - either DNA or RNA - radiolabeled in vitro to high specific activity with ^{32}P (>2 x 10^8 cpm/µg). Double-stranded DNA probes are two to three times less sensitive than single-stranded probes.
- RNA, immobilized on membrane
  Prepared as described in Chapter 7, Protocol 7.

METHOD

1. Incubate the membrane for 2 hours at 68°C in 10-20 ml of prehybridization solution.
2. If using a double-stranded probe, denature the ^{32}P-labeled double-stranded DNA by heating for 5 minutes at 100°C. Chill the probe rapidly in ice water.
   Alternatively, denature the probe by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris-Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.
   Single-stranded probes need not be denatured.
3. Add the denatured or single-stranded radiolabeled probe directly to the prehybridization solution. Continue incubation for 12-16 hours at the appropriate temperature.
   To detect low-abundance mRNAs, use at least 0.1 µg of probe whose specific activity exceeds 2 x 10^8 cpm/µg. Low-stringency hybridization, in which the probe is not homologous to the target gene, is best carried out at lower temperatures (37-42°C) in a hybridization buffer containing 50% deionized formamide, 0.25 M sodium phosphate (pH 7.2), 0.25 M NaCl, and 7% SDS.
4. After hybridization, remove the membrane from the plastic bag and transfer it as quickly as possible to a plastic box containing 100-200 ml of 1x SSC, 0.1% SDS at room temperature. Place the closed box on a platform shaker and agitate the fluid gently for 10 minutes.
   IMPORTANT *Do not allow the membrane to dry out at any stage during the washing procedure.*
   Increase the concentration of SDS in the washing buffer to 1% if single-stranded probes are used.
   Following low-stringency hybridization in formamide-containing buffers, rinse the membrane in 2x SSC at 23°C and then successively wash in 2x SSC, 0.5x SSC with 0.1% SDS, and 0.1x SSC with 0.1% SDS for 15 minutes each at 23°C. A final wash containing 0.1x SSC and 1% SDS is carried out at 50°C.
5. Transfer the membrane to another plastic box containing 100-200 ml of 0.5x SSC, 0.1% SDS, prewarmed to 68°C. Agitate the fluid gently for 10 minutes at 68°C.
6. Repeat the washing in Step 5 twice more for a total of three washes at 68°C.
7. Dry the membrane on blotting paper and establish an autoradiograph by exposing the membrane for 24-48 hours to X-ray film (Kodak XAR-5 or equivalent) at -70°C with an intensifying screen. Tungstate-based intensifying screens are more effective than the older rare-earth screens. Alternatively, an image of the membrane can be obtained by scanning in a phosphorimager.

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Protocol 9

Dot and Slot Hybridization of Purified RNA

Dot blotting of RNA is best carried out using purified preparations of RNA that are denatured with glyoxal or formaldehyde immediately before loading onto a nylon membrane through a vacuum manifold. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ NaOH (10 N)

Prehybridization solution for northern hybridization

RNA denaturation solution

0.1x SSC with 0.1% (w/v) SDS

0.1x SSC with 1% (w/v) SDS
Optional, please see Step 18.

0.5x SSC with 0.1% (w/v) SDS

1x SSC with 0.1% (w/v) SDS

2x SSC
Optional, please see Step 18.

20x SSC

Nucleic Acids and Oligonucleotides

⚠ Probe, radiolabeled and denatured
Denature just before use as described in Step 2 of Chapter 7, Protocol 8.
High-specific-activity (>5 x 10^8 cpm/µg) strand-specific probes (either DNA or RNA) can easily detect mRNAs that are present at medium to high abundance when 5 µg of total cellular RNA is loaded per slot. RNAs of the lowest abundance (1-5 copies/cell) are difficult to detect in dot blots of total mammalian cellular RNA. Such RNAs are best detected by loading >1 µg of purified poly(A)$^+$ RNA per slot and hybridizing with strand-specific probes of high specific activity (>5 x 10^8 cpm/µg).

RNA test samples, standards and negative controls
Prepare samples by one of the methods described in Chapter 7, Protocol 1, Chapter 7, Protocol 2, Chapter 7, Protocol 3, and Chapter 7, Protocol 4. All samples should contain the same amount of RNA dissolved in 10 µl of sterile, DEPC-treated H_2O. Standards are generated by mixing varying quantities of unlabeled sense-strand RNA synthesized in vitro (please see Chapter 9) to aliquots of a "negative" RNA preparation that lacks sequences complementary to the radiolabeled probe.

METHOD

1. Cut a piece of positively charged nylon membrane to a suitable size. Mark the membrane with a soft pencil or ballpoint pen to indicate the orientation. Wet the membrane briefly in H_2O and soak it in 20x SSC for 1 hour at room temperature.
2. While the membrane is soaking, clean the blotting manifold carefully with 0.1 N NaOH and then rinse it well with sterile H_2O.
3. Wet two sheets of thick blotting paper with 20x SSC, and place them on top of the vacuum unit of the apparatus.
4. Place the wet nylon membrane on the bottom of the sample wells cut into the upper section of the manifold. Roll a pipette across the surface of the membrane to smooth away any air bubbles trapped between the upper section of the manifold and the nylon membrane.
5. Clamp the two parts of the manifold together, and connect the unit to a vacuum line.
6. Fill all of the slots/dots with 10x SSC, and apply gentle suction until the fluid has passed through the nylon membrane. Turn off the vacuum, and refill the slots with 10x SSC.
7. Mix each of the RNA samples (dissolved in 10 µl of H_2O) with 30 µl of RNA denaturation solution.
8. Incubate the mixture for 5 minutes at 65°C, and then cool the samples on ice.
9. Add an equal volume of 20x SSC to each sample.
10. Apply gentle suction to the manifold until the 10x SSC in the slots has passed through the membrane. Turn off the vacuum.
11. Load all of the samples into the slots, and then apply gentle suction. After all of the samples have passed through the membrane, rinse each of the slots twice with 1 ml of 10x SSC.
12. After the second rinse has passed through the nylon membrane, continue suction for 5 minutes to dry the membrane.
13. Remove the membrane from the manifold, and fix the RNA to the membrane by either UV irradiation, baking, or microwaving, as described in Step 16 of Chapter 7, Protocol 7 .
    Before setting up the prehybridization and hybridization reactions, please see Chapter 7, Protocol 8.
14. Incubate the membrane for 2 hours at 68°C in 10-20 ml of prehybridization solution in a baking dish or hybridization chamber.
15. Add the denatured radiolabeled probe directly to the prehybridization solution. Continue the incubation for 12-16 hours at the appropriate temperature.
    To detect low-abundance mRNAs, use at least 0.1 µg of probe whose specific activity exceeds 5 x 10^8 cpm/µg. Low-stringency hybridization, in which the probe is not homologous to the target gene, is best carried out at lower temperatures (37-42°C) in a hybridization buffer containing 50% deionized formamide, 0.25 M sodium phosphate (pH 7.2), 0.25 M NaCl, and 7% SDS.
16. After hybridization, remove the membrane from the plastic bag and transfer it as quickly as possible to a plastic box containing 100-200 ml of 1x SSC, 0.1% SDS at room temperature. Place the closed box on a platform shaker and agitate the fluid gently for 10 minutes.
    IMPORTANT *Do not allow the membrane to dry out at any stage during the washing procedure.*
    Increase the concentration of SDS in the washing buffer to 1% if single-stranded probes are used.
    Following low-stringency hybridization in formamide-containing buffers, rinse the membrane in 2x SSC at 23°C and then successively wash in 2x SSC, 0.5x SSC with 0.1% SDS, and 0.1x SSC with 0.1% SDS for 15 minutes each at 23°C. A final wash containing 0.1x SSC and 1% SDS is carried out at 50°C.
17. Transfer the membrane to another plastic box containing 100-200 ml of 0.5x SSC, 0.1% SDS, prewarmed to 68°C. Agitate the fluid gently for 10 minutes at 68°C.
18. Repeat the washing in Step 17 twice more for a total of three washes at 68°C.
19. Dry the membrane on filter paper and establish an autoradiograph by exposing the membrane for 24-48 hours to X-ray film (Kodak XAR-5 or equivalent) at -70°C with an intensifying screen. Tungstate-based intensifying screens are more effective than the older rare-earth screens. Alternatively, an image of the membrane can be obtained by scanning in a phosphorimager.

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Protocol 10

Mapping RNA with Nuclease S1

Preparations of RNA containing an mRNA of interest are hybridized to a complementary single-stranded DNA probe. At the end of the reaction, nuclease S1 is used to degrade unhybridized regions of the probe, and the surviving DNA-RNA hybrids are then separated by gel electrophoresis and visualized by either autoradiography or Southern hybridization. The method can be used to quantitate RNAs, to map the positions of introns, and to identify the locations of 5´ and 3´ ends of mRNAs on cloned DNA templates. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium persulfate (10%)

Ethanol

Nuclease S1 stop mixture

⚠ Phenol:chloroform (1:1, v/v)

10x RNA annealing buffer

RNA gel elution buffer

RNA gel-loading buffer

RNA hybridization buffer without formamide

Sodium acetate (3 M, pH 5.2)

TE (pH 7.6)

⚠ TEMED (N,N,N´,N´-tetramethylethylene diamine)

⚠ TCA (trichloroacetic acid) (1% and 10%)
Dilute 100% stock solution 1/10 and 1/100 just before use. Chill the working solutions in ice.

Enzymes and Buffers

Bacteriophage T4 polynucleotide kinase

Klenow fragment of *E. coli* DNA polymerase (10 units/µl)

Nuclease S1 (for use in nuclease S1 digestion buffer, please see below)
It is necessary to titrate the nuclease S1 each time a new probe or RNA preparation is used.

Nuclease S1 digestion buffer

Restriction endonucleases

Nucleic Acids and Oligonucleotides

Carrier RNA (yeast tRNA)

DNA probe, uniformly labeled and single-stranded
The DNA probe is prepared in Steps 1-15 of this protocol. Use single-stranded probes uniformly labeled to high specific activity within a few days to avoid problems caused by radiochemical degradation.

⚠ dNTPs (20 mM) containing all four dNTPs
Dissolve the dNTPs in 25 mM Tris-Cl (pH 8.0) and store as small aliquots at -20°C.

RNA, for use as a standard
Synthesize in vitro by transcription of the appropriate strand of a recombinant plasmid containing the DNA sequences of interest and a bacteriophage promoter. Methods to synthesize and purify the RNA are outlined in Chapter 9, Protocol 6 .

Synthetic oligonucleotide (10 pmoles/µl) in distilled H_2O
The oligonucleotide used to prime synthesis of the probe from a single-stranded DNA template should be 20-25 nucleotides in length and complementary to the RNA strand to be analyzed. It should hybridize to the template DNA strand 250-500 nucleotides 3´ of the position that will be cleaved by the chosen restriction enzyme. Store oligonucleotides in aliquots at -20°C.

Template DNA (1 µg/µl), single-stranded
Use standard procedures (Chapter 3, Protocol 5) to prepare single-stranded DNA from a recombinant bacteriophage M13 carrying the insert DNA strand in the same sense as the test RNA.

Test RNA
Poly(A)$^+$ or total RNA prepared by one of the methods described in Chapter 7, Protocol 1, Chapter 7, Protocol 2, Chapter 7, Protocol 3 and Chapter 7, Protocol 4.

Radioactive Compounds

[γ-^{32}P]ATP (10 mCi/ml, 3000 Ci/mmole)

METHOD

1. Prepare a polyacrylamide minigel containing 8 M urea (13 cm x 15 cm x 0.75 mm) (e.g., Bio-Rad Mini-Protean).
   a. Mix the following reagents:

   7.2 g of urea

   1.5 ml of 10x TBE

   Add the appropriate amounts of 40% acrylamide (acrylamide:bisacrylamide 19:1) to generate a gel containing the desired concentration of polyacrylamide.
   b. Add H_2O to a final volume of 15 ml.
   c. Stir the mixture at room temperature on a magnetic stirrer until the urea dissolves. Then add:

   120 µl of 10% ammonium persulfate

   16 µl of TEMED

   Mix the solution quickly and then pour the gel into the mold of a minigel apparatus.
2. While the gel is polymerizing, mix the following reagents:

   10 pmoles (1 µl) of unlabeled oligonucleotide

   10 µl of [γ-^{32}P]ATP (3000 Ci/mmole, 10 mCi/ml)

   2 µl of 10x polynucleotide kinase buffer

6 µl of H_2O

10 units (1 µl) of polynucleotide kinase

Incubate the reaction mixture for 45 minutes at 37°C, and then for 3 minutes at 95°C to inactivate the polynucleotide kinase.

3. Add to the kinase reaction:

   2 µl (2 µg) of single-stranded DNA template

   4 µl of 10x annealing buffer

   14 µl of H_2O

   Incubate the reaction mixture for 10 minutes at 65°C and then allow it to cool to room temperature.
4. Add to the reaction mix from Step 3:

   4 µl of dNTP mixture

   1 µl (10 units) of the Klenow fragment of *E. coli* DNA polymerase I

   Incubate the reaction mixture for 15 minutes at room temperature and then inactivate the DNA polymerase by incubation for 3 minutes at 65°C.
5. Adjust the ionic composition and pH of the reaction mixture to suit the restriction enzyme. Add 20 units of restriction enzyme and incubate the reaction mixture for 2 hours at the appropriate temperature.
6. Add to the restriction endonuclease digestion reaction:

   2 µl of carrier RNA

   5 µl of 3 M sodium acetate (pH 5.2)

   Recover the DNA probe by standard precipitation with ethanol.
7. Dissolve the DNA in 20 µl of gel-loading buffer. Heat the solution to 95°C for 5 minutes to denature the DNA, and then cool the DNA quickly to 0°C.
8. While the DNA is incubating at 95°C, wash the loading slots of the gel to remove urea and then, without delay, load the probe into one of the slots of the gel.
9. Run the gel until the bromophenol blue reaches the bottom of the gel (200 mA for approx. 30 minutes).
10. Dismantle the gel apparatus, leaving the gel attached to the bottom glass plate. Wrap the gel and plate in a piece of plastic wrap (e.g., Saran Wrap). Make sure that there are no bubbles between the gel and the plastic film.
11. Expose the gel to X-ray film. Mark the location of the corners and sides of the plate on the film with a permanent marker. Also mark the position of the bromophenol blue and xylene cyanol.
12. Realign the glass plate with the film and excise the radiolabeled band with a scalpel. Reexpose the mutilated gel to a fresh piece of film to ensure that the region of the gel containing the band of the correct molecular weight has been accurately excised.
13. Transfer the fragment of gel to a fresh sterile microfuge tube and add just enough gel elution buffer to cover the fragment (250-500 µl). Incubate the closed tube on a rotating wheel overnight at room temperature.
14. Centrifuge the tube at maximum speed for 5 minutes in a microfuge.
15. Taking care to avoid the pellet of polyacrylamide, use an automatic pipetting device to transfer the supernatant to a fresh microfuge tube. The labeled probe should emit approx. 1 x 10^4 cpm/µl as measured by liquid scintillation spectroscopy.
16. Store the probe at -70°C.
17. Transfer 0.5-150 µg aliquots of RNA (test and standard) into sterile microfuge tubes. Add an excess of uniformly labeled single-stranded DNA probe to each tube.
18. Precipitate the RNA and DNA by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold ethanol. After storage for 30 minutes at 0°C, recover the nucleic acids by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge. Discard the ethanolic supernatant, rinse the pellet with 70% ethanol, and centrifuge the sample. Carefully remove all of the ethanol, and store the pellet containing RNA and DNA at room temperature until the last visible traces of ethanol have evaporated.
19. Dissolve the nucleic acid pellet in 30 µl of hybridization buffer. Pipette the solution up and down many times to ensure that the pellet is completely dissolved.
20. Close the lid of the tube tightly, and incubate the hybridization reaction in a water bath set at 85°C for 10 minutes to denature the nucleic acids.
21. Rapidly transfer the tube to a water bath set at the desired hybridization temperature (usually 65°C). Do not allow the tube to cool below the hybridization temperature during transfer. Hybridize the DNA and RNA for 12-16 hours at the chosen temperature.
22. Taking care to keep the body of the tube submerged, open the lid of the hybridization tube. Rapidly add 300 µl of ice-cold nuclease S1 digestion buffer, and immediately remove the tube from the water bath. Quickly mix the contents of the tube by vortexing gently, and then transfer the tube to a water bath set at the temperature appropriate for digestion with nuclease S1. Incubate for 1-2 hours, depending on the degree of digestion desired.
23. Chill the reaction to 0°C. Add 80 µl of nuclease S1 stop mixture and vortex the tube to mix the solution.
24. Extract the reaction once with phenol:chloroform. After centrifugation at maximum speed for 2 minutes at room temperature in a microfuge, transfer the aqueous supernatant to a fresh tube. Add 2 volumes of ethanol, mix, and store the tube for 1 hour at -20°C.
25. Recover the nucleic acids by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge. Carefully remove all of the supernatant, and store the open tube at room temperature until the last visible traces of ethanol have evaporated.
26. Dissolve the pellet in 4 µl of TE (pH 7.6). Add 6 µl of gel-loading buffer and mix well.
27. Heat the nucleic acids for 5 minutes at 95°C, and then immediately transfer the tube to an ice bath. Centrifuge the tubes briefly in a microfuge to consolidate the samples at the bottoms of the tubes.
28. Analyze the radiolabeled DNA by electrophoresis through a polyacrylamide/8 M urea gel.
29. After the tracking dyes have migrated an appropriate distance through the gel, turn off the power supply and disassemble the electrophoresis set up. Gently pry up one corner of the larger glass plate and slowly remove the plate from the gel. Cut off one corner of the gel for orientation purposes.
30. Transfer the glass plate containing the gel to a tray containing an excess of 10% TCA. Gently rock or rotate the tray for 10 minutes at room temperature.
31. Pour off the 10% TCA solution and replace with an excess of 1% TCA. Gently rock or rotate the tray for 5 minutes at room temperature.
32. Pour off the 1% TCA solution and briefly rinse the fixed gel with distilled deionized H_2O. Lift the glass plate together with the gel out of the tray and place them on a flat bench top. Use paper towels or Kimwipes to remove excess H_2O.
33. Cut a piece of Whatman 3MM filter paper (or equivalent) that is 1 cm larger than the gel on all sides. Transfer the gel to the filter paper by laying the paper on top of the gel and inverting the glass plate.
34. Remove the plate and dry the gel on a gel dryer for 1.0-1.5 hours at 60°C.
35. Establish an autoradiographic image of the dried gel. Scan the image by densitometry or phosphorimaging, or excise the segments of the gel containing the fragments and count them by liquid scintillation spectroscopy.

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Protocol 11

Ribonuclease Protection: Mapping RNA with Ribonuclease and Radiolabeled RNA Probes

Preparations of RNA containing an mRNA of interest are hybridized to a radiolabeled single-stranded RNA probe. At the end of the reaction, a mixture of RNase A and RNase T1 is used to degrade unhybridized regions of the probe, and the surviving molecules are then separated by denaturing gel electrophoresis and visualized by autoradiography. The method can be used to quantitate RNAs, to map the positions of introns, and to identify the locations of 5′ and 3′ ends of mRNAs on cloned DNA templates. **IMPORTANT:** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)

DTT (dithiothreitol) (0.2 M)

Ethanol

⚠ Phenol:chloroform (1:1, v/v)

RNA gel-loading buffer

⚠ RNA hybridization buffer with formamide

⚠ SDS (10% w/v)

Sodium acetate (3 M, pH 5.2)

⚠ TCA (trichloroacetic acid) (1% and 10%)
Dilute 100% stock solution 1/10 and 1/100 just before use. Chill the working solutions in ice.

TE (pH 7.6)

10x Transcription buffer

Enzymes and Buffers

Bacteriophage-encoded DNA-dependent RNA polymerases
T3, SP6, or T7, depending on the plasmid vector and on the strand of DNA to be transcribed. If, as is often the case, the bacteriophage RNA polymerase supplied by the manufacturer is highly concentrated, prepare an appropriate dilution of the enzyme in polymerase dilution buffer.

DNase I (1 mg/ml, RNase-free pancreatic DNase)
This enzyme is available from several manufacturers (e.g., RQ1 from Promega).

Polymerase dilution buffer
Prepare solution fresh for each use.

Proteinase K (10 mg/ml)

Protein inhibitor of RNase, chill in ice
These inhibitors are sold by several manufacturers under various trade names (e.g., RNAsin, Promega; Prime Inhibitor, 5 Prime->3 Prime).

RNase digestion mixture

Nucleic Acids and Oligonucleotides

Carrier RNA (1 mg/ml)

Plasmid DNA or linearized target DNA for preparing templates
If a plasmid DNA is used to prepare templates (please see Step 1), clone the DNA segment of interest into a plasmid vector of the pGEM (Promega) or Bluescript (Stratagene) series. pGEM plasmids contain promoters recognized by RNA polymerases from bacteriophages SP6 and T7, whereas Bluescript plasmids contain bacteriophage T7 and T3 promoters. Purify large-scale preparations from alkaline lysates of bacterial cultures by precipitation with polyethylene glycol (please see Chapter 1, Protocol 8).

Ribonucleotides
Prepare a solution containing GTP, CTP, and ATP, each at a concentration of 5 mM.

Riboprobe
The riboprobe is prepared in Steps 1-7 of this protocol.

RNA, for use as standards
RNA standards are synthesized in vitro by transcription of the appropriate strand of a recombinant plasmid containing DNA sequences of interest and a bacteriophage promoter (please see Chapter 9, Protocol 6). By cloning an appropriate fragment of the gene of interest, it is possible to distinguish by size between authentic mRNA in the test sample and the signal from the RNA standard.

Test RNA
Poly(A)$^+$ or total RNA prepared by one of the methods described in Chapter 7, Protocol 1, Chapter 7, Protocol 2, Chapter 7, Protocol 3, and Chapter 7, Protocol 4. In some cases, treatment of total RNA with RNase-free DNase may improve the accuracy of RNase protection.

⚠ UTP (100 µm)

Radioactive Compounds

⚠ [α-^{32}P]UTP (10 mCi/ml, 800 Ci/mmole)
[α-^{32}P]UTP is the radiolabel of choice because it is specific to RNA. However, some investigators prefer to use [α-^{32}P]GTP because bacteriophage SP6 RNA polymerase tolerates low concentrations of this ribonucleotide slightly better than it tolerates low concentrations of any of the other three.

METHOD

1. Prepare the linearized template DNA.

To prepare template from plasmid DNA

a. Linearize 5-20 µg of plasmid DNA by digestion with a fivefold excess of an appropriate restriction enzyme that cleaves either within the cloned DNA sequence or downstream from the DNA sequence. The distance from the promoter to the newly created terminus should be 200-400 bp. Make sure not to use an enzyme that separates the promoter from the sequence of interest. Because bacteriophage-encoded RNA polymerases may initiate transcription at 3′-protruding termini, choose a restriction enzyme that generates a blunt terminus or a 5′ extension.

b. At the end of the digestion, analyze an aliquot (approx. 200 ng) of the reaction by agarose gel electrophoresis. No trace of circular plasmid DNA should be visible. If necessary, add more restriction enzyme and continue digestion until no more circular plasmid DNA can be detected.

c. Purify the linear DNA by extracting twice with phenol:chloroform and then recover the DNA by standard precipitation with ethanol. After washing the precipitate with 70% ethanol, dissolve the DNA in TE (pH 7.6) at a concentration of 1 µg/µl.

To prepare template by amplification of target DNA

a. Carry out PCR to synthesize double-stranded DNA templates, 100-400 bp in length (please see Chapter 8, Protocol 1 for details).
The template should be either linearized plasmid DNA or a DNA fragment encoding the sequence of interest. Either one or both of the oligonucleotide primers are designed to contain the consensus sequence of a bacteriophage promoter at their 5´ termini. Amplification in PCR yields double-stranded DNA fragments carrying bacteriophage promoters at one or both ends.

b. Analyze the products of the PCR by electrophoresis through an agarose or a polyacrylamide gel to ensure that a DNA fragment of the appropriate size has been amplified.

c. Purify the linear amplification product by extracting twice with phenol:chloroform and then recover the DNA by standard precipitation with ethanol. After washing with 70% ethanol, dissolve the DNA in TE (pH 7.6) at a concentration of 1 µg/µl.

2. Mix the following in order, prewarmed to room temperature except when noted otherwise.
0.5 µg of linearized template DNA (from Step 1)
1 µl of 0.2 M dithiothreitol
2 µl of ribonucleotide solution
1 µl of 100 µM UTP
50-100 µCi [α-^{32}P]UTP (800 Ci/mmole, 10 mCi/ml)
H_2O to a volume of 16 µl
2 µl of 10x transcription buffer
24 units of protein inhibitor of RNase (on ice)
15-20 units of bacteriophage RNA polymerase (on ice)

Adding the reagents in the order shown at room temperature prevents both precipitation of the DNA by spermidine and Mg^{2+} in the transcription buffer and inactivation of the RNase inhibitor by high concentrations of dithiothreitol.
If, as is often the case, the bacteriophage RNA polymerase supplied by the manufacturer is highly concentrated, prepare an appropriate dilution of the enzyme in polymerase dilution buffer.
Incubate the reaction mixture for 60 minutes at 37°C.
The specific activity of the RNA synthesized in the reaction will be high (approx. 10^9 cpm/µg) since 60-80% of the radiolabeled UTP will be incorporated. The total yield of RNA should be approx. 100 ng.
3. At the end of the incubation period, add 1 unit of RNase-free DNase equivalent to approx. 1 µg of the enzyme and continue incubation for a further 10 minutes at 37°C.
4. Carry out steps 4 and 5 simultaneously. Dilute the reaction mixture to 100 µl with TE (pH 7.6) and measure the total radioactivity and the amount of TCA-precipitable radioactivity in 1-µl aliquots of the diluted mixture. From the fraction of radioactivity incorporated in TCA-precipitable material, calculate the weight and specific activity of the RNA probe synthesized in the reaction.
5. After removing 1-µl aliquots in Step 4, add 1 µl of 1 mg/ml carrier RNA to the remainder of the diluted reaction mixture. Extract the diluted reaction mixture once with phenol:chloroform. Transfer the aqueous phase to a fresh tube and precipitate the RNA by adding 10 µl of 10 M ammonium acetate and 300 µl of ethanol. Store the tube at -20°C until Step 4 has been completed.
A solution of carrier RNA (1 mg/ml) is prepared by diluting the stock solution 1:10 with DEPC-treated H_2O.
6. Recover the RNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge. Wash the RNA pellet with 75% ethanol and centrifuge again. Remove the supernatant and allow the pellet of RNA to dry in the air until no visible trace of ethanol remains. Dissolve the RNA in 20 µl of gel-loading buffer if the probe is to be purified by gel electrophoresis (Step 7) or in 20 µl of TE (pH 7.6) if the probe is to be used without further purification.
7. Following the instructions given in Steps 8-16 of Chapter 7, Protocol 10 , purify the probe by electrophoresis using the previously prepared polyacrylamide/8 M urea gel.
8. Combine each of the test RNAs and RNA standards with the riboprobe (2×10^5 to 10×10^5 cpm, 0.1-0.5 ng). Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold ethanol. Store the mixtures for 10 minutes at -20°C and then recover the RNAs by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge. Wash the pellet in 75% ethanol. Carefully remove all of the ethanol and store the pellet at room temperature until the last visible traces of ethanol have evaporated.
9. Dissolve the RNAs in 30 µl of hybridization buffer. Pipette the solution up and down numerous times to ensure that the pellet is completely dissolved.
10. Incubate the hybridization mixture for 10 minutes at 85°C to denature the RNAs. Quickly transfer the hybridization mixture to an incubator or water bath set at the annealing temperature. Incubate the mixture for 8-12 hours.
11. Cool the hybridization mixture to room temperature, and add 300 µl of RNase digestion mixture. Digest the hybridization reaction for 60 minutes at 30°C.
12. Add 20 µl of 10% SDS and 10 µl of 10 mg/ml proteinase K to stop the reaction. Incubate the reaction mixture for 30 minutes at 37°C.
13. Add 400 µl of phenol:chloroform, vortex the mixture for 30 seconds, and separate the phases by centrifugation at maximum speed for 5 minutes at room temperature in a microfuge.
14. Transfer the upper aqueous phase to a fresh tube, carefully avoiding the interface between the organic and aqueous phases.
15. Add 20 µg of carrier RNA and 750 µl of ice-cold ethanol. Mix the solution well by vortexing, and then store the solution for 30 minutes at -20°C.
16. Recover the RNA by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge. Carefully remove the ethanol and wash the pellet with 500 µl of 70% ethanol. Centrifuge as before.
17. Carefully remove all of the ethanol, and store the open tube at room temperature until the last visible traces of ethanol have evaporated.
18. Resuspend the precipitate in 10 µl of gel-loading buffer.
19. Heat the nucleic acids for 5 minutes at 95°C, and then immediately transfer the tube to an ice bath. Centrifuge the tubes briefly in a microfuge to consolidate the samples at the bottom of the tubes.
20. Analyze the radiolabeled RNA by electrophoresis through a "thin" polyacrylamide/8 M urea gel.
21. After the tracking dyes have migrated an appropriate distance through the gel, turn off the power supply and dismantle the electrophoresis set up. Gently pry up one corner of the larger glass plate and slowly remove the plate from the gel. Cut off one corner of the gel for orientation purposes.
22. Transfer the glass plate containing the gel to a tray containing an excess of 10% TCA. Gently rock or rotate the tray for 10 minutes at room temperature.
23. Pour off the 10% TCA solution and replace with an excess of 1% TCA. Gently rock or rotate the tray for 5 minutes at room temperature.
24. Pour off the 1% TCA solution and briefly rinse the fixed gel with distilled deionized H_2O. Lift the glass plate together with the gel out of the tray and place them on a flat bench top. Apply paper towels or Kimwipes to the sides of the gel to remove excess H_2O.
25. Cut a piece of Whatman 3MM filter paper (or equivalent) that is 1 cm larger than the gel on all sides. Transfer the gel to the filter paper by laying the paper on top of the gel and inverting the glass plate.
26. Remove the plate and dry the gel on a gel dryer for 1.0-1.5 hours at 60°C.
27. Establish an autoradiographic image of the dried gel. Scan the image by densitometry or phosphorimaging, or excise the segments of the gel containing the fragments and count them by liquid scintillation spectroscopy.

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1. Melton D.A., Krieg P.A., Rebagliati M.R., Maniatis T., Zinn K., and Green M.R. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035-7056.

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Protocol 12

Analysis of RNA by Primer Extension

Primer extension is used chiefly to map the 5´ termini of mRNAs. A preparation of polyadenylated mRNA is first hybridized with an excess of a single-stranded oligodeoxynucleotide primer, which is complementary to the target RNA and radiolabeled at its 5´ terminus. Reverse transcriptase is then used to extend the 3´ end of the primer. The size of the resulting cDNA, measured by denaturing polyacrylamide gel electrophoresis, is equal to the distance between the 5´ end of the priming oligonucleotide and the 5´ terminus of the target mRNA.
IMPORTANT: Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
⚠ Chloroform
DTT (dithiothreitol) (1 M)
Ethanol
⚠ Formamide loading buffer
KCl (1.25 M)
⚠ Phenol
Primer extension mix
Sodium acetate (3 M, pH 5.2)
TCA (trichloroacetic acid) (1% and 10%)
Dilute 100% stock solution 1/10 and 1/100 just before use. Chill the working solutions in ice.
TE (pH 7.6)

Enzymes and Buffers

Polynucleotide kinase
Protein inhibitor of RNase
These inhibitors are sold by several manufacturers under various trade names (e.g., RNAsin, Promega, Prime Inhibitor, 5 Prime->3 Prime).
Reverse transcriptase
A cloned version of reverse transcriptase encoded by the Moloney murine leukemia virus (Mo-MLV) is the enzyme of choice in this protocol. Mutants of the enzyme that lack RNase H activity (e.g., StrataScript, Stratagene) have some advantages over the wild-type enzyme since they produce higher yields of full-length extension product and work equally well at both 47°C and 37°C.

Nucleic Acids and Oligonucleotides

Carrier RNA (yeast tRNA)
DNA markers, radiolabeled, for gel electrophoresis
Input RNA to be analyzed
Preparations of poly(A)$^+$ RNA are preferred, especially when setting up primer extension reactions for the first time or when preparations of total RNA produce extension products of different lengths.
Oligonucleotide primer
These primers should be 20-30 nucleotides in length and preferentially purified through Sep-Pak chromatography and by gel electrophoresis (please see Chapter 10, Protocol 1). Crude preparations of oligonucleotides give rise to higher backgrounds on the autoradiogram, especially in the area of the film corresponding to the low-molecular-weight region of the polyacrylamide gel. Resuspend the purified oligonucleotide at a concentration of approx. 60 ng/µl (5-7 pmoles/µl) in TE (pH 7.6).

Radioactive Compounds

[γ-^{32}P]ATP (10 mCi/ml, 7000 Ci/mmole)

METHOD

1. Phosphorylate the oligonucleotide primer in a reaction containing:

oligonucleotide primer (5-7 pmoles or 60 ng)	1 µl
distilled deionized H_2O	6.5 µl
10x kinase buffer	1.5 µl
polynucleotide kinase (approx. 10 units)	1 µl
[γ-^{32}P]ATP (7000 Ci/mmole)	2 µl

   Incubate the reaction for 60 minutes at 37°C.
   The final concentration of radiolabeled ATP in the reaction should be approx. 30 nM.
2. Stop the kinase reaction with the addition of 500 µl of TE (pH 7.6). Add 25 µg of carrier RNA.
3. Add 400 µl of equilibrated phenol (pH 8.0) and 400 µl of chloroform (or 800 µl of commercial phenol:chloroform [1:1]). Vortex vigorously for 20 seconds. Separate the aqueous and organic phases by centrifugation for 2 minutes in a microfuge.
4. Transfer the aqueous layer to a fresh sterile microfuge tube and extract with 800 µl of chloroform. Vortex vigorously for 20 seconds. Separate the aqueous and organic phases by centrifugation for 2 minutes in a microfuge. Again transfer the aqueous layer to a fresh sterile microfuge tube.
5. Repeat Step 4.
6. Add 55 µl of sterile 3 M sodium acetate (pH 5.2) and 1 ml of ethanol to the aqueous layer from Step 5. Mix by vortexing and store the solution for at least 1 hour at -70°C.
7. Collect the precipitated oligonucleotide primer by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge. Remove and discard the radioactive supernatant. Wash the pellet in 70% ethanol and centrifuge again. Discard the supernatant and dry the precipitate in the air. Dissolve the precipitate in 500 µl of TE (pH 7.6).
8. Count 2 µl of radiolabeled oligonucleotide primer in 10 ml of scintillation fluid in a liquid scintillation counter. Calculate the specific activity of the radiolabeled primer assuming 80% recovery. The specific activity should be approx. 2 x 10^6 cpm/pmole of primer.
9. Mix 10^4 to 10^5 cpm (20-40 fmoles) of the DNA primer with 0.5-150 µg of the RNA to be analyzed. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. Store the solution for 60 minutes at -70°C, and then recover the RNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge. Wash the pellet with 70% ethanol and centrifuge again. Carefully remove all of the ethanol, and store the pellet at room temperature until the last visible

traces of ethanol have evaporated.

10. Resuspend the pellets in 8 µl of TE (pH 7.6) per tube. Pipette the samples up and down several times to dissolve pellets.
11. Add 2.2 µl of 1.25 M KCl. Vortex the samples gently and then deposit the fluid in the base of the tubes by centrifuging for 2 seconds in a microfuge.
12. Place the oligonucleotide/RNA mixtures in a water bath set at the appropriate annealing temperature. Incubate the samples for 15 minutes at the optimum temperature, as determined in preliminary experiments.
13. While the oligonucleotide and RNA are annealing, supplement an aliquot of primer extension mix with dithiothreitol and reverse transcriptase as follows: Thaw a 300-µl aliquot of primer extension mix on ice and then add 3 µl of 1 M dithiothreitol and reverse transcriptase to a concentration of 1-2 units/µl. Add 0.1 unit/µl of protein inhibitor of RNase, gently mix by inverting the tube several times, and store it on ice.
14. Remove the tubes containing the oligonucleotide primer and RNA from the water bath and deposit the fluid in the base of the tubes by centrifuging for 2 seconds in a microfuge.
15. Add 24 µl of supplemented primer extension mix to each tube. Gently mix the solution in the tubes and again deposit the liquid at the tube bottoms by centrifugation.
16. Incubate the tubes for 1 hour at 42°C to allow the primer extension reaction to proceed.
17. Terminate the primer extension reactions by the addition of 200 µl of TE (pH 7.6), 100 µl of equilibrated phenol (pH 8.0), and 100 µl of chloroform. Vortex for 20 seconds. Separate aqueous and organic phases by centrifugation for 4 minutes at room temperature in a microfuge.
18. Precipitate the nucleic acids by the addition of 50 µl of 10 M ammonium acetate and 700 µl of ethanol. Mix well by vortexing and incubate ethanol precipitations for at least 1 hour at -70°C.
19. Collect the precipitated nucleic acids by centrifugation for 10 minutes at 4°C in a microfuge. Carefully rinse the pellets with 400 µl of 70% ethanol. Centrifuge again for 5 minutes at 4°C and remove the 70% ethanol rinse with a pipette. Store the open tubes at room temperature until all visible traces of ethanol have evaporated.
20. Dissolve the nucleic acid precipitates in 10 µl of formamide loading buffer. Pipette the samples up and down to assist resuspension.
21. Heat the samples for 8 minutes at 95°C. Then plunge the tubes into an ice-water bath and immediately analyze the primer extension products by electrophoresis through a denaturing polyacrylamide gel.
22. After the tracking dyes have migrated an appropriate distance through the gel, turn off the power supply and dismantle the electrophoresis setup. Gently pry up one edge of the larger glass plate and slowly remove the plate from the gel. Cut off one corner of the gel for orientation purposes.
23. If a polyacrylamide gel 1.0 mm in thickness was used, fix the gel in TCA. Transfer the glass plate containing the gel to a tray containing an excess of 10% TCA. Gently rock or rotate the tray for 10 minutes at room temperature.
    This step is not necessary if a thin gel (0.4-mm thickness) was used. In this case, proceed to Step 26.
24. Pour off the 10% TCA solution and replace it with an excess of 1% TCA. Gently rock or rotate the tray for 5 minutes at room temperature.
25. Pour off the 1% TCA solution and briefly rinse the fixed gel with distilled deionized H_2O. Lift the glass plate together with the gel out of the tray and place them on a flat bench top. Apply paper towels or Kimwipes to the sides of the gel to remove excess H_2O.
26. Cut a piece of Whatman 3MM filter paper (or equivalent) that is 1 cm larger than the gel on all sides. Transfer the gel to the filter paper by laying the paper on top of the gel and inverting the glass plate.
27. Remove the plate and dry the gel on a heat-assisted vacuum-driven gel dryer for 1.0-1.5 hours at 60°C.
28. Establish an image of the gel using autoradiography or phosphorimaging.

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1. Boorstein W.R. and Craig E.A. 1989. Primer extension anlaysis of RNA. *Methods Enzymol.* 180:347-369.
2. Calzone F.J., Britten R.J., and Davidson E.H. 1987. Mapping of gene transcripts by nuclease protection assays and cDNA primer extension. *Methods Enzymol.* 157:611-632.

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Chapter 8 In Vitro Amplification of DNA by the Polymerase Chain Reaction

Protocol 1: The Basic Polymerase Chain Reaction

This protocol describes how to amplify a segment of double-stranded DNA in a chain reaction catalyzed by a thermostable DNA polymerase. It is the foundation for all subsequent variations of the polymerase chain reaction. For details on primer design and troubleshooting amplification reactions, please see Tables 8-3, 8-4, and 8-5 on pages 8.14, 8.23, and 8.24, respectively, in the print version of the manual.

Protocol 2: Purification of PCR Products in Preparation for Cloning

The residual enzymatic activity of thermostable DNA polymerases that survive the rigors of PCR can compromise subsequent enzymatic reactions. This protocol describes how to use proteinase K to destroy thermostable enzymes and to purify amplified DNA in preparation for cloning.

Protocol 3: Removal of Oligonucleotides and Excess dNTPs from Amplified DNA by Ultrafiltration

In this protocol, ultrafiltration through Centricon or Microcon concentrators is used to remove unused primers, primer-dimers, and NTPs from preparations of amplified DNA.

Protocol 4: Blunt-end Cloning of PCR Products

Incubation of a blunt-end ligation reaction in the presence of an excess amount of an appropriate restriction enzyme can dramatically increase the yield of recombinant plasmids. The role of the restriction enzyme is to cleave circular and linear concatemers at restriction sites that are re-formed when linear, blunt-ended plasmid molecules ligate to themselves. In almost all cases, ligation of the PCR product to the plasmid destroys the restriction site. The constant reclamation of vector molecules drives the equilibrium of the ligation reaction strongly in favor of the recombinants between vector and blunt-ended PCR product.

Protocol 5: Cloning PCR Products into T Vectors

This method of direct cloning takes advantage of the unpaired adenosyl residue added to the 3' terminus of amplified DNAs by *Taq* and other thermostable polymerases.

Protocol 6: Cloning PCR Products by Addition of Restriction Sites to the Termini of Amplified DNA

Pairs of oligonucleotide primers used in PCR are often designed with restriction sites in their 5' regions. In many cases, the sites are different in the two primers. In this case, amplification generates a target fragment whose termini now carry new restriction sites that can be used for directional cloning into plasmid vectors. The purified fragment and the vector are digested with the appropriate restriction enzymes, ligated together, and transformed into *E. coli.*

Protocol 7: Genetic Engineering with PCR

This method describes how to modify the termini of PCR products by introducing restriction sites and other features. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

Protocol 8: Amplification of cDNA Generated by Reverse Transcription of mRNA

In this method, an oligodeoxynucleotide primer hybridized to mRNA is extended by an RNA-dependent DNA polymerase to create a cDNA copy that can be amplified by PCR. Depending on the purpose of the experiment, the primer for first-strand cDNA synthesis can be specifically designed to hybridize to a particular target gene, or a general primer such as oligo(dT) can be used to prime cDNA synthesis from essentially all mammalian mRNAs. Similarly, the reverse primer used in the subsequent amplification reaction can be gene-specific or general (e.g., random hexamers). To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

Protocol 9: Rapid Amplification of 5´ cDNA Ends (5´-RACE)

This method is used to extend partial cDNA clones by amplifying the 5´ sequences of the corresponding mRNAs. The technique requires knowledge of a small region of sequence within the partial cDNA clone. During PCR, the thermostable DNA polymerase is directed to the appropriate target RNA by a single primer derived from the region of known sequence; the second primer required for PCR is complementary to a general feature of the target -- in the case of 5´-RACE, to a homopolymeric tail added (via terminal transferase) to the 3´ termini of cDNAs transcribed from a preparation of mRNA. This synthetic tail provides a primer-binding site upstream of the unknown 5´ sequence of the target mRNA. The products of the amplification reaction are cloned into a plasmid vector for sequencing and subsequent manipulation. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

Protocol 10: Rapid Amplification of 3´cDNA Ends (3´-RACE)

3´-RACE reactions are used to isolate unknown 3´ sequences or to map the 3´ termini of mRNAs onto a gene sequence. 3´-RACE requires knowledge of a small region of sequence within either the target RNA or a partial clone of cDNA. A population of mRNAs is transcribed into cDNA with an adaptor-primer consisting at its 3´ end of a poly(T) tract and at its 5´ end of an arbitrary sequence of 30-40 nucleotides. Reverse transcription is usually followed by two successive PCRs. The first is primed by a gene-specific sense oligonucleotide and an antisense primer complementary to the arbitrary sequence in the (dT)adaptor-primer. If necessary, the products of the first PCR can be used as templates for a second nested PCR, which is primed by a gene-specific sense oligonucleotide internal to the first, and a second antisense oligonucleotide complementary to the central region of the (dT)adaptor-primer. The products amplified in the second PCR are isolated from an agarose gel, cloned, and characterized. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

Protocol 11: Mixed Oligonucleotide-primed Amplification of cDNA (MOPAC)

In MOPAC, the amino-terminal and carboxy-terminal sequences of a peptide are used to design two redundant families of oligonucleotides encoding the amino- and carboxy-terminal sequences of the peptide. The primers are used either to amplify a segment of cDNA prepared by RT-PCR from a tissue known to express the protein or to amplify a segment of DNA from an established genomic or cDNA library. Because the length of the peptide is known, the size of the expected PCR product can be predicted exactly. After gel electrophoresis to resolve the amplification products, DNAs of the correct size are isolated, cloned, and sequenced. At least some of the clones should contain a DNA segment of the correct length that specifies the sequence of the starting peptide. Once identified, the entire cloned segment or the unique sequence lying between the two oligonucleotide primers is used as a probe to screen a cDNA library. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

Protocol 12: Rapid Characterization of DNAs Cloned in Prokaryotic Vectors

In this method, sequences cloned in standard bacteriophage or plasmid vectors are amplified in PCRs containing primers targeted to flanking vector sequences. The amplified fragments can be analyzed by gel electrophoresis, DNA sequencing, and/or restriction mapping. Many colonies or plaques can be assayed simultaneously. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

Protocol 13: Long PCR

The following protocol can be used to amplify DNA up to 25 kb in length. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

Protocol 14: Inverse PCR

Inverse PCR is used to amplify and clone unknown DNA that flanks one end of a known DNA sequence and for which no primers are available. The technique involves digestion by a restriction enzyme of a preparation of DNA containing the known sequence and its flanking region. The individual restriction fragments (many thousands in the case of total mammalian genomic DNA) are converted into circles by intramolecular ligation, and the circularized DNA is then used as a template in the PCR. The unknown sequence is amplified by two primers that bind specifically to the known sequence and point in opposite directions. The product of the amplification reaction is a linear DNA fragment containing a single site for the restriction enzyme originally used to digest the DNA. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

Protocol 15: Quantitative PCR

Quantitative PCR involves co-amplification of two templates: a constant amount of a preparation containing the desired target sequence and serial dilutions of a reference template that is added in known amounts to a series of amplification reactions. The concentration of the target sequence is determined by simple interpolation into a standard curve. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

Protocol 16: Differential Display-PCR

This method uses PCR to amplify and display many cDNAs derived from the mRNAs of a given cell or tissue type. The method relies on two different types of synthetic oligonucleotides: anchored antisense primers and arbitrary sense primers. A typical anchored primer is complementary to approx. 13 nucleotides of the poly(A) tail of mRNA and the adjacent two nucleotides of the transcribed sequence. Anchored primers therefore anneal to the junction between the poly(A) tail and the 3´-untranslated region of mRNA templates, from where they can prime synthesis of first-strand cDNA. A second primer, an arbitrary sequence of approx. 10 nucleotides, is then added to the reaction mixture, and double-stranded cDNAs are produced by conventional PCR, carried out at low stringency. The products of the amplification reaction are separated by electrophoresis through a denaturing polyacrylamide gel and visualized by autoradiography. By comparing the banding patterns of cDNA products derived from two different cell types, or from the same cell type grown under different conditions, it is sometimes possible to identify the products of differentially expressed genes. Bands of interest can then be recovered from the gel, amplified further, and cloned and/or used as probes to screen northern blots, cDNA libraries, etc. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

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Protocol 1

The Basic Polymerase Chain Reaction

This protocol describes how to amplify a segment of double-stranded DNA in a chain reaction catalyzed by a thermostable DNA polymerase. It is the foundation for all subsequent variations of the polymerase chain reaction. For details on primer design and troubleshooting amplification reactions, please see Tables 8-3, 8-4, and 8-5 on pages 8.14, 8.23, and 8.24, respectively, in the print version of the manual.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- ⚠ Chloroform
- dNTP solution (20 mM) containing all four dNTPs (pH 8.0)

Enzymes and Buffers

Thermostable DNA polymerase

Nucleic Acids and Oligonucleotides

Forward primer (20 µM) in H_2O

Reverse primer (20 µM) in H_2O

Template DNA.
Dissolve template DNA in 10 mM Tris-Cl (pH 7.6) containing a low concentration of EDTA (<0.1 mM) at the following concentrations: mammalian genomic DNA, 100 µg/ml; yeast genomic DNA, 1 µg/ml; bacterial genomic DNA, 0.1 µg/ml; and plasmid DNA, 1-5 ng/ml.

Additional Reagents

Step 4 of this protocol may require the reagents listed in Chapter 6, Protocol 10 , and/or Chapter 12, Protocol 6.

METHOD

1. In a sterile 0.5-ml microfuge tube, amplification tube, or the well of a sterile microtiter plate, mix in the following order:

10x amplification buffer	5 µl
20 mM solution of four dNTPs (pH 8.0)	1 µl
20 µM forward primer	2.5 µl
20 µM reverse primer	2.5 µl
1-5 units/µl thermostable DNA polymerase	1-2 units
H_2O	28-33 µl
template DNA	5-10 µl
Total volume	50 µl

The table below provides standard reaction conditions for PCR.

Mg^{2+}	KCl	dNTPs	Primers	DNA polymerase	Template DNA
1.5 mM	50 mM	200 µM	1 µM	1-5 units	1 pg to 1 µg

The amount of template DNA required varies according to the complexity of its sequence. In the case of mammalian DNA, up to 1.0 µg is used per reaction. Typical amounts of yeast, bacterial, and plasmid DNAs used per reaction are 10 ng, 1 ng, and 10 pg, respectively.

2. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Alternatively, place a bead of wax into the tube if using a hot start protocol. Place the tubes or the microtiter plate in the thermal cycler.
3. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed below.

Cycle Number	Denaturation	Annealing	Polymerization
30 Cycles	30 sec at 94°C	30 sec at 55°C	1 min at 72°C
Last cycle	1 min at 94°C	30 sec at 55°C	1 min at 72°C

Times and temperatures may need to be adapted to suit the particular reaction conditions.
Polymerization should be carried out for 1 minute for every 1000 bp of length of the target DNA.

4. Withdraw a sample (5-10 µl) from the test reaction mixture and the four control reactions, analyze them by electrophoresis through an agarose or polyacrylamide gel, and stain the gel with ethidium bromide or SYBR Gold to visualize the DNA.
A successful amplification reaction should yield a readily visible DNA fragment of the expected size. The identity of the band can be confirmed by DNA sequencing (please see Chapter 12, Protocol 6), Southern hybridization (please see Chapter 6, Protocol 10), and/or restriction mapping.
If all has gone well, lanes of the gel containing samples of the two positive controls (Tubes 1 and 2) and the template DNA under test should contain a prominent band of DNA of the appropriate molecular weight. This band should be absent from the lanes containing samples of the negative controls (Tubes 3 and 4).
5. If mineral oil was used to overlay the reaction (Step 2), remove the oil from the sample by extraction with 150 µl of chloroform.
The aqueous phase, which contains the amplified DNA, will form a micelle near the meniscus. The micelle can be transferred to a fresh tube with an automatic micropipette.
IMPORTANT *Do not attempt chloroform extractions in microtiter plates. The plastic used in these plates is not resistant to organic solvents.*

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Protocol 2

Purification of PCR Products in Preparation for Cloning

The residual enzymatic activity of thermostable DNA polymerases that survive the rigors of PCR can compromise subsequent enzymatic reactions. This protocol describes how to use proteinase K to destroy thermostable enzymes and to purify amplified DNA in preparation for cloning.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)

⚠ Chloroform

Ethanol

⚠ Phenol:chloroform (1:1, v/v)

TE (pH 8.0)

Enzymes and Buffers

Proteinase K (20 mg/ml)

Nucleic Acids and Oligonucleotides

Amplified DNA from polymerase chain reactions

METHOD

1. Pool up to eight PCRs (400 µl) containing 1 µg of the desired amplification product.
   If nonspecific amplification products are present at significant levels (e.g., are detectable by gel electrophoresis), purify the desired product by electrophoresis through low-melting-temperature agarose before proceeding (please see Chapter 5, Protocol 6).
   If mineral oil was used to prevent evaporation during PCR, centrifuge the pooled samples briefly and transfer the lower (aqueous) phase to a fresh microfuge tube.
2. Add 0.2 volume of 5x proteinase K buffer and proteinase K to a final concentration of 50 µg/ml. Incubate the mixture for 60 minutes at 37°C.
3. Inactivate the proteinase K by heating to 75°C for 20 minutes.
4. Extract the reaction mixture once with phenol:chloroform and once with chloroform.
5. Add 0.2 volume of 10 M ammonium acetate and 2.5 volumes of ethanol. Mix the solution well and store it for 30 minutes at 4°C.
6. Recover the DNA by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge. Discard the supernatant and then wash the pellet with 70% ethanol. Centrifuge again, remove the supernatant, and then allow the DNA to dry.
   The DNA may be further purified by chromatography or by gel electrophoresis. This step is recommended when primers have been used to add restriction sites to the ends of the amplified DNA. Unused primers and primer-dimers should be removed before digesting the DNA with the appropriate restriction enzymes (please see Chapter 8, Protocol 3).
7. Dissolve the pellet in TE (pH 8.0). Assume that the recovery of amplified DNA is 50-80% and dissolve the DNA in TE (pH 8.0) at an estimated concentration of 25 µg/ml (25 ng/µl).
8. Analyze approx. 25 ng of the purified DNA by agarose-ethidium bromide gel electrophoresis, using markers of an appropriate size. Check that the amplified band fluoresces with the intensity expected of approx. 25 ng of DNA.

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Protocol 3

Removal of Oligonucleotides and Excess dNTPs from Amplified DNA by Ultrafiltration

In this protocol, ultrafiltration through Centricon or Microcon concentrators is used to remove unused primers, primer-dimers, and NTPs from preparations of amplified DNA.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

Ethanol

Sodium acetate (3 M)

TE (pH 8.0)

Nucleic Acids and Oligonucleotides

Amplification reaction products (20-200 µl)

METHOD

1. Place 2 ml of TE (pH 8.0) in the reservoir chamber of a Centricon-100 unit. Carefully separate the amplification reaction products from the upper mineral oil layer by pipetting or by extraction with chloroform. Transfer the amplification reaction products to the reservoir chamber of the Centricon-100 unit.
2. Place the entire unit into an appropriate rotor of a preparative centrifuge (e.g., a fixed-angle rotor). Insert the microconcentrator into the centrifuge with the filtrate cup (translucent portion) toward the bottom of the rotor. Use a concentrator filled with an equivalent amount of fluid or a standard balance tube as a counterbalance.
   IMPORTANT *Do not touch the membrane with pipette or pipette tips when loading the microconcentrator.*
3. Centrifuge the loaded concentrator at 1000*g* for 30 minutes at a temperature between 4°C and 25°C.
4. Remove the concentrator from the centrifuge, and discard the filtrate cup. Invert the unit, and replace it in the centrifuge (i.e., the retentate tube should now be placed toward the bottom of the rotor). Centrifuge at 300-1000*g* for 2 minutes.
5. Remove the concentrator from the centrifuge; remove the retentate cup and discard the rest of the device. Transfer the fluid in the retentate cup to a fresh microfuge tube.
6. If necessary, precipitate the sample by adding one-tenth volume of 3 M sodium acetate and 2-3 volumes of ethanol. The amplified sample is now ready for subsequent manipulation (DNA sequencing and ligation).
   A single purification step is usually sufficient for most subsequent manipulation steps. If necessary, trace oligonucleotide primers can be further removed by performing a second 30-minute centrifugation step. At Step 4 above, empty the translucent filtrate cup, reassemble the device, and add another 2-ml aliquot of TE (pH 8.0) to the reservoir chamber. Repeat Steps 2 through 4.

REFERENCES

1. Krowczynska A.M. and Henderson M.B. 1992. Efficient purification of PCR products using ultrafiltration. *BioTechniques* 13:286-289.

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Protocol 4

Blunt-end Cloning of PCR Products

Incubation of a blunt-end ligation reaction in the presence of an excess amount of an appropriate restriction enzyme can dramatically increase the yield of recombinant plasmids. The role of the restriction enzyme is to cleave circular and linear concatemers at restriction sites that are re-formed when linear, blunt-ended plasmid molecules ligate to themselves. In almost all cases, ligation of the PCR product to the plasmid destroys the restriction site. The constant reclamation of vector molecules drives the equilibrium of the ligation reaction strongly in favor of the recombinants between vector and blunt-ended PCR product.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)

dNTP solution (2 mM) containing all four dNTPs

10x Universal KGB buffer

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Bacteriophage T4 DNA polymerase
Do not use the Klenow fragment of E. coli *DNA polymerase.*

Restriction endonuclease for cloning
The restriction enzyme should generate blunt ends, cleave the vector once, and not cleave the amplified DNA (please see Step 1).

Restriction endonucleases
Please see Step 4.

Nucleic Acids and Oligonucleotides

Closed circular plasmid DNA (50 µg/ml)
Choose a plasmid vector containing a single site for a restriction enzyme that generates blunt ends (e.g., Sma*I*, Srf*I, and* Eco*RV). The plasmid vector and its bacterial host should carry a blue/white screening system.*

Target DNA (25 µg/ml), amplified by PCR.
When the PCR mixture contains more than one or two bands of amplified DNA, purify the target fragment by electrophoresis through low melting/gelling temperature agarose (please see Chapter 5, Protocol 6). If not purified by gel electrophoresis, PCR-amplified DNA should be prepared for ligation by extraction with phenol:chloroform and ultrafiltration through a Centricon-100 filter (please see Chapter 8, Protocol 3).

Additional Reagents

Step 3 of this protocol requires the reagents listed in Chapter 1, Protocol 25 or Chapter 1, Protocol 26 and Chapter 1, Protocol 27

Step 4 of this protocol may require the reagents listed in Chapter 8, Protocol 12 .

Step 6 of this protocol requires the reagents listed in Chapter 12, Protocol 6 or Chapter 6, Protocol 10 .

METHOD

1. In a microfuge tube, mix the following in the order shown:

50 µg/ml closed circular plasmid vector	1 µl
25 µg/ml amplified target DNA	8 µl
10x universal KGB buffer	2 µl
H_2O (please see note below)	5 µl
10 mM ATP	1 µl
2 mM dNTPs	1 µl
restriction enzyme	2 units
T4 DNA polymerase	1 unit
T4 DNA ligase	3 units

Adjust the amount of H_2O added so that the final reaction volume is 20 µl.
Set up a control reaction that contains all of the reagents listed above except the amplified target DNA.
2. Incubate the ligation mixture for 4 hours at 22°C.
3. Dilute 5 µl of each of the two ligation mixtures with 10 µl of H_2O and transform a suitable strain of competent *E. coli* to antibiotic resistance as described in Chapter 1, Protocol 25 or Chapter 1, Protocol 26 . Plate the transformed cultures on media containing IPTG and X-gal (please see Chapter 1, Protocol 27) and the appropriate antibiotic.
4. Calculate the number of colonies obtained from each of the ligation mixtures. Pick a number of white colonies obtained by transformation with the ligation reaction containing the target DNA. Confirm the presence of the amplified fragment by (i) isolating the plasmid DNAs and digesting them with restriction enzymes whose sites flank the insert in the multiple cloning site or (ii) colony PCR (Chapter 8, Protocol 12).
5. Fractionate the restricted DNA by electrophoresis through an agarose gel using appropriate DNA size markers. Measure the size of the cloned fragments.
6. Confirm the identity of the cloned fragments by DNA sequencing, restriction mapping, or Southern hybridization.

REFERENCES

1. Chuang S.E., Wang K.C., and Cheng A.L. 1995. Single-step direct cloning of PCR products. *Trends Genet* 11:7-8.
2. Liu Z.G. and Schwartz L.M. 1992. An efficient method for blunt-end ligation of PCR products. *BioTechniques* 12:28-30.
3. Weiner M.P. 1993. Directional cloning of blunt-ended PCR products. *BioTechniques* 15:502-505.

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Protocol 5

Cloning PCR Products into T Vectors

This method of direct cloning takes advantage of the unpaired adenosyl residue added to the 3' terminus of amplified DNAs by *Taq* and other thermostable polymerases.

MATERIALS

CAUTION: Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Nucleic Acids and Oligonucleotides

Target DNA (25 µg/ml), amplified by PCR

When the PCR mixture contains more than one or two bands of amplified DNA, purify the target fragment by electrophoresis through low melting/gelling temperature agarose (please see Chapter 5, Protocol 6). If not purified by gel electrophoresis, PCR-amplified DNA should be prepared for ligation by extraction with phenol:chloroform and ultrafiltration through a Centricon-100 filter (please see Chapter 8, Protocol 3).

Vectors

T vector

Additional Reagents

Step 3 of this protocol requires the reagents listed in Chapter 1, Protocol 25 or Chapter 1, Protocol 26 and Chapter 1, Protocol 27

Step 6 of this protocol requires the reagents listed in Chapter 12, Protocol 6 or Chapter 6, Protocol 10 .

METHOD

1. In a microfuge tube, set up the following ligation mixture:

25 µg/ml amplified target DNA	1 µl
T-tailed plasmid	20 ng
10x ligation buffer	1 µl
bacteriophage T4 DNA ligase	3 units
H_2O	to 10 µl

   If necessary, add ATP to a final concentration of 1 mM. A 1:5 molar ratio of vector:amplified DNA fragment is recommended.
   Set up a control reaction that contains all the reagents listed above except the amplified target DNA.
2. Incubate the ligation mixture for 4 hours at 14°C.
3. Dilute 5 µl of each of the two ligation mixtures with 10 µl of H_2O and transform a suitable strain of competent *E. coli* to antibiotic resistance as described in Chapter 1, Protocol 25 or Chapter 1, Protocol 26. Plate the transformed cultures on media containing IPTG and X-gal (please see Chapter 1, Protocol 27) and the appropriate antibiotic.
4. Calculate the number of colonies obtained from each of the ligation mixtures. Pick a number of white colonies obtained by transformation with the ligation reaction containing the target DNA. Confirm the presence of the amplified fragment by (i) isolating the plasmid DNAs and digesting them with restriction enzymes whose sites flank the insert in the multiple cloning site or (ii) colony PCR (Chapter 8, Protocol 12)
   The ratio of blue:white colonies varies between 1:5 and 2:1.
5. Fractionate the restricted DNA by electrophoresis through an agarose gel using appropriate DNA size markers. Measure the size of the cloned fragments.
6. Confirm the identity of the cloned fragments by DNA sequencing, restriction mapping, or Southern hybridization.

REFERENCES

1. Holton T.A. and Graham M.W. 1991. A simple and efficient method for direct cloning of PCR products using ddT-tailed vectors. *Nucleic Acids Res.* 19:1154.
2. Marchuk D., Drumm M., Saulino A., and Collins F.S. 1991. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Res.* 19:1156.

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Protocol 6

Cloning PCR Products by Addition of Restriction Sites to the Termini of Amplified DNA

Pairs of oligonucleotide primers used in PCR are often designed with restriction sites in their 5' regions. In many cases, the sites are different in the two primers. In this case, amplification generates a target fragment whose termini now carry new restriction sites that can be used for directional cloning into plasmid vectors. The purified fragment and the vector are digested with the appropriate restriction enzymes, ligated together, and transformed into *E. coli.*

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

EDTA (0.5 M, pH 8.0)

Ethanol

⚠ Phenol:chloroform (1:1, v/v)

Sodium acetate (3 M, pH 5.2)

TE (pH 7.5)

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Restriction endonucleases

Nucleic Acids and Oligonucleotides

Forward primer (20 µM) in H_2O

Reverse primer (20 µM) in H_2O

Target DNA

Vectors

Plasmid DNA cleaved with the appropriate restriction enzyme(s) and purified by gel electrophoresis
If the linearized plasmid DNA carries compatible termini that can be ligated to each other, use alkaline phosphatase to remove the 5´-phosphate groups and suppress self-ligation (please see Chapter 1, Protocol 20).

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 8, Protocol 1 .

Step 10 of this protocol requires the reagents listed in Chapter 1, Protocol 25 or Chapter 1, Protocol 26 and Chapter 1, Protocol 27

Step 11 of this protocol may require the reagents listed in Chapter 8, Protocol 12 .

Step 13 of this protocol requires the reagents listed in Chapter 12, Protocol 6 or Chapter 6, Protocol 10 .

METHOD

1. Design and synthesize the appropriate oligonucleotide primers. Use these forward and reverse primers to set up and carry out four identical amplification reactions (50-µl volume) to amplify the target fragment (please see Chapter 8, Protocol 1). Combine the four PCRs, which, in aggregate, should contain 200-500 ng of the desired amplification product.
   Design forward and reverse primers carrying the appropriate restriction sites. The 3´ end of each primer should be an exact complement of approx. 15 consecutive bases at a selected site in the target DNA. The 5´ terminus of each primer serves as a clamp to hold together the termini of the amplified DNA and to provide a landing site for the restriction enzyme. The clamp should be 3-10 nucleotides in length. The mid-portion of the primer contains the recognition site for the restriction enzyme. Each primer should therefore be 24-31 nucleotides in length and contain approximately equal numbers of the four bases, with a balanced distribution of G and C residues and a low propensity to form stable secondary structures.
2. If the PCR mixture contains more than one or two bands of amplified DNA, purify the target fragment by electrophoresis through low melting/gelling temperature agarose (please see Chapter 5, Protocol 6). If not purified by gel electrophoresis, prepare PCR-amplified DNA for ligation by extraction with phenol:chloroform and ultrafiltration through a Centricon-100 filter (please see Chapter 8, Protocol 3). Dissolve in TE (pH 7.5) at a concentration of 25 µg/ml.
3. In a reaction volume of 20 µl, digest approx. 100 ng of purified PCR product with 1.0-2.0 units of the relevant restriction enzyme(s). Incubate the reactions for 1 hour at the optimum temperature for digestion.
4. At the end of the digestion, adjust the volume to 100 µl with H_2O, and add 0.5 M EDTA to a final concentration of 5 mM. Extract once with phenol:chloroform and once with chloroform.
5. Transfer the aqueous phase to a fresh tube and add 3 M sodium acetate (pH 5.2) to achieve a final concentration of 0.3 M. Add 2 volumes of ethanol. Store the mixture for 30 minutes at 0°C.
6. Recover the precipitated DNA by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge. Discard the supernatant, and then wash the pellet with 70% ethanol. Centrifuge again, remove the supernatant, and allow the DNA to dry.
7. Dissolve the DNA in 10 µl of H_2O.
8. In a microfuge tube, set up the following ligation mixture:

25 µg/ml amplified target DNA	1.0 µl
plasmid DNA	20 ng
10x ligation buffer	1.0 µl
T4 DNA ligase	1 unit
H_2O	to 10 µl

   If necessary, add ATP to a final concentration of 1 mM.
   When directional cloning is used, the ligation mixture should contain an approx. 1:1 molar ratio of purified target DNA to cleaved plasmid vector.
   Set up a control reaction that contains all the reagents listed above except the amplified target DNA.
9. Incubate the ligation mixtures for 4 hours at 16°C.
10. Dilute 5 µl of each of the two ligation mixtures with 10 µl of H_2O and transform a suitable strain of competent *E. coli* to antibiotic resistance as described in Chapter 1, Protocol 25 or Chapter 1, Protocol 26 . Plate the transformed cultures on media containing IPTG and X-gal (please see Chapter 1, Protocol 27) and the appropriate antibiotic.
11. Calculate the number of colonies obtained from each of the ligation mixtures. Pick a number of white colonies obtained by transformation with the ligation reaction containing the target DNA. Confirm the presence of the amplified fragment

by (i) isolating the plasmid DNAs and digesting them with restriction enzymes whose sites flank the insert in the multiple cloning site or (ii) colony PCR (Chapter 8, Protocol 12).

In different experiments, the ratio of blue:white colonies can vary between 1:5 and 2:1.

12. Fractionate the restricted DNA by electrophoresis through an agarose gel using appropriate DNA size markers. Measure the size of the cloned fragments.
13. Confirm the identity of the cloned fragments by DNA sequencing, restriction mapping, or Southern hybridization.

REFERENCES

1. Kaufman D.L. and Evans G.A. 1990. Restriction endonuclease cleavage at the termini of PCR products. *BioTechniques* 9:304-306.
2. Scharf S.J., Horn G.T., and Erlich H.A. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science* 233:1076-1078.

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Protocol 7

Genetic Engineering with PCR

This method describes how to modify the termini of PCR products by introducing restriction sites and other features. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- dNTP solution (20 mM) containing all four dNTPs (pH 8.0)

Enzymes and Buffers

Restriction endonucleases

Thermostable DNA polymerase

Nucleic Acids and Oligonucleotides

Oligonucleotide primer 1 (10 µM) in TE (pH 8.0)

Oligonucleotide primer 2 (10 µM) in TE (pH 8.0)

Positive control DNA

Template DNA
The template DNA could be a cloned gene or cDNA in a vector or genomic DNA.

Vectors

Vector DNA cleaved with the appropriate restriction enzyme(s) and purified by gel electrophoresis.
If the restricted vector DNA carries compatible termini that can be ligated to each other, use alkaline phosphatase to remove the 5´-phosphate groups and suppress self-ligation (please see Chapter 1, Protocol 20).

Additional Reagents

Step 5 of this protocol requires the reagents listed in Chapter 5, Protocol 2, Chapter 12, Protocol 6, or Chapter 6, Protocol 10 .

Step 6 of this protocol requires the reagents listed in Chapter 8, Protocol 3 .

Step 7 of this protocol requires the reagents listed in Chapter 8, Protocol 6 .

METHOD

1. Design and synthesize the appropriate oligonucleotide primers for the end modifications desired.
   In this example, two primers derived from the 5´ sequence (5´ dATCATATGGCTCTGGATGA ACTGTGCCTGCTGGACATGCT 3´) and the 3´ sequence (5´ dATAAGCTTTTATTAAGACAGAC TCAGCTCATGGGAGGCAA 3´) of the starting cDNA template are used to introduce an NdeI *(CATATG) site at the 5´ end of the cDNA and to change several codons to those preferentially used in* E. coli*. The underlined nucleotides indicate differences between the oligonucleotide primers and the cDNA template. The number of perfectly matched nucleotides required at the 3´ end of the oligonucleotide primers for a successful amplification has not been rigorously determined; however, eight to ten generally work well.*
2. In a sterile 0.5-ml microfuge tube, amplification tube, or the well of a sterile microtiter plate, mix in the following order:

100 ng template DNA	10 µl
10x amplification buffer	5 µl
20 mM solution of four dNTPs	5 µl
10 µM primer 1 (50 pmoles)	5 µl
10 µM primer 2 (50 pmoles)	5 µl
1-2 units of thermostable DNA polymerase	1 µl
H_2O	to 50 µl

   Set up two control reactions. In one reaction, include all of the above additions, except the template DNA. In the other reaction, include a DNA template that has previously yielded a positive result in the PCR. Carry the controls through all subsequent steps of the protocol.
3. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. This prevents evaporation of the samples during repeated cycles of heating and cooling. Alternatively, place a bead of wax into the tube if using hot start PCR. Place the tubes or the microtiter plate in the thermal cycler.
4. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing/Polymerization
20 cycles	1 min at 94°C	3 min at 68°C
Last Cycle	1 min at 94°C	15 min at 68°C

   Times and temperatures may need to be adapted to suit the particular reaction conditions.
5. Analyze 5-10% of the amplification on an agarose or polyacrylamide gel and estimate the concentration of the amplified target DNA. Include DNA markers of an appropriate size. Stain the gel with ethidium bromide or SYBR Gold to visualize the DNA.
   A successful amplification reaction should yield a readily visible DNA fragment of the expected size. The identity of the band can be confirmed by DNA sequencing (please see Chapter 12), Southern hybridization (please see Chapter 6), and/or restriction mapping.
6. If mineral oil was used to overlay the reaction (Step 3), remove the oil from the sample by extraction with 150 µl of chloroform.
   The aqueous phase, which contains the amplified DNA, will form a micelle near the meniscus. The micelle can be transferred to a fresh tube with an automatic micropipette.
   IMPORTANT *Do not attempt chloroform extractions in microtiter plates. The plastic used in these plates is not resistant to organic solvents.*
7. For subsequent cloning, cleave the DNA fragment at the restriction sites placed (or located) at the 5´ ends of the primers (with *Nde*I and *Hin*dIII in the above example). Purify the digested fragment using gel electrophoresis or ultrafiltration (please see Chapter 8, Protocol 3).
8. Set up the appropriate ligation reaction with the desired vector DNA. Use a molar ratio of insert to vector of 3:1 in the ligation reactions.

Because of the error rate of thermostable DNA polymerases, it is very important to verify the sequence of the amplified DNA after cloning into the expression vector.

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Protocol 8

Amplification of cDNA Generated by Reverse Transcription of mRNA

In this method, an oligodeoxynucleotide primer hybridized to mRNA is extended by an RNA-dependent DNA polymerase to create a cDNA copy that can be amplified by PCR. Depending on the purpose of the experiment, the primer for first-strand cDNA synthesis can be specifically designed to hybridize to a particular target gene, or a general primer such as oligo(dT) can be used to prime cDNA synthesis from essentially all mammalian mRNAs. Similarly, the reverse primer used in the subsequent amplification reaction can be gene-specific or general (e.g., random hexamers). To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

10x Amplification buffer

⚠ Chloroform

dNTP solution (20 mM) containing all four dNTPs (pH 8.0)

Ethanol

$MgCl_2$ (50 mM)

⚠ Phenol:chloroform (1:1, v/v)

Placental RNase inhibitor (20 units/µl)

Enzymes and Buffers

Reverse transcriptase (RNA-dependent DNA polymerase)

Thermostable DNA-dependent DNA polymerase
Taq *DNA polymerase is the standard and appropriate enzyme for the amplification stage of most forms of RT-PCR. However, where elongation from 3´-mismatched primers is suspected, a thermostable DNA polymerase with 3´->5´ proofreading activity may be preferred.*

Nucleic Acids and Oligonucleotides

Exogenous reference RNA
Please see Step 2.

Gene-specific oligonucleotide (20 µM) in H_2O (20 pmoles/µl)
The gene-specific oligonucleotide should be complementary to a known sequence in the target mRNA.

$Oligo(dT)_{12-18}$ (100 µg/ml) in TE (pH 8.0)

Random hexanucleotides (1 mg/ml) in TE (pH 8.0)

Sense and antisense oligonucleotide primers for amplification of cDNA by PCR
The primer used to generate cDNA may also be used as the antisense primer in the amplification stage of standard RT-PCR. However, the specificity of amplification can be improved by using an antisense primer that binds to an upstream sequence in the target transcript. Both sense and antisense primers are gene-specific synthetic oligonucleotides, which should be 20-30 nucleotides in length and contain approximately equal numbers of the four bases, with a balanced distribution of G and C residues, and a low propensity to form stable secondary structures.

Template RNA
The template may be total RNA (100 µg/ml) in H_2O or poly(A)$^+$ RNA (10 µg/ml) in H_2O. Total RNA extracted from cells with chaotropic agents is generally the template of choice for RT-PCR for mRNAs that are expressed at moderate to high abundance. Poly(A)$^+$ is preferred as a template for all forms of RT-PCR when the target mRNA is expressed at low abundance.

Additional Reagents

Step 8 of this protocol requires the reagents listed in Chapter 5, Protocol 2 , Chapter 12, Protocol 6, or Chapter 6, Protocol 10 .

Step 10 of this protocol requires the reagents listed in Chapter 8, Protocol 3 and Chapter 8, Protocol 4, Chapter 8, Protocol 5 , or Chapter 8, Protocol 6 .

METHOD

1. Transfer 1 pg to 100 ng of poly(A)$^+$ mRNA or 10 pg to 1 µg of total RNA to a fresh microfuge tube. Adjust the volume to 10 µl with H_2O. Denature by heating at 75°C for 5 minutes, followed by chilling on ice.
2. To the denatured RNA add:

10x amplification buffer	2 µl
20 mM solution of four dNTPs (pH 8.0)	1 µl
primers (optimized, please see below)	1 µl
approx. 20 units/µl placental RNase inhibitor	1 µl
50 mM $MgCl_2$	1 µl
100-200 units/µl reverse transcriptase	1 µl
H_2O	to 20 µl

Incubate the reaction for 60 minutes at 37°C.
Depending on the experiment, $oligo(dT)_{12-18}$, random hexanucleotides, or gene-specific antisense oligonucleotides can be used as primers for synthesis of first-strand cDNA.
The optimum ratio of primer to template should be ascertained empirically for each preparation of RNA. As a starting point for optimization, we recommend adding varying amounts of primers to 20-µl reactions:

synthetic oligonucleotide complementary to the target RNA:	5-20 pmoles
$oligo(dT)_{12-18}$:	0.1-0.5 µg
random hexanucleotides:	1-5 µg

Set up three negative control reactions. In one reaction, include all components of the first-strand reaction except the RNA template. In another reaction, include all components except the reverse transcriptase. Omit primers from the third reaction. Carry the controls through all subsequent steps of the protocol.
3. Inactivate the reverse transcriptase and denature the template-cDNA complexes by heating the reaction to 95°C for 5 minutes or by phenol extraction and ethanol precipitation.

4. Adjust the reaction mixture so that it contains 20 pmoles of the sense and antisense primers.
5. Add to the reaction mixture:

1x amplification buffer (or volume required to bring reaction mixture to 99 µl)	77 µl
1-2 units thermostable DNA polymerase	1 µl

6. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Alternatively, place a bead of paraffin wax into the tube if using a hot start protocol. Place the tubes or the microtiter plate in the thermal cycler.
7. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
35 Cycles	45 sec at 94°C	45 sec at 55°C	1 min 15 sec at 72°C
Last cycle	1 min at 94°C	45 sec at 55°C	1 min 15 sec at 72°C

Times and temperatures may need to be adapted to suit the particular reaction conditions.
Polymerization should be carried out for 1 minute for every 1000 bp of length of the target DNA.

8. Withdraw a sample (5-10 µl) from the test reaction mixture and the four control reactions and analyze them by electrophoresis through an agarose or polyacrylamide gel. Include DNA markers of an appropriate size. Stain the gel with ethidium bromide or SYBR Gold to visualize the DNA.
A successful amplification reaction should yield a readily visible DNA fragment of the expected size. The identity of the band can be confirmed by DNA sequencing (please see Chapter 12, Protocol 6), Southern hybridization (please see Chapter 6, Protocol 10), and/or restriction mapping.
If no product is visible after 30 cycles of amplification, add fresh Taq *polymerase and continue the amplification reaction for a further 15-20 cycles.*
9. If mineral oil was used to overlay the reaction (in Step 6), remove the oil from the sample before cloning by extraction with 150 µl of chloroform.
The aqueous phase, which contains the amplified DNA, will form a micelle near the meniscus. The micelle can then be transferred to a fresh tube with an automatic micropipette.
IMPORTANT *Do not attempt chloroform extractions in microtiter plates. The plastic used in these plates is not resistant to organic solvents.*
10. Clone the amplified products into an appropriately prepared vector by any of the methods described in Chapter 8, Protocol 4 , Chapter 8, Protocol 5 , or Chapter 8, Protocol 6 . Before cloning, separate the amplified DNA from the residual thermostable DNA polymerase and dNTPs (please see Chapter 8, Protocol 3). The DNA can then be ligated to a blunt-ended vector or a T vector, or it can digested with restriction enzymes and ligated to a vector with compatible termini.

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Protocol 9

Rapid Amplification of 5´ cDNA Ends (5´-RACE)

This method is used to extend partial cDNA clones by amplifying the 5´ sequences of the corresponding mRNAs. The technique requires knowledge of a small region of sequence within the partial cDNA clone. During PCR, the thermostable DNA polymerase is directed to the appropriate target RNA by a single primer derived from the region of known sequence; the second primer required for PCR is complementary to a general feature of the target -- in the case of 5´-RACE, to a homopolymeric tail added (via terminal transferase) to the 3´ termini of cDNAs transcribed from a preparation of mRNA. This synthetic tail provides a primer-binding site upstream of the unknown 5´ sequence of the target mRNA. The products of the amplification reaction are cloned into a plasmid vector for sequencing and subsequent manipulation. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- ⚠ Chloroform
- dNTP solution (20 mM) containing all four dNTPs (pH 8.0)

  Placental RNase inhibitor (20 units/µl)
- TE (pH 7.6)

Enzymes and Buffers

- 5x Reverse transcriptase buffer

  Reverse transcriptase (RNA-dependent DNA polymerase)

  Terminal deoxynucleotidyl transferase (terminal transferase)
- 5x Terminal transferase buffer

  Thermostable DNA polymerase

  Taq *DNA polymerase is the standard and appropriate enzyme for the amplification stage of most forms of RT-PCR. However, where elongation from 3´-mismatched primers is suspected, a thermostable DNA polymerase with 3´->5´ proofreading activity may be preferred.*

Nucleic Acids and Oligonucleotides

Adaptor-primer (10 µM) (5´ GACTCGAGTCGACATCG 3´) in H_2O (10 pmoles/µl)

The adaptor-primer is used in conjunction with a gene-specific sense primer to amplify a particular target cDNA.

$(dT)_{17}$-Adaptor-primer (10 µM) (5´ GACTCGAGTCGACATCGA$(T)_{17}$ 3´) in H_2O (10 pmoles/µl)

The $(dT)_{17}$-adaptor-primer binds to the poly(A)$^+$ tract added to the 5´ terminus of cDNAs by terminal transferase. In the example given in this protocol, one of the termini of the amplified DNAs becomes equipped with recognition sites for Xho*I,* Sal*I,* Acc*I,* Hinc*II, and* Cla*I restriction enzymes.*

Gene-specific antisense oligonucleotide primers (10 µM) in H_2O (10 pmoles/µl)

The gene-specific antisense primers should be complementary to the known sequence of the target mRNA, should be 20-30 nucleotides in length, and should contain approximately equal numbers of the four bases, with a balanced distribution of G and C residues and a low propensity to form stable secondary structures. Gene-specific primer 1 is used in the reverse transcriptase reaction (Step 2) to prime synthesis of gene-specific first-strand cDNA. Gene-specific primer 2 is complementary to a sequence in the target mRNA that is 5´ to primer 1 and is used in the amplification stage of the reaction. The presence of restriction sites simplifies cloning of the amplified cDNAs.

Random hexanucleotides (1 mg/ml) in TE (pH 8.0) (optional)

In an emergency, random hexanucleotides can be used in place of the gene-specific antisense oligonucleotide to prime synthesis of cDNA. Subsequent use of a gene-specific sense primer in the PCR phase of the 5´-RACE reaction generates the desired product.

Template RNA

The template may be total RNA (100 µg/ml) in H_2O or poly(A)$^+$ RNA (10 µg/ml) in H_2O. Total RNA extracted from cells with chaotropic agents is generally the template of choice for RT-PCR for mRNAs that are expressed at moderate to high abundance. Poly(A)$^+$ is preferred as a template for all forms of RT-PCR when the target mRNA is expressed at low abundance.

Additional Reagents

Step 9 of this protocol requires the reagents listed in Chapter 5, Protocol 2 , Chapter 12, Protocol 6, or Chapter 6, Protocol 10 .

Step 11 of this protocol requires the reagents listed in Chapter 8, Protocol 3 and Chapter 8, Protocol 4, Chapter 8, Protocol 5 , or Chapter 8, Protocol 6 .

METHOD

1. Transfer 1 pg to 100 ng of poly(A)$^+$ mRNA or 10 pg to 1 µg of total RNA to a fresh microfuge tube. Adjust the volume to 9 µl with H_2O. Denature the RNA by heating for 5 minutes at 75°C, followed by rapid chilling on ice.
2. To the denatured RNA add:

5x reverse transcriptase buffer	4 µl
20 mM solution of four dNTPs (pH 8.0)	1 µl
10 µM gene-specific antisense primer 1	4 µl
approx. 20 units/µl placental RNase inhibitor	1 µl
100-200 units/µl reverse transcriptase	1 µl
H_2O	to 20 µl

Incubate the reaction for 60 minutes at 37°C. Set up three negative control reactions. In one reaction, include all of the components of the first-strand reaction, except the RNA template. In another reaction, include all of the components, except the reverse transcriptase. Omit primer from the third reaction. Carry the controls through all subsequent steps of the protocol.

Total *cDNA synthesis can be estimated from the proportion of trichloroacetic acid (TCA)-precipitable radioactivity incorporated in reverse transcription reactions supplemented with 10-20 µCi of [^{32}P]dCTP (sp. act. 3000 Ci/mmole). Be sure to set up a control reaction that contains no oligonucleotide primer.*

The success or failure of 5´-RACE is determined here. If the reverse transcription step works efficiently, the chance of

Isolating clones that contain the 5´-terminal sequences of the target mRNA is high. On the other hand, no amount of work on the later steps of the protocol can compensate for ineffective reverse transcription. It is therefore worthwhile to take the time to optimize the reverse transcriptase reaction by determining the optimum ratio of primer to template for each preparation of RNA and by varying the concentration of Mg^{2+} in the reaction.

3. Remove excess primer by diluting the reverse transcriptase reaction to a final volume of 2 ml with H_2O and then applying the solution to a Centricon-100 microconcentrator (please see Chapter 8, Protocol 3). Centrifuge the solution at 500-1100*g* (2000-3000 rpm in a Sorvall SS-34 rotor) for 20 minutes at a temperature between 4°C and 25°C. Repeat the dilution step and centrifuge again. Transfer the retentate to a fresh 0.5-ml microfuge tube and reduce the volume to approx. 10 µl in a rotary vacuum evaporator.
   Alternatively, remove the dNTPs and unused primers by precipitating the cDNA twice in 2.5 M ammonium acetate and 3 volumes of ethanol.
4. To the cDNA in a volume of 10 µl add:

5x terminal transferase buffer	4 µl
1 mM dATP	4 µl
terminal transferase	10-25 units

   Incubate the reaction for 15 minutes at 37°C.
   The tailing reaction can be optimized by setting up mock reactions containing approx. 50 ng of a control DNA fragment, 100-200 nucleotides in length. After tailing, the size of the fragment should increase by 20-100 nucleotides as measured by electrophoresis through a 1% neutral agarose gel.
5. Inactivate the terminal transferase by heating the reaction for 3 minutes at 80°C. Dilute the dA-tailed cDNA to a final volume of 1 ml with TE (pH 7.6).
6. In a sterile 0.5-ml microfuge tube, amplification tube, or the well of a sterile microtiter plate, set up a series of PCRs containing the following:

diluted cDNA	0-20 µl
10x amplification buffer	5 µl
20 mM solution of four dNTPs	5 µl
10 µM $(dT)_{17}$-adaptor-primer (16 pmoles)	1.6 µl
10 µM adaptor-primer (32 pmoles)	3.2 µl
10 µM gene-specific primer 2 (32 pmoles)	3.2 µl
1-2 units of thermostable DNA polymerase	1 µl
H_2O	to 50 µl

   It is essential to set up a series of amplification reactions to find the amount of tailed cDNA that generates the largest quantity of amplified 5´ termini. The control reaction containing no cDNA template serves as a control for contamination.
7. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Alternatively, place a bead of paraffin wax into the tube if using a hot start protocol. Place the tubes or the microtiter plate in the thermal cycler.
8. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
First Cycle	5 min at 94°C	5 min at 50-58°C	40 min at 72°C
Subsequent cycles (30)	40 sec at 94°C	1 min at 50-58°C	3 min at 72°C
Last cycle	40 sec at 94°C	1 min at 50-58°C	15 min at 72°C

   Times and temperatures may need to be adapted to suit the particular reaction conditions.
9. Withdraw a sample (5-10 µl) from the test reaction mixture and the four control reactions and analyze them by electrophoresis through an agarose or polyacrylamide gel. Include DNA markers of an appropriate size. Stain the gel with ethidium bromide or SYBR Gold to visualize the DNA.
   A successful amplification reaction should yield a readily visible DNA fragment of the expected size. The identity of the band can be confirmed by DNA sequencing (please see Chapter 12, Protocol 6), Southern hybridization (please see Chapter 6, Protocol 10), and/or restriction mapping.
10. If mineral oil was used to overlay the reaction (in Step 7), remove the oil from the sample before cloning by extraction with 150 µl of chloroform.
    The aqueous phase, which contains the amplified DNA, will form a micelle near the meniscus. The micelle can be transferred to a fresh tube with an automatic micropipette.
    IMPORTANT *Do not attempt chloroform extractions in microtiter plates. The plastic used in these plates is not resistant to organic solvents.*
11. Separate the amplified DNA from the residual thermostable DNA polymerase and dNTPs (please see Chapter 8, Protocol 3).
    The DNA can now be ligated to a blunt-ended vector or a T vector, or it can digested with restriction enzymes and ligated to a vector with compatible termini (please see Chapter 8, Protocol 4, Chapter 8, Protocol 5, and Chapter 8, Protocol 6).

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1. Frohman M.A., Dush M.K., and Martin G.R. 1988. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci.* 85:8998-9002.
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Protocol 10

Rapid Amplification of 3´cDNA Ends (3´-RACE)

3´-RACE reactions are used to isolate unknown 3´ sequences or to map the 3´ termini of mRNAs onto a gene sequence. 3´-RACE requires knowledge of a small region of sequence within either the target RNA or a partial clone of cDNA. A population of mRNAs is transcribed into cDNA with an adaptor-primer consisting at its 3´ end of a poly(T) tract and at its 5´ end of an arbitrary sequence of 30-40 nucleotides. Reverse transcription is usually followed by two successive PCRs. The first is primed by a gene-specific sense oligonucleotide and an antisense primer complementary to the arbitrary sequence in the (dT)adaptor-primer. If necessary, the products of the first PCR can be used as templates for a second nested PCR, which is primed by a gene-specific sense oligonucleotide internal to the first, and a second antisense oligonucleotide complementary to the central region of the (dT)adaptor-primer. The products amplified in the second PCR are isolated from an agarose gel, cloned, and characterized. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- ⚠ Chloroform
- dNTP solution (20 mM) containing all four dNTPs (pH 8.0)
- Placental RNase inhibitor (20 units/µl)
- TE (pH 7.6)

Enzymes and Buffers

- Reverse transcriptase (RNA-dependent DNA polymerase)
- 5x Reverse transcriptase buffer
- Thermostable DNA polymerase
  Taq *DNA polymerase is the standard and appropriate enzyme for the amplification stage of most forms of RT-PCR. However, where elongation from 3´-mismatched primers is suspected, a thermostable DNA polymerase with 3´->5´ proofreading activity may be preferred.*

Nucleic Acids and Oligonucleotides

Adaptor-primer (10 µM) (5´ GACTCGAGTCGACATCG 3´) in H_2O (10 pmoles/µl).

The adaptor-primer is used in conjunction with a gene-specific sense primer to amplify a particular target cDNA.

$(dT)_{17}$-Adaptor-primer (10 µM) (5´ GACTCGAGTCGACATCGA$(T)_{17}$ 3´) in H_2O (10 pmoles/µl).

The oligo(dT) region of the $(dT)_{17}$-adaptor antisense primer binds to the poly(A)$^+$ tract at the 3´ end of mRNA, leaving the adaptor sequences unpaired. During amplification, the adaptor sequences are converted to double-stranded cDNA that, in the example given in this protocol, is equipped with recognition sites for Xho*I*, Sal*I*, Acc*I*, Hinc*II*, and Cla*I* restriction enzymes.

Gene-specific sense oligonucleotide primer (10 µM) in H_2O (10 pmoles/µl).

The gene-specific sense primer should encode a known region of sequence of the target mRNA, should be 20-30 nucleotides in length, and contain approximately equal numbers of the four bases, with a balanced distribution of G and C residues and a low propensity to form stable secondary structures. Restriction sites can be incorporated into the gene-specific oligonucleotides to facilitate cloning.

Template RNA.

Template RNA may be total RNA (100 µg/ml) in H_2O or poly(A)$^+$ RNA (10 µg/ml) in H_2O. Total RNA extracted from cells with chaotropic agents is generally the template of choice for RT-PCR for mRNAs that are expressed at moderate to high abundance. Poly(A)$^+$ is preferred as a template for all forms of RT-PCR when the target mRNA is expressed at low abundance.

Additional Reagents

Step 6 of this protocol requires the reagents listed in Chapter 5, Protocol 2 , Chapter 12, Protocol 6, or Chapter 6, Protocol 10 .

Step 8 of this protocol requires the reagents listed in Chapter 8, Protocol 3 and Chapter 8, Protocol 4 , Chapter 8, Protocol 5 , or Chapter 8, Protocol 6.

METHOD

1. Transfer 1 pg to 100 ng of poly(A)$^+$ mRNA or 10 pg to 1 µg of total RNA to a fresh microfuge tube. Adjust the volume to 10 µl with H_2O. Denature the RNA by heating at 75°C for 5 minutes, followed by rapid chilling on ice.
2. To the denatured RNA add:

5x reverse transcriptase buffer	10 µl
20 mM solution of four dNTPs	1.5 µl
10 µM $(dT)_{17}$-adaptor-primer (80 pmoles)	8.0 µl
approx. 20 units/µl placental RNase inhibitor	1 µl
100-200 units/µl reverse transcriptase	1 µl
H_2O	to 50 µl

   Incubate the reaction for 60 minutes at 37°C. Set up three negative control reactions. In one reaction, include all of the components of the first-strand reaction, except the RNA template. In another reaction, include all of the components, except the reverse transcriptase. Omit primer from the third reaction. Carry the controls through all subsequent steps of the protocol.
   Total cDNA synthesis can be estimated from the proportion of TCA-precipitable radioactivity incorporated in reverse transcriptase reactions supplemented with 10-20 µCi of [^{32}P]dCTP (sp. act. 3000 Ci/mmole). Be sure to set up a control reaction that contains no $(dT)_{17}$ adaptor antisense primer.
   The success or failure of 3´-RACE is determined here. If the reverse transcription step works efficiently, the chance of isolating clones that contain the 3´-terminal sequences of the target mRNA is high. On the other hand, no amount of work on the later steps of the protocol can compensate for ineffective reverse transcription. It is therefore worthwhile to take the time to optimize the reverse transcriptase reaction by determining the optimum ratio of primer to template for each preparation of RNA and by varying the concentration of Mg^{2+} in the reaction.
3. Dilute the reverse transcriptase reaction (cDNA) to a final volume of 1 ml with TE (pH 7.6).
   It may be necessary to remove excess oligonucleotide and random hexamer primers from the cDNA preparation and

then to optimize the concentrations of the sense and antisense primers in the amplification reaction (please see Step 3 of Chapter 8, Protocol 9).

4. In a sterile 0.5-ml microfuge tube, amplification tube, or the well of a sterile microtiter plate, set up a series of PCRs containing the following:

diluted cDNA	0-20 µl
10x amplification buffer	5 µl
20 mM solution of four dNTPs	5 µl
10 µM $(dT)_{17}$-adaptor-primer (16 pmoles)	1.6 µl
10 µM adaptor-primer (32 pmoles)	3.2 µl
10 µM gene-specific, sense oligonucleotide primer (32 pmoles)	3.2 µl
1-2 units thermostable DNA polymerase	1 µl
H_2O	to 50 µl

It is essential to set up a series of amplification reactions to find the amount of cDNA that generates the largest quantity of amplified 3´ termini. The control containing no cDNA template serves as a control for contamination.

5. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Alternatively, place a bead of paraffin wax into the tube if using a hot start protocol. Place the tubes or the microtiter plate in the thermal cycler. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
First Cycle	5 min at 94°C	5 min at 50-58°C	40 min at 72°C
Subsequent cycles (20)	40 sec at 94°C	1 min at 50-58°C	3 min at 72°C
Last cycle	40 sec at 94°C	1 min at 50-58°C	15 min at 72°C

Times and temperatures may need to be adapted to suit the particular reaction conditions.

6. Withdraw a sample (5-10 µl) from the test reaction mixture and the four control reactions and analyze them by electrophoresis through an agarose or polyacrylamide gel. Include DNA markers of an appropriate size. Stain the gel with ethidium bromide or SYBR Gold to visualize the DNA.
A successful amplification reaction should yield a readily visible DNA fragment of the expected size. The identity of the band can be confirmed by DNA sequencing (please see Chapter 12, Protocol 6), Southern hybridization (please see Chapter 6, Protocol 10), and/or restriction mapping.

7. If mineral oil was used to overlay the sample, remove the oil from the sample before cloning by extraction with 150 µl of chloroform.
The aqueous phase, which contains the amplified DNA, will form a micelle near the meniscus. The micelle can be transferred to a fresh tube with an automatic micropipette.
IMPORTANT *Do not attempt chloroform extractions in microtiter plates. The plastic used in these plates in not resistant to organic solvents.*

8. Separate the amplified DNA from the residual thermostable DNA polymerase and dNTPs (please see Chapter 6, Protocol 10).
The DNA can now be ligated to a blunt-ended vector or a T vector, or it can digested with restriction enzymes and ligated to a vector with compatible termini (please see Chapter 8, Protocol 4 , Chapter 8, Protocol 5 , and Chapter 8, Protocol 6).

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2. Frohman M.A., Dush M.K., and Martin G.R. 1988. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci.* 85:8998-9002.
3. Schaefer B.C. 1995. Revolutions in rapid amplification of cDNA ends: New strategies for polymerase chain reaction cloning of full-length cDNA ends. *Anal. Biochem.* 227:255-273.

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Protocol 11

Mixed Oligonucleotide-primed Amplification of cDNA (MOPAC)

In MOPAC, the amino-terminal and carboxy-terminal sequences of a peptide are used to design two redundant families of oligonucleotides encoding the amino- and carboxy-terminal sequences of the peptide. The primers are used either to amplify a segment of cDNA prepared by RT-PCR from a tissue known to express the protein or to amplify a segment of DNA from an established genomic or cDNA library. Because the length of the peptide is known, the size of the expected PCR product can be predicted exactly. After gel electrophoresis to resolve the amplification products, DNAs of the correct size are isolated, cloned, and sequenced. At least some of the clones should contain a DNA segment of the correct length that specifies the sequence of the starting peptide. Once identified, the entire cloned segment or the unique sequence lying between the two oligonucleotide primers is used as a probe to screen a cDNA library. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- dNTP solution (20 mM) containing all four dNTPs (pH 8.0)

Enzymes and Buffers

Thermostable DNA polymerase.
Taq *DNA polymerase is the standard and appropriate enzyme for the amplification stage of most forms of RT-PCR. However, where elongation from 3´-mismatched primers is suspected, a thermostable DNA polymerase with 3´->5´ proofreading activity may be preferred.*

Nucleic Acids and Oligonucleotides

DNA template (100 µg/ml) in TE (pH 8.0).
Any DNA (cDNA, genomic DNA, or cDNA library) that contains the target sequence(s) can be used as a template in MOPAC reactions. Before carrying out the MOPAC, it is essential to remove any primers (such as oligo[dT]) that were used to prime synthesis of cDNA templates (for details, please see Chapter 8, Protocol 3).

Family of antisense oligonucleotide primers (10 µM) in TE (pH 8.0)

Family of sense oligonucleotide primers (10 µM) in TE (pH 8.0)

Additional Reagents

Step 4 of this protocol requires the reagents listed in Chapter 5, Protocol 2 and Chapter 12, Protocol 6.

METHOD

1. In a sterile 0.5-ml microfuge tube, amplification tube, or the well of a sterile microtiter plate, mix in the following order:

0.5 µg of template DNA	5 µl
10x amplification buffer	5 µl
20 mM solution of four dNTPs	5 µl
10 µM sense primer family (70 pmoles)	7 µl
10 µM antisense primer family (70 pmoles)	7 µl
1-2 units thermostable DNA polymerase	1 µl
H_2O	to 50 µl

   Set up several control reactions. In one reaction, include all of the above components, except the template DNA. In two other reactions, include all the components minus one or the other of the oligonucleotide primers.
2. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Alternatively, place a bead of paraffin wax into the tube if using a hot start protocol. Place the tubes or the microtiter plate in the thermal cycler.
3. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
First Cycle	5 min at 94°C	2.5 min at 40°C	5 min at 50°C
Subsequent cycles (35)	1.5 min at 94°C	2.5 min at 40°C	5 min at 50°C

   Times and temperatures may need to be adapted to suit the particular reaction conditions.
   The amplification conditions described above are based on 32-member degenerate families of oligonucleotide primers that are 17-20 bases in length. Higher annealing and polymerization temperatures can be used with longer and/or less complex mixtures of oligonucleotides.
   The specificity of MOPAC can be increased (i) by using a thermal cycler program that includes a temperature ramping protocol, (ii) by using touchdown PCR, or (iii) by incorporating cosolvents into the amplification reactions.
4. Withdraw a sample (5-10 µl) from the test reaction mixture and the control reactions and analyze them by electrophoresis through a neutral polyacrylamide gel. Include DNA markers of an appropriate size. Stain the gel with ethidium bromide or SYBR Gold to visualize the DNA.
   If a single contiguous amino acid sequence was used to design both sense and antisense primers for the MOPAC reaction, the exact size of the desired product will be known. In most cases, thin polyacrylamide gels (≤ 6%) should allow size fractionation at nucleotide resolution and unambiguous identification of the desired product. Once identified, the product can be end-labeled (Chapter 9, Protocol 14) and subjected to chemical sequencing (Chapter 12, Protocol 7) to determine a unique sequence linking the two families of oligonucleotide probes. This unique sequence can in turn be synthesized and used as a probe to isolate longer cDNAs. Alternatively, the MOPAC product itself can be radiolabeled during a second round of PCR and used as a probe.
5. If mineral oil was used to overlay the reaction (Step 2), remove the oil from the sample by extraction with 150 µl of chloroform.
   The aqueous phase, which contains the amplified DNA, will form a micelle near the meniscus. The micelle can be transferred to a fresh tube with an automatic micropipette.
   IMPORTANT *Do not attempt chloroform extractions in microtiter plates. The plastic used in these plates is not resistant to organic solvents.*

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Protocol 12

Rapid Characterization of DNAs Cloned in Prokaryotic Vectors

In this method, sequences cloned in standard bacteriophage or plasmid vectors are amplified in PCRs containing primers targeted to flanking vector sequences. The amplified fragments can be analyzed by gel electrophoresis, DNA sequencing, and/or restriction mapping. Many colonies or plaques can be assayed simultaneously. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- dNTP solution (20 mM) containing all four dNTPs (pH 8.0)
- Tris-Cl (10 mM, pH 7.6)

Enzymes and Buffers

Thermostable DNA polymerase

Nucleic Acids and Oligonucleotides

Forward primers (20 µM) in H_2O

Reverse primers (20 µM) in H_2O (20 pmoles/µl)

Template DNAs.
This method uses unpurified templates obtained by cracking bacteriophage λ particles or transformed bacterial cells. Essentially the same methods can be used to analyze viral DNAs in productively infected mammalian or yeast cells transformed by multicopy vectors.

Additional Reagents

Step 9 of this protocol may require the reagents listed in Chapter 5, Protocol 2 and Chapter 6, Protocol 10 .

METHOD

1. Calculate the number of bacterial colonies or bacteriophage λ plaques that are to be screened. Prepare the appropriate amount of master mix; analysis of each colony or plaque requires 25 µl of master mix; *1 ml of master mix contains:*

10x amplification buffer	100 µl
20 mM solution of four dNTPs	50 µl
forward primer	1 nmole
reverse primer	1 nmole
H_2O	to 1 ml

2. Dispense 25-µl aliquots of the master mix into the appropriate number of amplification tubes.
3. Use a sterile 200-µl pipette tip (NOT a toothpick) to touch each bacterial colony or bacteriophage λ plaque. Working quickly, wash the pipette tip in 25 µl of master mix.
4. Close the caps of the tubes. Incubate the closed tubes in a boiling water bath for 10 minutes (bacterial colonies) or 2 minutes (bacteriophage λ plaques).
5. Dilute the required amount of *Taq* DNA polymerase to a concentration of 1 unit/µl in 10 mM Tris (pH 7.6). Store the diluted enzyme on ice.
6. Allow the samples (from Step 4) to cool to room temperature. Centrifuge the tubes briefly and then add 1 µl of the diluted *Taq* DNA polymerase to each tube.
7. Set up two control reactions. In one reaction, include all of the components, except the template DNA. In the other reaction, include a recombinant bacteriophage λ plaque or transformed bacterial lysate that has previously produced a positive result in this assay.
8. If the thermal cycler does not have a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Place the tubes in the thermal cycler. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
30 cycles	1 min at 94°C	2 min at 50°C	2 min at 72°C

Times and temperatures may need to be adapted to suit the particular reaction conditions.

9. Withdraw a sample (5-10 µl) from the test reaction mixture and the control reactions and analyze them by electrophoresis through an agarose gel. Include DNA markers of an appropriate size. Stain the gel with ethidium bromide or SYBR Gold to visualize the DNA.
A successful amplification reaction should yield a readily visible DNA fragment. Nonrecombinant PCR products will be equal to the length of DNA between the locations of the 5´ termini of the two primers in the cloning vector. Recombinant PCR products will be the sum of (i) the length of the insert and (ii) the distance between the 5´ termini of two primers in the vector. If necessary, the identity of the band can be confirmed by restriction mapping and Southern hybridization.

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Protocol 13

Long PCR

The following protocol can be used to amplify DNA up to 25 kb in length. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

dNTP solution (20 mM) containing all four dNTPs (pH 8.0)

10x Long PCR buffer

Enzymes and Buffers

Thermostable DNA polymerase mix
Klentaq1 can be obtained from AB Peptides (St. Louis, Missouri), and Pfu *polymerase can be obtained from Stratagene. A typical mixture contains 0.187 unit of* Pfu *and 33.7 units of Klentaq1 in a total volume of 1.2 µl.*

Nucleic Acids and Oligonucleotides

Forward primers (20 µM) in H_2O

Reverse primers (20 µM) in H_2O (20 pmoles/µl)

Template DNA
Long PCR works well on a variety of templates including recombinant PACs, BACs, cosmids, and bacteriophage λ clones, as well as high-molecular-weight genomic DNAs. However, the quality of the DNA is paramount. The average length of the template DNAs (assayed by agarose gel or pulsed-field gel electrophoresis) should be at least three times greater than the length of the desired PCR product. The DNAs should also be extensively purified by equilibrium density centrifugation in CsCl gradients, followed by dialysis against TE (pH 8.0) (please see Chapter 1, Protocol 10).

Additional Reagents

Step 4 of this protocol requires the reagents listed in Chapter 5, Protocol 2 and Chapter 6, Protocol 10.

METHOD

1. In a thin-walled amplification tube, add and mix in the following order:

10x long PCR buffer	5 µl
20 mM solution of four dNTPs	5 µl
20 mM forward primer	1 µl
20 mM reverse primer	1 µl
thermostable DNA polymerase mix	0.2 µl
template DNA	100 pg to 2 µg
H_2O	to 50 µl

Templates purified from individual recombinant clones constructed in bacteriophage λ, cosmid, bacteriophage P1, PAC, and BAC vectors should be used in amounts ranging from 100 pg to 300 ng. Larger amounts of total genomic DNAs are required, usually between 100 ng and 1 µg per reaction. The optimum amount of template and the optimum ratio of primers:template should be ascertained empirically for each new preparation of DNA.
Primers used for long PCR are generally slightly longer (25-30 nucleotides) than those used for standard PCR. It is particularly important to strive for equality between the melting temperatures of the two primers. If the difference in melting temperatures exceeds one centigrade degree, mispriming and preferential amplification of one strand may become a problem.
2. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Alternatively, place a bead of paraffin wax into the tube if using a hot start PCR. Place the tubes or the microtiter plate in the thermal cycler. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
24 cycles	1 min at 94°C	1 min at 60-67°C	5-20 min at 68°C

Times and temperatures may need to be adapted to suit the particular reaction conditions.
The temperature used for the annealing step depends on the melting temperature of the oligonucleotide primers. Because the primers used in long PCR are generally 27-30 nucleotides in length, the annealing temperatures used in long PCR can be considerably higher than those used in standard PCR.
3. If mineral oil was used to overlay the reaction (Step 2), remove the oil from the sample by extraction with 150 µl of chloroform.
The aqueous phase, which contains the amplified DNA, will form a micelle near the meniscus. The micelle can be transferred to a fresh tube with an automatic micropipette.
IMPORTANT *Do not attempt chloroform extractions in microtiter plates. The plastic used in these plates is not resistant to organic solvents.*
4. Analyze an aliquot of the aqueous phase by electrophoresis through an agarose gel using markers of an appropriate size. In many cases, the amount of amplified product may be too small to be detected by conventional staining with ethidium bromide. In this case, stain the DNA in the gel with SYBR Gold or transfer to a nylon or nitrocellulose filter and probe by Southern hybridization (please see Chapter 6, Protocol 10).

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Protocol 14

Inverse PCR

Inverse PCR is used to amplify and clone unknown DNA that flanks one end of a known DNA sequence and for which no primers are available. The technique involves digestion by a restriction enzyme of a preparation of DNA containing the known sequence and its flanking region. The individual restriction fragments (many thousands in the case of total mammalian genomic DNA) are converted into circles by intramolecular ligation, and the circularized DNA is then used as a template in the PCR. The unknown sequence is amplified by two primers that bind specifically to the known sequence and point in opposite directions. The product of the amplification reaction is a linear DNA fragment containing a single site for the restriction enzyme originally used to digest the DNA. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- ⚠ ATP (10 mM)
- ⚠ Chloroform
- dNTP solution (20 mM) containing all four dNTPs (pH 8.0)
- Ethanol
- ⚠ Phenol:chloroform (1:1, v/v)
- Sodium acetate (3 M)
- TE (pH 8.0)
- Tris-Cl (10 mM, pH 7.6)

Enzymes and Buffers

Bacteriophage T4 ligase (1 unit/µl)

Restriction endonucleases

Thermostable DNA polymerase

Nucleic Acids and Oligonucleotides

Oligonucleotide primer 1 (20 µM) in H_2O and Oligonucleotide primer 2 (20 µM) in H_2O.

Each primer should be 20-30 nucleotides in length and should contain approximately equal numbers of the four bases, with a balanced distribution of G and C residues and a low propensity to form stable secondary structures. Restriction sites can be added to the 5´ ends of the primers to facilitate subsequent cloning.

Template DNA in 10 mM Tris-Cl (pH 7.6) containing <0.1 mM EDTA.
Inverse PCR requires a circular DNA as template. Steps 1-4 of this protocol describe how such templates can be generated from conventional preparations of linear DNAs, which can be a purified fragment of DNA; a preparation of total genomic DNA, fractionated according to size; a bacteriophage λ cDNA library; an aliquot of a cosmid or bacteriophage P1 genomic library; or any other DNA whose sequence complexity is <10^9 bp.

Additional Reagents

Step 7 of this protocol requires the reagents listed in Chapter 5, Protocol 2 , Chapter 6, Protocol 10 , or Chapter 12, Protocol 6.

METHOD

1. Design and synthesize oligonucleotide primers 1 and 2 based on the known sequence of DNA.
2. Digest 2-5 µg of DNA template (sequence complexity <10^9 bp) with an appropriate restriction enzyme (please see note below). Extract the digested DNA with phenol:chloroform, and then with chloroform alone. Precipitate the DNA with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. Recover the precipitated DNA by centrifugation and dissolve it in TE (pH 8.0) at a concentration of 100 µg/ml.
   Alternatively, heat the digested DNA to 65°C for 15-20 minutes to inactivate the restriction enzyme.
3. In sterile 0.5-ml microfuge tubes, amplification tubes, or the wells of a sterile microtiter plate, set up a series of ligation reactions containing cleaved template DNA at concentrations ranging from 0.1 to 1 µg/ml.

template DNA	10 ng to 100 ng
10x ligation buffer	10 µl
1 unit/µl bacteriophage T4 DNA ligase	4 µl
10 mM ATP	10 µl
H_2O	to 100 µl

   Incubate the reactions for 12-16 hours at 16°C.
   Some commercial ligase buffers contain ATP. When using such buffers, the addition of ATP to the ligation reaction is no longer required.
4. Extract the ligated DNA with phenol:chloroform, and then with chloroform alone. Precipitate the DNA with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. Recover the precipitated DNA by centrifugation and dissolve it in 10 mM Tris (pH 7.6) or H_2O at a concentration of 100 µg/ml.
5. In a sterile 0.5-ml thin-walled amplification tube, add and mix in the following order:

10x amplification buffer	5 µl
20 mM solution of four dNTPs (pH 8.0)	1 µl
20 µM oligonucleotide primer 1	2.5 µl
20 µM oligonucleotide primer 2	2.5 µl
1-5 units/µl thermostable DNA polymerase	1.0 µl
H_2O	28-33 µl
ligated template DNA	5-10 µl
Total volume	50 µl

   Set up two control reactions. In one reaction, include all of the above reagents, except the template DNA. In the other reaction, replace the template with a plasmid of known size, containing the DNA insert from which the oligonucleotide primers were derived. Carry each control reaction through all subsequent steps of the protocol.
6. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Alternatively, place a bead of wax into the tube if using hot start PCR. Place the tubes or the microtiter plate

in the thermal cycler. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
30 cycles	30 sec at 94°C	30 sec at 60°C	2.5 min at 72°C
Last cycle	30 sec at 94°C	30 sec at 60°C	10 min at 72°C

Times and temperatures may need to be adapted to suit the particular reaction conditions.
The exact annealing temperature should be established empirically for the primer pairs used in a given amplification reaction. An extended polymerization time (up to 10 minutes per cycle) should be tried if the target DNA is long (>4kb). Alternatively, the use of mutant thermostable DNA polymerases that lack and/or that contain a low level of 3'-exonuclease activity may produce longer templates.

7. Withdraw a sample (5-10 µl) from the test reactions and the control reactions and analyze them by electrophoresis through an agarose or polyacrylamide gel. Include DNA markers of an appropriate size. Stain the gel with ethidium bromide or SYBR Gold.
A successful amplification reaction should yield a readily visible DNA. The identity of the band can be confirmed by DNA sequencing (please see Chapter 12, Protocol 6), and/or restriction mapping and/or Southern hybridization using probes homologous to the known DNA sequence (please see Chapter 6, Protocol 10).

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Protocol 15

Quantitative PCR

Quantitative PCR involves co-amplification of two templates: a constant amount of a preparation containing the desired target sequence and serial dilutions of a reference template that is added in known amounts to a series of amplification reactions. The concentration of the target sequence is determined by simple interpolation into a standard curve. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- dNTP solution (20 mM) containing all four dNTPs (pH 8.0)

$MgCl_2$ (50 mM)

Placental RNase inhibitor (20 units/µl)

Enzymes and Buffers

Appropriate restriction endonuclease
Please see Step 7.

Reverse transcriptase (100-200 units/µl)

Thermostable DNA polymerase.
Taq *DNA polymerase is the standard and appropriate enzyme for the amplification stage of most forms of quantitative PCR.*

Radioactive Compounds

⚠ [α-^{32}P]dCTP (sp. act. 3000 Ci/mmole) at 10 mCi/ml

Nucleic Acids and Oligonucleotides

Externally added reference
Use a DNA or RNA reference of known concentration to measure the concentration of DNA sequences by conventional PCR and, whenever possible, an RNA reference to measure the concentration of a target RNA by quantitative RT-PCR (Chapter 15, Protocol 2).

Sense primer (20 µM) in H_2O

Antisense primer (20 µM) in H_2O.
Each primer should be 20-30 nucleotides in length and contain approximately equal numbers of the four bases, with a balanced distribution of G and C residues and a low propensity to form stable secondary structures.

Target nucleic acid
The target can be a preparation of DNA or RNA, either total or poly(A)$^+$. Dissolve preparations of total RNA at a concentration of 0.5-1 mg/ml and preparations of poly(A)$^+$ RNA at a concentration of 10-100 µg/ml. Dissolve DNA targets in 10 mM Tris-Cl (pH 7.6) at the following concentrations: mammalian genomic DNA, 100 µg/ml; yeast genomic DNA, 1 µg/ml; bacterial genomic DNA, 0.1 µg/ml; and plasmid DNA, 1-5 ng/ml.

METHOD

1. Design and prepare a reference template suitable for the task at hand. Measure the concentration of the reference template as carefully as possible, preferably by fluorometry. Alternatively, estimate the amount of reference template after gel electrophoresis and ethidium bromide staining.
2. Make a series of tenfold dilutions (in H_2O) containing concentrations of the reference template ranging from 10^{-6} to 10^{-12} M. After using the dilutions in Step 3, they should be stored at -70°C for use in Step 8.
3. If starting from RNA, denature the target RNA by incubating aliquots for 5 minutes at 75°C, followed by rapid chilling in ice water. Then, without delay, set up a series of reverse transcription reactions containing increasing amounts of reference template in sterile 0.5-ml microfuge tubes. For each reaction in the series, prepare the following:

10x amplification buffer	2 µl
20 mM solution of four dNTPs (pH 8.0)	1 µl
20 µM antisense primer	2.5 µl
approx. 20 units/µl placental RNase inhibitor	1 µl
50 mM $MgCl_2$	1 µl
denatured target RNA	10 pg to 1.0 µg
100-200 units/µl reverse transcriptase	1 µl
tenfold dilution of reference template	1 µl
H_2O	to 20 µl

$MgCl_2$ is added to meet the needs of the reverse transcriptase.
Incubate the reaction for 60 minutes at 37°C and then denature the reverse transcriptase by heating to 95°C for 20 minutes.
4. In sterile 0.5-ml microfuge tubes, amplification tubes, or the wells of a sterile microtiter plate, set up amplification reactions with each reaction in the series from Step 3:

reverse transcriptase reaction (Step 3) or target DNA	5 µl
20 µM sense primer	1.5 µl
20 µM antisense primer	1.25 µl
10x amplification buffer	5 µl
[α-^{32}P]dCTP (3000 Ci/mmole)	10 µCi
20 mM solution of four dNTPs	1 µl
Taq DNA polymerase	2 units
H_2O	to 50 µl

IMPORTANT *Do not reduce the concentration of unlabeled dCTP in the reaction mixture to increase the specific activity of the precursor pool. There is a danger that the amount of the nucleotide could become limiting at late stages in the amplification reaction.*
5. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Alternatively, place a bead of wax into the tube if using hot start PCR. Place the tubes or the microtiter plate

in the thermal cycler.

6. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
30 cycles	30 sec at 95°C	30 sec at 55°C	1 min at 72°C
Last cycle	1 min at 95°C	30 sec at 55°C	1 min at 72°C

Times and temperatures may need to be adapted to suit the particular reaction conditions.

7. Analyze and quantitate the amplified products.

When using a reference template that differs from the target sequence in size

a. Analyze the sizes of the amplified products in a 20-µl aliquot of each of the reactions by gel electrophoresis and autoradiography.
b. Excise the amplified bands of the control template and target sequences from the gel, and measure the amount of radioactivity in each band in a liquid scintillation counter. Alternatively, scan the gel with the appropriate detector (e.g., Ambis scanner or phosphorimager).
c. Calculate the relative amounts of the two radiolabeled DNAs in each of the PCRs.

Correct the amount of radioactivity to allow for differences in the molecular weights of the two radiolabeled DNAs.

When using a reference template that contains a novel restriction site or lacks a naturally occurring site

a. Heat the samples to 94°C for 5 minutes following the final round of amplification.
b. Allow the samples to cool gradually to room temperature and then digest a 20-µl aliquot of each of the reactions with the appropriate restriction enzyme.
c. Analyze the sizes of the amplified DNA fragments by gel electrophoresis and autoradiography.
d. Excise the amplified bands of the control template and target sequences from the gel, and measure the amount of radioactivity in each band in a liquid scintillation counter. Alternatively, scan the gel with the appropriate detector (e.g, Ambis scanner or phosphor-imager).
e. Calculate the relative amounts of the two radiolabeled DNAs in each of the PCRs.

Correct the amount of radioactivity to allow for differences in the molecular weights of the two radiolabeled DNAs.

8. Examine the results to determine the concentration of reference template that yields approximately the same amount of amplified product as the target sequence. Set up a second series of amplification reactions (please see Step 4) containing a narrower range of concentrations of reference template.
It is best to generate this series of dilutions from the appropriate tenfold dilution of the reference template (Step 2).
9. Repeat Steps 5, 6, and 7. For each amplification reaction, measure the ratio of the yield of amplified reference template to the yield of amplified target sequence. Plot this ratio against the amount of reference template added to each amplification reaction. From the resulting straight line, determine the equivalence point (i.e., the amount of reference template that gives exactly the same quantity of amplified product as the target sequence in the reaction). Calculate the concentration of the target sequence in the original sample.

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Protocol 16

Differential Display-PCR

This method uses PCR to amplify and display many cDNAs derived from the mRNAs of a given cell or tissue type. The method relies on two different types of synthetic oligonucleotides: anchored antisense primers and arbitrary sense primers. A typical anchored primer is complementary to approx. 13 nucleotides of the poly(A) tail of mRNA and the adjacent two nucleotides of the transcribed sequence. Anchored primers therefore anneal to the junction between the poly(A) tail and the 3´-untranslated region of mRNA templates, from where they can prime synthesis of first-strand cDNA. A second primer, an arbitrary sequence of approx. 10 nucleotides, is then added to the reaction mixture, and double-stranded cDNAs are produced by conventional PCR, carried out at low stringency. The products of the amplification reaction are separated by electrophoresis through a denaturing polyacrylamide gel and visualized by autoradiography. By comparing the banding patterns of cDNA products derived from two different cell types, or from the same cell type grown under different conditions, it is sometimes possible to identify the products of differentially expressed genes. Bands of interest can then be recovered from the gel, amplified further, and cloned and/or used as probes to screen northern blots, cDNA libraries, etc. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- 5x DD-PCR reverse transcriptase buffer
- DTT (dithiothreitol) (100 mM)
- dNTP solution (20 mM) containing all four dNTPs (pH 8.0)
- Placental RNase inhibitor (20 units/ml)
- ⚠ 5x Formamide loading buffer

Enzymes and Buffers

Reverse transcriptase (RNA-dependent DNA polymerase).
For DD-PCR, a reverse transcriptase deficient in RNase H is required (e.g., Superscript from Life Technologies or StrataScript from Stratagene).

Thermostable DNA polymerase
Taq *DNA polymerase is the standard and appropriate enzyme for the amplification stage of most forms of DD-PCR.*

Nucleic Acids and Oligonucleotides

Anchoring 3´ oligonucleotide primers (300 µg/ml) in 10 mM Tris-Cl (pH 7.6), 0.1 mM EDTA
*The anchoring 3´ oligonucleotides are a family of 12 primers with the general structure 5´-d(T)$_{12}$*VN*-3´, where* V *is either C, A, or G, and* N *is C, T, A, or G. For example, one primer in the series is 5´-d(T)$_{12}$CC-3´, the next is 5´-d(T)$_{12}$CT-3´, etc.*

Arbitrary 5´ oligodeoxynucleotide primers (50 µg/ml) in 10 mM Tris-Cl (pH 7.6), 0.1 mM EDTA
Sixteen arbitrary 5´ oligonucleotide primers are required, each ten nucleotides in length. The sequence of each primer is chosen at random, but it should contain approximately equal numbers of the four bases, with a balanced distribution of G and C residues, and a low propensity to form stable secondary structures.

Total RNA (100 µg/ml)
Total RNA extracted from cells with chaotropic agents is generally the template of choice for DD-PCR. RNAs to be compared by DD-PCR should be prepared in an identical fashion. Poly(A)$^+$ RNA is not ideal as a template in differential display.

Radioactive Compounds

⚠ Radiolabeled dATP (10 µCi/µl, sp. act. 3000 Ci/mmole)
DNA labeled with ∝-^{35}S or ∝-^{33}P generates sharper bands on autoradiographs than does DNA labeled with ∝-^{32}P.

Additional Reagents

Steps 10 and 13 of this protocol require the reagents listed in Chapter 12, Protocol 10 and Chapter 12, Protocol 11.

Step 22 of this protocol requires the reagents listed in Chapter 8, Protocol 5 .

Step 23 of this protocol requires the reagents listed in Chapter 8, Protocol 12 .

Step 24 of this protocol requires the reagents listed in Chapter 7, Protocol 8 or Chapter 7, Protocol 11, and Chapter 8, Protocol 15.

METHOD

1. In sterile 0.5-ml microfuge tubes, set up a series of trial reactions to establish the optimum concentrations of "control" and "test" RNAs required to produce a pattern of 100-300 amplified cDNA bands after gel electrophoresis and autoradiography. Make fivefold serial dilutions in H_2O of the RNA preparations to produce concentrations of between 1 µg/ml and 100 µg/ml.
2. Choose one or more primers from the collection of anchored 3´ oligonucleotides and set up a series of annealing reactions that contain different amounts of diluted RNA templates:

template RNA	8.0 µl
anchored 3´ oligonucleotide primer	2.0 µl

Incubate the reactions for 10 minutes at 65°C and then place them in a 37°C water bath.
The total amount of RNA in the annealing reactions should vary between 8 ng and 800 ng.

3. Add the following to the annealing reactions:

5x DD-PCR reverse transcriptase buffer	4 µl
100 mM dithiothreitol	2 µl
200 µM solution of four dNTPs	2 µl
approx. 25 units/µl placental RNase inhibitor	0.25 µl
200 units/µl reverse transcriptase	0.25 µl
H_2O	to 20 µl

Incubate the tubes at 37°C for 1 hour.

To test for contaminating genomic DNA, set up one or more control reactions that contain no reverse transcriptase enzyme and carry these through Step 10 of the protocol. If necessary, the RNA preparation can be treated with RNase-free DNase I either as a separate step during purification or in the same reaction tube that will later be used to synthesize cDNA.

4. Inactivate the reverse transcriptase by incubating the reaction mixtures for 10 minutes at 94°C.
   Steps 3 and 4 can be carried out in a thermal cycler programmed with a single cycle of 37°C for 1 hour/94°C for 10 minutes, followed by a 4°C hold.
5. Set up two series of eight 0.5-ml amplification tubes. Each tube should contain:

10x amplification buffer	2 µl
anchored 3´ oligonucleotide primer	2 µl
20 mM solution of four dNTPs (pH 8.0)	1 µl
[α-^{33}P]dATP or [α-^{35}S]dATP (3000 Ci/mmole)	1 µl
H_2O	9 µl
5 units/µl thermostable DNA polymerase	1 unit

   To each tube, add 2 µl of a different arbitrary 5´ primer. Mix the contents by tapping the sides of the tubes.
   Wherever possible, use the 10x amplification buffer supplied by the manufacturer of the Taq *DNA polymerase.*
6. Into one series of eight tubes, dispense approx. 3-µl aliquots of the reverse transcriptase reaction containing the test RNA. Into the other series of eight tubes dispense approx. 3-µl aliquots of the reverse transcriptase reaction containing the preparation of control RNA. Close the tubes and mix the contents gently.
7. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Place the tubes in the thermal cycler.
8. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
30 cycles	15 sec at 94°C	30 sec at 42°C	15 sec at 72°C
Last cycle	15 sec at 94°C	30 sec at 42°C	2 min at 72°C

   Times and temperatures may need to be adapted to suit the particular reaction conditions.
9. At the end of the program, remove the tubes from the thermal cycler and add 5 µl of 5x formamide loading buffer to each.
10. Separate the radiolabeled products of the reactions by electrophoresis through an electrolyte gradient polyacrylamide gel of the type used for DNA sequencing. Electrophoresis is carried at constant electrical power until the xylene cyanol tracking dye has migrated about two thirds of the length of the gel (please see Chapter 12, Protocol 10 and Chapter 12, Protocol 11). Dry the gel and expose it to autoradiographic film.
11. Examine the pattern of DNA bands arising from reactions containing different concentrations of control and test RNAs. A good differential display contains between 100 and 250 well-resolved bands. Select the concentration of test and control RNAs that work well with the largest number of primer pairs.
12. Repeat the annealing, reverse transcriptase, and amplification reactions using all combinations of primer pairs and the optimum amount of RNA templates. Set up the reactions in 96-well microtiter plates designed for use in a thermal cycler.
13. Separate the products of the amplification reactions by electrophoresis through polyacrylamide sequencing gels, as in Steps 9 and 10.
    Load the reactions generated with each primer pair in adjacent lanes on the gel, i.e., load the reaction obtained with primer pair A + B from one RNA preparation next to the reaction obtained with primer pair A + B from the other RNA preparation.
14. Compare the patterns of bands obtained with each primer pair from the different RNA populations.
    When a differentially expressed band is identified, it is advisable to repeat the experiment to make sure that the initial finding is reproducible. Ideally, different batches of the two RNAs should be used, although this precaution may not always be practicable.
15. Recover target bands from the dried polyacrylamide gel. Lay the autoradiogram on top of the gel and use a soft pencil to lightly mark the position of the desired band on the autoradiogram. Cutting through the autoradiogram with a clean razor blade, excise each target band and the attached Whatman 3MM paper. Soak each sliver of dried gel/paper overnight at room temperature in a separate 0.5-ml microfuge tube containing 50 µl of sterile H_2O.
16. Puncture the bottom of each 0.5-ml tube with a small-gauge needle. Place each punctured tube inside a 1.5-ml microfuge tube. Centrifuge for 20 seconds to transfer the eluate to the larger tube. Discard the amplification tube containing the residue of the Whatman 3MM paper and polyacrylamide.
17. Amplify the eluted fragment in a reaction containing the following:

10x amplification buffer	2 µl
DNA eluted from polyacrylamide gel	3 µl
arbitrary 5´ oligonucleotide primer	2 µl
anchoring 3´ oligonucleotide primer	2 µl
20 mM solution of four dNTPs (pH 7.0)	1 µl
H_2O	9.5 µl
5 units/µl *Taq* thermostable DNA polymerase	2 units

    Wherever possible, use the 10x amplification buffer supplied by the manufacturer of the Taq *DNA polymerase.*
18. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Place the tubes in a thermal cycler.
19. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
30 cycles	15 sec at 94°C	30 sec at 42°C	15 sec at 72°C
Last cycle	15 sec at 94°C	30 sec at 42°C	2 min at 72°C

    Polymerization should be carried out for 1 minute for every 1000 bp of length of the target DNA.
20. Estimate the concentration of the reamplified DNA fragment by electrophoresis of 5-10% of the reaction through a 1% (w/v) agarose gel.
21. If mineral oil was used to overlay the reaction (Step 18), remove the oil from the sample by extraction with 150 µl of chloroform.
    The aqueous phase, which contains the amplified DNA, will form a micelle near the meniscus. The micelle can be transferred to a fresh tube with an automatic micropipette.
    IMPORTANT *Do not attempt chloroform extractions in microtiter plates. The plastic used in these plates is not resistant to organic solvents.*
22. Ligate the DNA into a vector that has been tailed with dT (e.g., pGEM T vector from Promega) (please see Chapter 8, Protocol 5) and transform *E. coli* with aliquots of the ligation reaction.
23. Isolate plasmid DNA from six or more recombinants and compare the sizes of the inserts released by restriction enzyme digestion.
    The sequence of the insert DNA can be established by using universal primers that bind to the flanking regions of the vector. These oligonucleotides can also be used as primers to check the size of the inserts by PCR (please see Chapter 8, Protocol 12).
    It is important to isolate and sequence more than one plasmid recombinant from the ligation reaction. Compare the cDNA sizes and sequences to each other and to those in the various databases.
24. Confirm the differential expression of a candidate cDNA/mRNA in as many ways as possible, including northern hybridization (Chapter 7, Protocol 8), RNase protection (Chapter 7, Protocol 11), or quantitative PCR (Chapter 8, Protocol 15). In situ mRNA hybridization can be used to localize the transcript to a diseased or developing tissue.

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1. Liang P. and Pardee A.B. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain

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Chapter 9 Preparation of Radiolabeled DNA and RNA Probes

Protocol 1: Random Priming: Radiolabeling of Purified DNA Fragments by Extension of Random Oligonucleotides

Short oligonucleotides of random sequence can serve as primers for the initiation of DNA synthesis at multiple sites on single-stranded DNA templates. During extension of the primers by DNA polymerase, the complement of every nucleotide in the template (except those at the extreme 5´ terminus) will be incorporated into the product at approximately equal frequency. The DNA synthesized can be labeled by using one [α-^{32}P]dNTP and three unlabeled dNTPs as precursors, generating probes with specific activities of 5 x 10^8 to 5 x 10^9 dpm/µg. For details on separating labeled probe from unincorporated dNTPs, please see Appendix 8 in the print version of the manual.

Protocol 2: Random Priming: Radiolabeling of DNA by Extension of Random Oligonucleotides in the Presence of Melted Agarose

A variation of the technique described in Chapter 9, Protocol 1 can be used to radiolabel DNA in slices cut from gels cast with low-melting-temperature agarose. For details on separating labeled probe from unincorporated dNTPs, please see Appendix 8 in the print version of the manual.

Protocol 3: Radiolabeling of DNA Probes by the Polymerase Chain Reaction

In this protocol, double-stranded DNA probes, labeled in each strand, are produced in conventional PCRs containing equal concentrations of two primers, a double-stranded DNA template, three unlabeled dNTPs at concentrations exceeding the K_m, and one [α-^{32}P]dNTP at a concentration at or slightly above the K_m (2-3 µm) for a thermostable DNA polymerase such as *Taq*.

Protocol 4: Synthesis of Single-stranded DNA Probes of Defined Length from Bacteriophage M13 Templates

A synthetic oligonucleotide annealed to single-stranded DNA derived from a recombinant bacteriophage M13 or phagemid template is used to prime the synthesis of complementary radiolabeled DNA. Synthesis is catalyzed by the Klenow fragment of *E. coli* DNA polymerase I, which extends the annealed primer for various distances along the single-stranded template DNA. The products of the reaction, which are heterogeneous both in length and in the amount of incorporated radiolabeled dNTPs, are digested with a restriction enzyme to create double-stranded DNA fragments of uniform length, which are subsequently purified by agarose gel electrophoresis.
The oligonucleotide primer is usually complementary to a region of the *lac* gene immediately 3´ to the polycloning site in the mp series of bacteriophage M13 vectors. This "universal" primer, which is sold by several companies, has the advantage that it can be used to prepare probes complementary to any segment of DNA that has been cloned into any restriction site in the polylinker of the vector. However, custom-made oligonucleotides complementary to specific sequences within the cloned DNA can also be used to prepare probes that represent only a portion of the cloned sequence.

Protocol 5: Synthesis of Single-stranded DNA Probes of Heterogeneous Length from Bacteriophage M13 Templates

The following technique yields a heterogeneous population of short radiolabeled molecules 200-300 nucleotides in length. These probes are synthesized, as in Protocol 4 (Chapter 9), by extension of an oligonucleotide primer on a single-stranded DNA template. The radiolabeled products of the reaction are then separated from the template by electrophoresis through a denaturing gel from which they are eluted directly into hybridization buffer. The method is useful for synthesizing single-stranded DNA probes of very high specific activity for Southern analysis of single-copy genes in complex genomes or for northern analysis of rare species mRNAs.

Protocol 6: Synthesis of Single-stranded RNA Probes by In Vitro Transcription

This protocol describes a procedure for synthesizing RNA probes of high specific activity (1 x 10^9 dpm/mg) from double-stranded linear DNAs containing promoters for bacteriophage-encoded RNA polymerases. **IMPORTANT** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 7: Synthesis of cDNA Probes from mRNA Using Random Oligonucleotide Primers

This protocol describes the generation of cDNA probes from poly(A)$^+$ mRNA using random oligonucleotide primers. Probes of this type are used for differential screening of cDNA libraries. **IMPORTANT** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 8: Synthesis of Radiolabeled, Subtracted cDNA Probes Using Oligo(dT) as a Primer

This protocol describes the preparation of subtracted cDNA probes by hybridization to an mRNA driver, followed by purification of the single-stranded radiolabeled cDNA by hydroxyapatite chromatography. For further information, please see page 9.89 in the print version of the manual. Before preparing the probe, it is a good idea to have filters (which contain the cDNA library to be screened) ready to hybridize. **IMPORTANT** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 9: Radiolabeling of Subtracted cDNA Probes by Random Oligonucleotide Extension

In this procedure, synthesis of cDNA is carried out in the presence of saturating concentrations of all four dNTPs and trace amounts of a single radiolabeled dNTP. After subtraction hybridization, the enriched single-stranded cDNA is radiolabeled to high specific activity in a second synthetic reaction by extension of random oligonucleotide primers using the Klenow fragment of *E. coli* DNA polymerase. Because the concentrations of dNTP in the first reaction are nonlimiting, both the amounts and size of cDNA generated are greater than those achieved in standard labeling protocols. For further information, please see page 9.89 in the print version of the manual. **IMPORTANT** Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 10: Labeling 3´ Termini of Double-stranded DNA Using the Klenow Fragment of *E. coli* DNA Polymerase I

Templates for the end-filling reaction are produced by digestion of DNA with a restriction enzyme that creates a recessed 3´-hydroxyl terminus. The Klenow enzyme is then used to catalyze the incorporation of one or more [α-^{32}P]dNTPs into the recessed 3´ terminus in a template-dependent fashion.

Protocol 11: Labeling 3´ Termini of Double-stranded DNA with Bacteriophage T4 DNA Polymerase

Bacteriophage T4 DNA polymerase enzyme, unlike the Klenow enzyme, rapidly digests 3´-protruding termini and then continues at a slower pace to remove 3´ nucleotides from the double-stranded portion of the DNA substrate. Consequently, in the absence of dNTPs, the enzyme will degrade double-stranded molecules to about half-length single strands. However, in the presence of high concentrations of dNTPs, recessed 3´-hydroxyl termini generated by exonucleolytic activity act as primers for template-directed addition of mononucleotides by the 5´->3´ polymerase. Because the synthetic capacity of bacteriophage T4 DNA polymerase exceeds its exonucleolytic abilities, protruding 3´ termini are converted to termini with flush ends. The reaction therefore consists of cycles of removal and replacement of the 3´-terminal nucleotides from recessed or blunt-ended DNA (O'Farrell et al. 1980). If one of the four dNTPs is radiolabeled, the resulting blunt-ended double-stranded molecules will be labeled at or near their 3´ termini.

Protocol 12: End Labeling Protruding 3´ Termini of Double-stranded DNA with [α-^{32}P]Cordycepin 5´-Triphosphate or [α-^{32}P]dideoxyATP

The 3´-protruding termini of DNA, generated by cleavage with restriction enzymes such as *Pst*I or *Sac*I, can be labeled using calf thymus terminal transferase to catalyze the transfer of [α-^{32}P]dideoxyATP or [α-^{32}P]cordycepin triphosphate. Because neither of these nucleotide analogs carries a 3´-hydroxyl group, no additional nucleotides can be added to the modified protruding 3´ terminus.

Protocol 13: Dephosphorylation of DNA Fragments with Alkaline Phosphatase

Essentially any protein phosphatase (e.g., bacterial alkaline phosphatase [BAP], calf intestinal phosphatase [CIP], placental alkaline phosphatase, and shrimp alkaline phosphatase [SAP]) will catalyze the removal of 5´ phosphates from nucleic acid templates. Because CIP and SAP are readily inactivated, they are the most widely used phosphatases in molecular cloning. Although CIP is cheaper per unit of activity, SAP enzyme has the advantage of being readily inactivated in the absence of chelators.

Protocol 14: Phosphorylation of DNA Molecules with Protruding 5'-Hydroxyl Termini

Bacteriophage T4 polynucleotide kinase catalyzes the transfer of γ-^{32}P of ATP to the terminal 5´-hydroxyl groups of single- or double-stranded nucleic acids. When [γ-^{32}P]ATP of high specific activity (3000-7000 Ci/mmole) is used as a substrate, approximately 40-50% of the protruding 5´ termini in the reaction become radiolabeled.

Protocol 15: Phosphorylation of DNA Molecules with Dephosphorylated Blunt Ends or Recessed 5' Termini

Blunt ends, recessed 5´ termini, or internal nicks in DNA are labeled less efficiently in reactions catalyzed by T4 DNA kinase than are protruding 5´ termini. However, the efficiency of phosphorylation of blunt-ended DNAs greater than 300 bp in length can be increased by including a condensing reagent such as polyethylene glycol in the reaction.

Protocol 16: Phosphorylation of DNA Molecules with Protruding 5' Termini by the Exchange Reaction

The exchange reaction catalyzed by bacteriophage T4 polynucleotide kinase does not require that the 5´ termini of DNA substrates be dephosphorylated. However, the efficiency of the reaction is poor unless crowding reagents such as polyethylene glycol are included in the reaction mixture.

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Protocol 1

Random Priming: Radiolabeling of Purified DNA Fragments by Extension of Random Oligonucleotides

Short oligonucleotides of random sequence can serve as primers for the initiation of DNA synthesis at multiple sites on single-stranded DNA templates. During extension of the primers by DNA polymerase, the complement of every nucleotide in the template (except those at the extreme 5´ terminus) will be incorporated into the product at approximately equal frequency. The DNA synthesized can be labeled by using one [α-^{32}P]dNTP and three unlabeled dNTPs as precursors, generating probes with specific activities of 5 x 10^8 to 5 x 10^9 dpm/µg. For details on separating labeled probe from unincorporated dNTPs, please see Appendix 8 in the print version of the manual.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
Optional, please see Step 5.

Ethanol
Optional, please see Step 5.

NA stop/storage buffer

5x Random primer buffer

Enzymes and Buffers

Klenow fragment of *E. coli* DNA polymerase I

Nucleic Acids and Oligonucleotides

dNTP solution containing three unlabeled dNTPs, each at 5 mM
The composition of this solution depends on the [α-^{32}P]dNTP to be used. If radiolabeled dATP is used, the mix should contain dCTP, dTTP, and dGTP each at a concentration of 5 mM. If two radiolabeled dNTPs are used, this solution should contain the other two dNTPs each at a concentration of 5 mM.

Random deoxynucleotide primers six or seven bases in length (125 ng/µl in TE, pH 7.6)

Template DNA (5-25 ng/µl) in TE (pH 7.6)

Radioactive Compounds

⚠ [α-^{32}P]dNTP (10 mCi/ml, sp. act. >3000 Ci/mmole)

METHOD

1. In a 0.5-ml microfuge tube, combine template DNA (25 ng) in 30 µl of H_2O with 1 µl of random deoxynucleotide primers (approx. 125 ng). Close the top of the tube tightly and place the tube in a boiling water bath for 2 minutes.
   Purify the DNA to be radiolabeled by one of the methods described in Chapter 5.
   This protocol works best when 25 ng of template DNA is used in a standard 50-µl reaction.
2. Remove the tube and place it on ice for 1 minute. Centrifuge for 10 seconds at 4°C in a microfuge. Return the tube to the ice bath.
3. To the mixture of primer and template, add:

5 mM dNTP solution	1 µl
5x random primer buffer	10 µl
10 mCi/ml [α-^{32}P]dNTP (sp. act. 3000 Ci/mmole)	5 µl
H_2O	to 50 µl

4. Add 5 units (approx. 1 µl) of the Klenow fragment. Mix the components gently. Centrifuge the tube at maximum speed for 1-2 seconds in a microfuge. Incubate the reaction mixture for 60 minutes at room temperature.
   To label larger amounts of DNA, assemble reaction mixtures as described in Steps 3 and 4 and then incubate the reaction for 60 minutes. To label smaller amounts of DNA, incubate the reactions for times that are in inverse proportion to the amount of template added.
   To monitor the course of the reaction, measure the proportion of radiolabeled dNTPs that either is incorporated into material precipitated by trichloroacetic acid (TCA) or adheres to a DE-81 filter.
   Under these reaction conditions, the length of the radiolabeled product is approx. 400-600 nucleotides, as determined by electrophoresis through an alkaline agarose gel (Chapter 5, Protocol 8) or a denaturing polyacrylamide gel (Chapter 12, Protocol 8).
5. Add 10 µl of NA stop/storage buffer to the reaction, and proceed with one of the following options as appropriate.
   - Store the radiolabeled probe at -20°C until it is needed for hybridization.

     or

   - Separate the radiolabeled probe from unincorporated dNTPs by either spun-column chromatography or selective precipitation of the radiolabeled DNA with ammonium acetate and ethanol (please see Appendix 8 in the print version of the manual). This step is generally not required if >50% of the radiolabeled dNTP has been incorporated during the reaction.

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1. Feinberg A.P. and Vogelstein B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
2. Feinberg A.P. and Vogelstein B. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Addendum. *Anal. Biochem.* 137:266-267.

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Protocol 2

Random Priming: Radiolabeling of DNA by Extension of Random Oligonucleotides in the Presence of Melted Agarose

A variation of the technique described in Chapter 9, Protocol 1 can be used to radiolabel DNA in slices cut from gels cast with low-melting-temperature agarose. For details on separating labeled probe from unincorporated dNTPs, please see Appendix 8 in the print version of the manual.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
Optional, please see Step 5.

Bovine serum albumin (10 mg/ml)

Ethanol
Optional, please see Step 5.

⚠ Ethidium bromide (10 mg/ml)
Alternatively, SYBR Gold may be used.

NA stop/storage buffer

5x Oligonucleotide labeling buffer

Enzymes and Buffers

Klenow fragment of *E. coli* DNA polymerase

Nucleic Acids and Oligonucleotides

Random deoxynucleotide primers six or seven bases in length (125 ng/µl in TE, pH 7.6)
Primers are incorporated into the 5x oligonucleotide labeling buffer.

Template DNA
The DNA to be labeled is recovered after electrophoresis through a low-melting-temperature agarose gel (please see Steps 1-3 of this protocol). For details on casting and running low-melting-temperature gels, please see Chapter 5.
This protocol works best when 25 ng of template DNA is used in a standard 50-µl reaction.

Radioactive Compounds

⚠ [α-^{32}P]dNTP (10 mCi/ml, sp. act. >3000 Ci/mmole)

METHOD

1. After electrophoresis, stain the gel with ethidium bromide (final concentration 0.5 µg/ml) or SYBR Gold, and excise the desired band, eliminating as much extraneous agarose as possible.
2. Place the band in a preweighed microfuge tube and measure its weight. Add 3 ml of H_2O for every gram of agarose gel.
3. Place the microfuge tube in a boiling water bath for 7 minutes to melt the gel and denature the DNA.
If radiolabeling is to be carried out immediately, store the tube at 37°C until the template is required. Otherwise, store the tube at -20°C. After each removal from storage, reheat the DNA/gel slurry to 100°C for 3-5 minutes and then store at 37°C until the radiolabeling reaction is initiated.
4. To a fresh microfuge tube in a 37°C water bath or heating block, add in the following order:

5x oligonucleotide labeling buffer	10 µl
10 mg/ml bovine serum albumin solution	2 µl
DNA in a volume no greater than 32 µl	20-50 ng
10 mCi/ml [α-^{32}P]dNTP (sp. act. >3000 Ci/mmole)	5 µl
Klenow fragment (5 units)	1 µl
H_2O	to 50 µl

Mix the components completely with a micropipettor. Incubate the reaction for 2-3 hours at room temperature or for 60 minutes at 37°C.
To label larger amounts of DNA, adjust the volume of the reaction mixture proportionately and incubate the reaction for 60 minutes. To label smaller amounts of DNA, incubate the reactions for times that are in inverse proportion to the amount of template added.
To monitor the course of the reaction, measure the proportion of radiolabeled dNTPs that either are incorporated into material precipitated by TCA or that adhere to a DE-81 filter.
Under these reaction conditions, the length of the radiolabeled product is approx. 400-600 nucleotides, as determined by electrophoresis through an alkaline agarose gel (Chapter 5, Protocol 8) or a denaturing polyacrylamide gel (Chapter 5, Protocol 9).
5. Add 50 µl of NA stop/storage buffer to the reaction, and proceed with one of the following options as appropriate:
   - Store the radiolabeled probe at -20°C until it is needed for hybridization.

   or

   - Separate the radiolabeled probe from unincorporated dNTPs by either spun-column chromatography or selective precipitation of the radiolabeled DNA with ammonium acetate and ethanol (please see Appendix 8 in the print version of the manual). This step is generally not required if >50% of the radiolabeled dNTP has been incorporated during the reaction.

REFERENCES

1. Feinberg A.P. and Vogelstein B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
2. Feinberg A.P. and Vogelstein B. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Addendum. *Anal. Biochem.* 137:266-267.

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Protocol 3

Radiolabeling of DNA Probes by the Polymerase Chain Reaction

In this protocol, double-stranded DNA probes, labeled in each strand, are produced in conventional PCRs containing equal concentrations of two primers, a double-stranded DNA template, three unlabeled dNTPs at concentrations exceeding the K_m, and one [α-^{32}P]dNTP at a concentration at or slightly above the K_m (2-3 µm) for a thermostable DNA polymerase such as *Taq*.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)

10x Amplification buffer

Carriers used during ethanol precipitation of radiolabeled probe (please see Step 5)
Use either *glycogen (stock solution = 50 mg/ml in H_2O)* or *yeast tRNA (stock solution 10 mg/ml in H_2O).*

⚠ Chloroform

Ethanol

TE (pH 7.6)

Enzymes and Buffers

Thermostable DNA polymerase (e.g., *Taq* DNA polymerase)

Nucleic Acids and Oligonucleotides

⚠ dCTP (0.1 mM)
Dilute 1 volume of a stock solution of 10 mM dCTP with 99 volumes of 10 mM Tris-Cl (pH 8.0). Store the diluted solution at -20°C in 50-µl aliquots.

dNTP solution containing dATP, dGTP, and dTTP, each at 10 mM

Forward primer (20 µM) in H_2O and Reverse primer (20 µM) in H_2O
Each primer should be 20-30 nucleotides in length and contain approximately equal numbers of the four bases, with a balanced distribution of G and C residues and a low propensity to form stable secondary structures. Store the stock solutions of primers at -20°C.

Template DNA (2-10 ng)
A variety of templates can be used in this protocol, including crude minipreparations of plasmid DNAs (please see Chapter 1, Protocol 1), purified bacteriophage λ DNA or plaque eluates (please see Chapter 2, Protocol 23), bacteriophage M13 single-stranded or double-stranded DNAs (please see Chapter 3, Protocol 5), purified DNA fragments isolated from agarose or polyacrylamide gels (please see Chapter 5, Protocol 1), and genomic DNAs from organisms with low complexity (e.g., bacteria and yeast).

Radioactive Compounds

⚠ [α-^{32}P]dCTP (10 mCi/ml, sp. act. 3000 Ci/mmole)

METHOD

1. In a 0.5-ml thin-walled microfuge tube, set up an amplification/radiolabeling reaction containing:

10x amplification buffer	5.0 µl
10 mM dNTP solution	1.0 µl
0.1 mM dCTP	1.0 µl
20 µM forward oligonucleotide primer	2.5 µl
20 µM reverse oligonucleotide primer	2.5 µl
template DNA (2-10 ng or approx. 1 fmole)	5-10 µl
10 mCi/ml [α-^{32}P]dCTP (sp. act. 3000 Ci/mmole)	5.0 µl
H_2O	to 48 µl

   Add 2.5 units of thermostable DNA polymerase to the reaction mixture. Gently tap the side of the tube to mix the ingredients.
   If more than one DNA fragment is to be radiolabeled using a single pair of primers, make up and dispense a master mix consisting of all the reaction components except the DNA templates to the PCR tubes. Individual DNA templates can then be added to each tube just before addition of enzyme and initiation of the reaction.
2. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixture with 1 drop (50 µl) of light mineral oil or a bead of paraffin wax. Place the tubes in a thermal cycler.
3. Amplify the samples using the denaturation, annealing, and polymerization times listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
30 cycles	30-45 sec at 94°C	30-45 sec at 55-60°C	1-2 min at 72°C
Last cycle	1 min at 94°C	30 sec at 55°C	1 min at 72°C

   Times and temperatures may need to be adapted to suit the particular reaction conditions.
   Polymerization should be carried out for 1 minute for every 1000 bp of length of the target DNA.
4. Remove the tubes from the thermal cycler. Use a micropipettor to remove as much mineral oil from the top of the reaction mixture as possible. Extract the reaction mix with 50 µl of chloroform. Separate the aqueous and organic layers by centrifugation for 1 minute at room temperature in a microfuge.
5. Remove the upper, aqueous layer to a fresh microfuge tube, add carrier tRNA (10-100 µg) or glycogen (5 µg), and precipitate the DNA with an equal volume of 4 M ammonium acetate and 2.5 volumes of ethanol. Store the tube for 1-2 hours at -20°C or for 10-20 minutes at -70°C. Collect the precipitated DNA by centrifugation at maximum speed for 5-10 minutes at 4°C.
6. Dissolve the DNA in 20 µl of TE (pH 7.6) and remove remaining unincorporated dNTPs and the oligonucleotide primers by spun-column chromatography through Sephadex G-75.
7. Use a liquid scintillation counter to measure the amount of radioactivity in 1.0 µl of the void volume of the spun column. Store the remainder of the radiolabeled DNA at -20°C until required.

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Protocol 4

Synthesis of Single-stranded DNA Probes of Defined Length from Bacteriophage M13 Templates

A synthetic oligonucleotide annealed to single-stranded DNA derived from a recombinant bacteriophage M13 or phagemid template is used to prime the synthesis of complementary radiolabeled DNA. Synthesis is catalyzed by the Klenow fragment of *E. coli* DNA polymerase I, which extends the annealed primer for various distances along the single-stranded template DNA. The products of the reaction, which are heterogeneous both in length and in the amount of incorporated radiolabeled dNTPs, are digested with a restriction enzyme to create double-stranded DNA fragments of uniform length, which are subsequently purified by agarose gel electrophoresis.
The oligonucleotide primer is usually complementary to a region of the *lac* gene immediately 3´ to the polycloning site in the mp series of bacteriophage M13 vectors. This "universal" primer, which is sold by several companies, has the advantage that it can be used to prepare probes complementary to any segment of DNA that has been cloned into any restriction site in the polylinker of the vector. However, custom-made oligonucleotides complementary to specific sequences within the cloned DNA can also be used to prepare probes that represent only a portion of the cloned sequence.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
DTT (Dithiothreitol) (1 M)
EDTA (0.5 M, pH 8.0)
Ethanol
NaCl (5 M)
⚠ Phenol:chloroform (1:1, v/v)
5x TBE
Optional, please see Step 16.
Tris-Cl (1 M, pH 7.6)
Optional, please see Step 16.

Enzymes and Buffers

10x Klenow basic buffer
Klenow fragment of *E. coli* DNA polymerase I
Restriction enzyme(s), as appropriate for generation of the desired probe
The restriction enzyme used to cleave the products of the labeling reaction should cleave between 200 bp and 1 kb downstream from the primer-binding site.

Nucleic Acids and Oligonucleotides

dNTP solution containing unlabeled dCTP, dGTP, and dTTP, each at 20 mM
The composition of this solution depends on the [α-^{32}P]dNTP to be used. If, as in this protocol, radiolabeled dATP is used, the mix should contain dCTP, dTTP, and dGTP each at a concentration of 20 mM. If two radiolabeled dNTPs are used, this solution should contain the other two dNTPs.
dATP (40 µM and 20 mM)
Oligonucleotide primer
The best results are obtained with a purified oligonucleotide (bacteriophage M13 Universal Primer or custom-synthesized oligonucleotide) (please see the purification strategies in Chapter 10, Protocol 1). Resuspend the oligonucleotide at a concentration of 5 pmoles/ml in TE (pH 7.6).
Template DNA
Purify the single-stranded bacteriophage M13 or phagemid DNA as described in Chapter 3, Protocol 4 or Chapter 3, Protocol 8 . Measure the concentration of the DNA by absorption spectrophotometry or staining with SYBR Gold. Approximately 1 µg of template DNA in a volume of 15 µl or less is required for each synthetic reaction.

Radioactive Compounds

⚠ [α-^{32}P]dATP (10 mCi/ml, sp. act. 800-3000 Ci/mmole)

Additional Reagents

Step 17 of this protocol requires the reagents listed in Chapter 5, Protocol 4 or Chapter 5, Protocol 12 .

METHOD

1. In a 0.5-ml microfuge tube, mix:

single-stranded template (bacteriophage M13 or phagemid DNA) (approx. 0.5 pmole)	1 µg
oligonucleotide primer	5 pmoles
10x Klenow basic buffer	3 µl
H_2O	to 20 µl

2. Heat the mixture to 85°C for 5 minutes and then let it cool slowly to 37°C.
This slow cooling can be achieved by floating the microfuge tube in a piece of Styrofoam in a 250-ml beaker filled with water equilibrated to 85°C. Place the beaker containing the primer-template mix at room temperature until the temperature of the water falls to 37°C (approx. 30 minutes). The same result can be achieved using a thermal cycler programmed to heat at 85°C for 5 minutes before ramping down to 37°C over a 30-minute period.
3. To the tube of annealed primer and template, add:

0.1 M dithiothreitol	2 µl
10 mCi/ml [α-^{32}P]dATP (sp. act. 3000 Ci/mmole)	5 µl
40 µM dATP	1 µl
20 mM solution of dTTP, dCTP, and dGTP	1 µl

Mix the reagents by gently tapping the side of the tube. Centrifuge the tube at maximum speed for 1-2 seconds in a microfuge to transfer all of the liquid to the bottom.
As an alternative, substitute [α-^{32}P]dNTPs of sp. act. 800 Ci/mmole in this reaction, in which case, omit the 1 µl of 40 µM dATP.
4. Transfer 0.5 µl of the mixture to a microfuge tube containing 15 µl of 20 mM EDTA (pH 8.0). Store the tube on ice.
5. Add 1 µl (5 units) of the Klenow fragment to the remainder of the mixture. Mix the components of the reaction by gently

tapping the side of the tube. Incubate the reaction for 30 minutes at room temperature.
Approximately 5 units of the Klenow fragment are required for each labeling reaction.

6. Transfer 0.5 µl of the reaction to a fresh microfuge tube containing 20 µl of 0.5 M EDTA (pH 8.0). Store the tube on ice.
7. Add 1 µl of 20 mM unlabeled dATP to the remainder of the reaction. Mix by gently tapping the side of the tube. Centrifuge the tube at maximum speed for 1-2 seconds in a microfuge to transfer all of the liquid to the bottom. Incubate the reaction mixture for a further 20 minutes at room temperature.
8. During the 20-minute incubation (Step 7), measure the fraction of radioactivity in the samples stored in Steps 4 and 6 that either has become insoluble in 10% TCA or that adheres to a DE-81 filter.
9. Heat the reaction to 68°C for 10 minutes to inactivate the Klenow fragment.
10. Adjust the concentration of NaCl in the reaction to achieve optimal conditions for cleavage of the product by the selected restriction enzyme.
    The concentration of NaCl in the primer-extension reaction is 50 mM.
    If the restriction enzyme works best in the absence of NaCl, or if the enzyme requires unusual conditions (e.g., high concentrations of Tris or the presence of detergent), transfer the radiolabeled DNA to the appropriate buffer.
11. Add 20 units of the desired restriction enzyme, and incubate the reaction for 1 hour at the appropriate temperature.
12. Purify the DNA by standard extraction with phenol:chloroform, and remove unincorporated dNTPs by spun-column chromatography or differential precipitation with 2.5 M ammonium acetate and ethanol (please see Appendix 8 in the print version of the manual). Add 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM.
    IMPORTANT *It is essential that free magnesium ions not be present when the DNA is exposed to alkali in the next step. Otherwise, the DNA will form an insoluble complex with $Mg(OH)_2$.*
13. Isolate the radiolabeled DNA by electrophoresis through a denaturing polyacrylamide gel (please see Chapter 12, Protocol 8) or an alkaline agarose gel (please see Chapter 5, Protocol 8), depending on the size of the fragment.
14. After electrophoresis, prepare the gel for autoradiography. Expose the gel to X-ray film for 5-10 minutes.
15. Align the developed film with the gel, using the images from the phosphorescent dots (Glogos) or radioactive ink spots. Tape the film to the gel and mark the position of the primer-extended radioactive DNA fragment on the back of the gel plate. Remove the film and cut out the segment of the gel containing the desired fragment of DNA.
16. If a polyacrylamide gel was used to separate the DNA fragment from the template, proceed to Step 17. If an alkaline agarose gel was used, neutralize the gel by gentle shaking for 45 minutes in 0.5 M Tris-Cl (pH 7.6), followed by shaking for an additional 45 minutes in 0.5x TBE before the radiolabeled DNA is eluted.
17. Extract the DNA from the gel by electroelution or by crushing and soaking the polyacrylamide gel slice in an appropriate buffer (please see Chapter 5, Protocol 4 and Chapter 5, Protocol 12 , respectively).
    Check the efficiency of the elution process with a hand-held minimonitor.
    The eluted DNA is ready for use as a probe and does not need to be denatured.

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Protocol 5

Synthesis of Single-stranded DNA Probes of Heterogeneous Length from Bacteriophage M13 Templates

The following technique yields a heterogeneous population of short radiolabeled molecules 200-300 nucleotides in length. These probes are synthesized, as in Protocol 4 (Chapter 9), by extension of an oligonucleotide primer on a single-stranded DNA template. The radiolabeled products of the reaction are then separated from the template by electrophoresis through a denaturing gel from which they are eluted directly into hybridization buffer. The method is useful for synthesizing single-stranded DNA probes of very high specific activity for Southern analysis of single-copy genes in complex genomes or for northern analysis of rare species mRNAs.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Elution buffer (9-5)
- ⚠ Formamide loading buffer
- $MgCl_2$ (1 M)
- ⚠ NaOH (10 N)
- 3x Probe synthesis buffer

Enzymes and Buffers

Klenow fragment of *E. coli* DNA polymerase I

Nucleic Acids and Oligonucleotides

Oligonucleotide primer (bacteriophage M13 Universal Primer or custom-synthesized oligonucleotide)
The best results are obtained with a purified oligonucleotide (please see the purification strategies in Chapter 10, Protocol 1). Resuspend the oligonucleotide at a concentration of 5 pmoles/µl in TE (pH 7.6).

Template DNA
Purify the single-stranded bacteriophage M13 or phagemid DNA as described in Chapter 3, Protocol 4 or Chapter 3, Protocol 8 , respectively. Measure the concentration of the DNA by absorption spectrophotometry.
Approximately 0.3 µg of template DNA in a volume of 2-3 µl is required for each synthesis.

Radioactive Compounds

⚠ [α-^{32}P]dCTP (10 mCi/ml, sp. act. 800-3000 Ci/mmole)

METHOD

1. In a 0.5-ml microfuge tube, mix:

single-stranded template (bacteriophage M13 or phagemid DNA) (approx. 0.15 pmole, approx. 0.1 µg/µl)	0.3 µg
oligonucleotide primer (5 pmoles/µl)	1 µl
25 mM $MgCl_2$	1 µl

   If possible, include as positive control in each experiment a reaction tube containing a control DNA template and an oligonucleotide that has worked well previously.
2. Cap the tube and incubate the reaction mixture for 5-10 minutes at 57°C in a heating block.
3. Transfer the tube from the heating block to a bucket of ice, and add to the reaction mixture:

3x probe synthesis buffer	4 µl
10 mCi/ml [α-^{32}P]dCTP (sp. act. 3000 Ci/mmole)	3 µl
Klenow fragment (approx. 2.5 units)	0.5 µl

   Mix the reagents by gently tapping the side of the tube. Centrifuge the tube at maximum speed for 1-2 seconds in a microfuge. Incubate the reaction at 37°C for 40 minutes.
4. While the primer reaction is incubating, pour a 5% polyacrylamide gel containing 7 M urea in 1x TBE buffer (please see Chapter 12, Protocol 8). The gel should be 1.5-mm thick, at least 15-cm long, with 2.5-cm wide slots. Prerun the gel for 15 minutes at 20-25 V/cm of gel length to remove ammonium persulfate from the wells.
5. Stop the primer-extension reaction by adding 25 µl of formamide loading buffer and heating the reaction mixture in a boiling water bath for 3-5 minutes. Transfer the tube to an ice bucket and add 1 µl of 1 N NaOH.
6. Wash urea and loose fragments of polyacrylamide from the wells of the gel using a syringe loaded with 1x TBE, and immediately load the DNA samples. Separate the radiolabeled probe from the template DNA by electrophoresis for 30 minutes at 20-25 V/cm of gel length.
   It is unnecessary to include DNA size standards on the polyacrylamide gel because the xylene cyanol dye migrates to a position comparable to that of a DNA fragment of approx. 125 bases.
7. After 30 minutes, separate the gel plates, cover the gel with Saran Wrap, and locate the radiolabeled DNA by autoradiography. Expose the gel to X-ray film for approx. 1 minute.
   The majority of the radiolabeled DNA should have migrated slightly slower than the xylene cyanol (green) tracking dye. Although a continuous smear of radiolabeled material is the expected result, in practice, between three and ten bands of discrete length are usually detected that may be spaced over a distance of 2-3 cm of the gel. The cause of the discrete bands is presumed to be secondary structures in the particular single-stranded DNA template used in the reaction.
8. Cut the radioactive bands out of the gel with a clean razor blade. Place them in the bottom of a 12 x 17-mm plastic snap-cap tube and crush with a disposable inoculating stick. Add 1-2 ml of elution buffer and shake the fragments of gel for >3 hours at 50°C.
   Elution of the radioactive probe is more efficient if individual bands are cut from the gel, rather than excising a wide swath of polyacrylamide that spans all the bands.
9. Place the eluate in a fritted column, place the column in a 12 x 17-mm plastic snap-cap tube, and centrifuge in a desktop clinical centrifuge for 1-2 minutes. Measure the amount of radioactivity in 1 µl of the radioactive probe by liquid scintillation spectroscopy.

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Protocol 6

Synthesis of Single-stranded RNA Probes by In Vitro Transcription

This protocol describes a procedure for synthesizing RNA probes of high specific activity (1 x 10^9 dpm/mg) from double-stranded linear DNAs containing promoters for bacteriophage-encoded RNA polymerases. **IMPORTANT** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
Optional, please see Step 8.

Bovine serum albumin (2 mg/ml, Fraction V, Sigma)

DTT (Dithiothreitol) (1 M)

Ethanol

⚠ Phenol:chloroform (1:1, v/v)

Placental RNase inhibitor (20 units/µl)

Sodium acetate (3 M, pH 5.2)

10x Transcription buffer (9-6)

Enzymes and Buffers

Appropriate restriction enzymes
Please see Step 1.

DNA-dependent RNA polymerase of bacteriophage T3, T7, or SP6
These enzymes of bacteriophage T3, T7, or SP6 are available from several companies and are usually supplied at concentrations of 10-20 units/µl.

RNase-free pancreatic DNase I (1 mg/ml)

Nucleic Acids and Oligonucleotides

rNTP solution containing rATP, rCTP, and rUTP, each at 5 mM

⚠ rGTP (0.5 mM)
Optional, please see Step 5.

Template DNA
The DNA fragment to be transcribed should be cloned into one of the commercially available plasmids containing bacteriophage RNA polymerase promoters on both sides of the polycloning sequence (e.g., pGEM from Promega or pBluescript from Stratagene). Purify the superhelical recombinant plasmid by one or more of the methods described in Chapter 1.

Radioactive Compounds

⚠ [α-^{32}P]rGTP (10 mCi/ml, sp. act. 400-3000 Ci/mmole)

Additional Reagents

Step 2 of this protocol requires the reagents listed in Chapter 9, Protocol 11 .

Step 8 of this protocol may require the reagents listed in Chapter 5, Protocol 9 , Chapter 5, Protocol 11 , and Chapter 5, Protocol 12 .

METHOD

1. Prepare 5 pmoles of linear template DNA by complete digestion of superhelical plasmid DNA with a suitable restriction enzyme. Analyze an aliquot (100 ng) of the digested DNA by agarose gel electrophoresis.
2. If restriction enzymes, such as *Pst*I or *Sst*I, that generate protruding 3´ termini must be used, treat the digested DNA with bacteriophage T4 DNA polymerase in the presence of all four dNTPs to remove the 3´ protrusion (please see Chapter 9, Protocol 11).
3. Purify the template DNA by extraction with phenol:chloroform and standard precipitation with ethanol. Dissolve the DNA in H_2O at a concentration of 100 nM (i.e., 200 µg/ml for a 3-kb plasmid).
4. Warm the first six components listed below to room temperature, and in a sterile 0.5-ml microfuge tube, mix *in the following order at room temperature:*

template DNA	0.2 pmole (400 ng for a 3-kb plasmid)
RNase-free H_2O	to 6 µl
5 mM rNTP solution	2 µl
100 mM dithiothreitol	2 µl
10x transcription buffer (9-6)	2 µl
2 mg/ml bovine serum albumin	1 µl
10 mCi/ml [α-^{32}P]rGTP (sp. act. 400-3000 Ci/mmole)	5 µl

Mix the components of the mixture by gently tapping the outside of the tube. Then add:

placental RNase inhibitor (10 units)	1 µl
bacteriophage DNA-dependent RNA polymerase (approx. 10 units)	1 µl

Mix the reagents by gently tapping the outside of the tube. Centrifuge the tube for 1-2 seconds to transfer all of the liquid to the bottom. Incubate the reaction for 1-2 hours at 37°C (bacteriophages T3 and T7 DNA-dependent RNA polymerases) or 40°C (bacteriophage SP6 DNA-dependent RNA polymerase).
The reaction may be scaled from 20 µl to 50 µl to accommodate more dilute reagents.
When the reaction is carried out as described above, 80-90% of the radiolabel will be incorporated into RNA. The yield of RNA will be approx. 20 ng (sp. act. 4.7 x 10^9 dpm/µg) when the specific activity of the [α-^{32}P]GTP is 3000 Ci/mmole and approx. 150 ng (sp. act. 6.2 x 10^8 dpm/µg) when the specific activity of the precursor is 400 Ci/mmole.
5. (*Optional*) If full-length transcripts are desired, add 2 µl of 0.5 mM rGTP and incubate the reaction mixture for an additional 60 minutes at the temperature appropriate for the polymerase.
6. Terminate the in vitro transcription reaction by adding 1 µl of 1 mg/ml RNase-free pancreatic DNase I to the reaction tube. Mix the reagents by gently tapping the outside of the tube. Incubate the reaction mixture for 15 minutes at 37°C.
7. Add 100 µl of RNase-free H_2O, and purify the RNA by extraction with phenol:chloroform.

If the probe will be used in experiments where length is important (e.g., RNase protection), purify the radiolabeled RNA by polyacrylamide gel electrophoresis (please see Chapter 5, Protocol 9).

8. Transfer the aqueous phase to a fresh microfuge tube, and separate the radiolabeled RNA from undesired small RNAs and rNTPs by one of three methods:

To purify RNA by ethanol precipitation

a. Add 30 µl of 10 M ammonium acetate to the aqueous phase. Mix, and then add 250 µl of ice-cold ethanol to the tube. After storage for 30 minutes on ice, collect the RNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge.

b. Remove as much of the ethanol as possible by gentle aspiration, and leave the open tube on the bench for a few minutes to allow the last visible traces of ethanol to evaporate. Dissolve the RNA in 100 µl of RNase-free H_2O.

c. Add 2 volumes of ice-cold ethanol to the tube and store the RNA at -70°C until needed.
To recover the RNA, transfer an aliquot of the ethanolic solution to a fresh microfuge tube. Add 0.25 volume of 10 M ammonium acetate, mix, and then store the tube for at least 15 minutes at -20°C. Centrifuge the solution at maximum speed for 10 minutes at 4°C in a microfuge, Remove the ethanol by aspiration, and dissolve the RNA in the desired volume of the appropriate RNase-free buffer.

To purify RNA by spun-column chromatography

a. Prepare a Sephadex G-50 spun column that has been autoclaved in 10 mM Tris-Cl (pH 7.5).

b. Purify the RNA by spun-column chromatography.

c. Store the eluate in a microfuge tube at -70°C until the RNA is needed.

To purify RNA by gel electrophoresis

a. Prepare a neutral polyacrylamide gel according to Chapter 5, Protocol 9 .

b. Add the appropriate gel-loading buffer to the aqueous phase and purify the RNA by gel electrophoresis.

c. Locate the RNA by autoradiography according to Chapter 5, Protocol 11 .

d. Purify the RNA from the gel slice using the crush and soak method according to Chapter 5, Protocol 12 .

e. Store the RNA at -70°C until needed.

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2. Melton D.A., Krieg P.A., Rebagliati M.R., Maniatis T., Zinn K., and Green M.R. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035-7056.

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Protocol 7

Synthesis of cDNA Probes from mRNA Using Random Oligonucleotide Primers

This protocol describes the generation of cDNA probes from poly(A)$^+$ mRNA using random oligonucleotide primers. Probes of this type are used for differential screening of cDNA libraries. **IMPORTANT** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)

DTT (Dithiothreitol) (1 M)

EDTA (0.5 M, pH 8.0)

Ethanol

⚠ HCl (2.5 N)

⚠ NaOH (3 N)

⚠ Phenol:chloroform (1:1, v/v)

Placental RNase inhibitor (20 units/µl)
These inhibitors are sold by several manufacturers under various trade names (e.g., RNAsin, Promega; Prime Inhibitor, 5 Prime->3 Prime; RNaseOUT, Life Technologies).

⚠ SDS (10% w/v)

Tris-Cl (1 M, pH 7.4)

Enzymes and Buffers

Reverse transcriptase
The cloned version of reverse transcriptase encoded by the Mo-MLV enzyme is the enzyme of choice in this protocol.

10x Reverse transcriptase buffer

Nucleic Acids and Oligonucleotides

dNTP solution containing dATP, dGTP, and dTTP, each at 20 mM

⚠ dCTP (125 µM)
Add 1 µl of a 20 mM stock solution of dCTP to 160 µl of 25 mM Tris-Cl (pH 7.6). Store the diluted solution in small aliquots at -20°C.

Random deoxynucleotide primers, six or seven bases in length

Template mRNA
Prepare poly(A)$^+$ RNA as described in Chapter 7, Protocol 3 or Chapter 7, Protocol 4 , and dissolve in RNase-free H_2O at a concentration of 250 µg/ml.

Radioactive Compounds

⚠ [α-^{32}P]dCTP (10 mCi/ml, sp. act. >3000 Ci/mmole)

METHOD

1. Transfer 1 µg of poly(A)$^+$ RNA to a sterile microfuge tube. Adjust the volume of the solution to 4 µl with RNase-free H_2O. Heat the closed tube for 5 minutes at 70°C, and then quickly transfer the tube to an ice-water bath.
2. To the chilled solution in the microfuge tube, add:

10 mM dithiothreitol	2.5 µl
placental RNase inhibitor	20 units
random deoxyoligonucleotide primers	5 µl
10x reverse transcriptase buffer	2.5 µl
20 mM solution of dGTP, dATP, and dTTP	1 µl
125 µM solution of dCTP	1 µl
10 mCi/ml [α-^{32}P]dCTP (sp. act. >3000 Ci/mmole)	10 µl
RNase-free H_2O	to 24 µl
reverse transcriptase (200 units)	1 µl

IMPORTANT *Add the reverse transcriptase last.*
Reverse transcriptase supplied by different manufacturers varies in its activity per unit. When using a new batch of enzyme, set up a series of extension reactions containing equal amounts of poly(A)$^+$ RNA and oligonucleotide primer and different amounts of enzyme. If possible, the primer should be specific for an mRNA present at moderate abundance in the preparation of poly(A)$^+$ RNA. Assay the products of each reaction by gel electrophoresis as described in this protocol. Use the minimal amount of enzyme required to produce the maximum yield of extension product. The units used in this protocol work well with most batches of StrataScript and Superscript II.
Mix the components by gently tapping the side of the tube. Remove bubbles by brief centrifugation in a microfuge. Incubate the reaction mixture for 1 hour at 45°C.
As an alternative, [α-^{32}P]dCTP of specific activity 800 Ci/mmole can be substituted in this reaction. If this substitution is made, then omit the 125 µM dCTP from the reaction mixture.

3. Stop the reaction by adding:

0.5 M EDTA (pH 8.0)	1 µl
10% (w/v) SDS	1 µl

Mix the reagents in the tube completely.

4. Add 3 µl of 3 N NaOH to the reaction tube. Incubate the mixture for 30 minutes at 68°C to hydrolyze the RNA.
5. Cool the reaction mixture to room temperature. Neutralize the solution by adding 10 µl of 1 M Tris-Cl (pH 7.4), mixing well, and then adding 3 µl of 2.5 N HCl. Check the pH of the solution by spotting a very small amount on pH paper.
6. Purify the cDNA by extraction with phenol:chloroform.
7. Separate the radiolabeled probe from the unincorporated dNTPs by either spun-column chromatography or selective precipitation by ethanol in the presence of 2.5 M ammonium acetate.
8. Measure the proportion of radiolabeled dNTPs that either are incorporated into material precipitated by TCA or adhere to a DE-81 filter.
If a larger amount of radiolabeled cDNA is required, then scale up the reaction by increasing the volumes of all components proportionally. It is important to maintain a ratio of 200 units of reverse transcriptase/µg of input mRNA to

ensure maximum yield.

The purified radiolabeled cDNA can be used for hybridization without denaturation. Use 5 x 10^7 dpm of radiolabeled cDNA for each 150-mm filter and 5 x 10^6 to 1 x 10^7 dpm for each 90-mm filter.

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Protocol 8

Synthesis of Radiolabeled, Subtracted cDNA Probes Using Oligo(dT) as a Primer

This protocol describes the preparation of subtracted cDNA probes by hybridization to an mRNA driver, followed by purification of the single-stranded radiolabeled cDNA by hydroxyapatite chromatography. For further information, please see page 9.89 in the print version of the manual. Before preparing the probe, it is a good idea to have filters (which contain the cDNA library to be screened) ready to hybridize. **IMPORTANT** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- ⚠ Ammonium acetate (10 M)
- DTT (Dithiothreitol) (1 M)
- EDTA (0.5 M, pH 8.0)
- Ethanol
- ⚠ HCl (2.5 N)
- ⚠ Isobutanol
- ⚠ NaOH (3 N)
- ⚠ Phenol:chloroform (1:1, v/v)
- Placental RNase inhibitor (20 units/µl)
  These inhibitors are sold by several manufacturers under various trade names (e.g., RNAsin, Promega Inc; Prime Inhibitor, 5 Prime->3 Prime; RNaseOUT, Life Technologies).
- ⚠ SDS (10% w/v)
- SDS/EDTA solution
- Sodium phosphate buffer (2 M, pH 6.8)
- SPS buffer
- Tris-Cl (1 M, pH 7.4)

Enzymes and Buffers

Reverse transcriptase
The cloned version of reverse transcriptase encoded by the Mo-MLV enzyme is the enzyme of choice in this protocol.

- 10x Reverse transcriptase buffer

Nucleic Acids and Oligonucleotides

- ⚠ dCTP (125 µM)
  Add 1 µl of a 20 mM stock solution of dCTP to 160 µl of 25 mM Tris-Cl (pH 7.6). Store the diluted solution in small aliquots at -20°C.
- dNTP solution containing dATP, dGTP, and dTTP, each at 20 mM

Driver mRNA
Please see Steps 9 and 19.

Oligo$(dT)_{12\text{-}18}$
Purchase and dissolve oligo$(dT)_{12\text{-}18}$ at 1 mg/ml in TE (pH 7.6). Store at -20°C. Random deoxynucleotide primers can also be substituted in the cDNA synthesis reaction (please see Chapter 9, Protocol 7). The choice of primer depends on the method used to construct the cDNA library to be screened. Use oligo(dT) as a primer in this protocol if the cDNA library was constructed with oligo(dT). Use random primers if the cDNA library was manufactured in any other way.

Template mRNA
Prepare poly(A)$^+$ RNA as described in Chapter 7, Protocol 3 or Chapter 7, Protocol 4 , and dissolve in RNase-free H_2O at a concentration of 250 µg/ml.

Radioactive Compounds

⚠ [α-^{32}P]dCTP (10 mCi/ml, sp. act. >3000 Ci/mmole)

METHOD

1. Transfer 5-10 µg of poly(A)$^+$ RNA to a sterile microfuge tube. Adjust the volume of the solution to 40 µl with RNase-free H_2O. Heat the closed tube to 70°C for 5 minutes, and then quickly transfer the tube to an ice water bath.
2. To the chilled microfuge tube, add:

0.1 M dithiothreitol	2.5 µl
placental RNase inhibitor	200 units
oligo$(dT)_{12\text{-}18}$	10 µl
10x reverse transcriptase buffer	25 µl
20 mM solution of dGTP, dATP, and dTTP	10 µl
125 µM dCTP	10 µl
10 mCi/ml [α-^{32}P]dCTP (sp. act. >3000 Ci/mmole)	100 µl
RNase-free H_2O	to 240 µl
reverse transcriptase (2000 units)	10 µl

   IMPORTANT *Add the reverse transcriptase last.*
   Mix the components by gently tapping the side of the tube. Collect the reaction mixture in the bottom of the tube by brief centrifugation in a microfuge. Incubate the reaction for 1 hour at 45°C.
   As an alternative, [α-^{32}P]dCTP of specific activity 800 Ci/mmole can be substituted in this reaction. If this substitution is made, then omit the 125 µM dCTP from the reaction mixture.
3. Stop the reaction by adding:

0.5 M EDTA (pH 8.0)	10 µl
10% (w/v) SDS	10 µl

   Mix the reagents in the tube well.
4. Add 30 µl of 3 N NaOH to the reaction tube. Incubate the mixture for 30 minutes at 68°C to hydrolyze the RNA.

5. Cool the mixture to room temperature. Neutralize the solution by adding 100 µl of 1 M Tris-Cl (pH 7.4), mixing well, and then adding 30 µl of 2.5 N HCl. Check the pH of the solution by spotting <1 µl on pH paper.
6. Purify the cDNA by extraction with phenol:chloroform.
7. Measure the proportion of radiolabeled dNTPs that either are incorporated into material precipitated by TCA or adhere to a DE-81 filter (please see Appendix 8 in the print version of the manual). Calculate the yield of cDNA as follows:
   In a reaction containing 1.5 nmoles of the limiting dNTP:
   cpm incorporated x 1.5 nmoles dCTP x 330 ng/nmole x 160 = ng of cDNA synthesized
   total cpm
8. Separate the radiolabeled probe from the unincorporated dNTPs by chromatography through a 5-ml column of Sephadex G-50.
   IMPORTANT *Perform this step and all subsequent steps with siliconized tubes.*
9. To the radiolabeled cDNA, add tenfold excess by weight of the driver RNA that will be used to subtract the cDNA probe, 0.2 volume of 10 M ammonium acetate, and 2.5 volumes of ice-cold ethanol. Incubate the mixture for 10-15 minutes at 0°C, and then recover the nucleic acids by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.
10. Remove all of the ethanol by aspiration, and store the open tube on the bench for a few minutes. Dissolve the nucleic acids in 6 µl of RNase-free H_2O.
11. To the dissolved nucleic acids, add:

2 M sodium phosphate (pH 6.8)	2 µl
SDS/EDTA solution	2 µl

12. *Either* cover the solution with a drop of light mineral oil *or* draw the mixture into a siliconized, disposable 20-µl glass capillary tube and seal the ends of the tube in the flame of a Bunsen burner.
13. Place the microfuge tube or sealed capillary tube in a boiling water bath for 5 minutes. Transfer to a water bath set at 68°C, and allow the nucleic acids to hybridize to $C_{r_o}t$ = 1000 moles seconds/liter.To calculate the time required to reach value, please see page 9.44 of the print version of the manual.
14. Remove the microfuge tube or capillary tube from the water bath. Use a drawn-out pipette tip attached to a micropipettor to remove the hybridization solution from the microfuge tube, or open the ends of the capillary tube with a file or diamond pen. Transfer the hybridization mixture into a tube containing 1 ml of SPS buffer.
15. Separate the single-stranded and double-stranded nucleic acids by chromatography on hydroxyapatite at 60°C.
    Measure the amount of radioactivity in each fraction by liquid scintillation counting. At least 90% of the input [^{32}P]cDNA should have hybridized to the mRNA and be present in the >0.36 M sodium phosphate wash.
16. Pool the fractions containing the single-stranded cDNA and concentrate them by *repeated* extractions with isobutanol extraction: Add an equal volume of isobutanol. Mix the two phases by vortexing, and centrifuge the mixture at maximum speed for 2 minutes at room temperature in a microfuge. Discard the upper (organic) phase. Repeat the extraction with isobutanol until the volume of the aqueous phase is <100 µl.
17. Remove salts from the cDNA by spun-column chromatography through Sephadex G-50 equilibrated in TE (pH 8.0) containing 0.1% SDS.
    IMPORTANT *Do not use ethanol precipitation to concentrate the cDNA as the presence of phosphate ions interferes with precipitation. Do not use dialysis to remove phosphate ions, as the cDNA will stick to the dialysis bag.*
18. Measure the amount of radioactivity in the sample and calculate the weight of DNA in the subtracted probe.
19. Repeat Steps 9-18.
    Between 10% and 30% of the cDNA will form hybrids with the driver RNA during the second round of hybridization.
    It is not necessary to concentrate or remove salts from the final preparation of cDNA if it is to be used to probe a cDNA library. The radiolabeled cDNA can be used for hybridization without denaturation. The subtractive hybridizations should be carried out as rapidly as practicable, and the probe should be used without delay. Use 5 x 10^7 dpm of radiolabeled cDNA for each 150-mm filter and 5 x 10^6 to 1 x 10^7 dpm for each 90-mm filter.
    If a genomic DNA library is screened with the radiolabeled subtracted probe, oligo(dA) can be added to the prehybridization and hybridization reactions at 1 µg/ml to prevent nonspecific hybridization between the oligo(dT) tails of the cDNA and oligo(dA) tracts in the genomic DNA.

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Protocol 9

Radiolabeling of Subtracted cDNA Probes by Random Oligonucleotide Extension

In this procedure, synthesis of cDNA is carried out in the presence of saturating concentrations of all four dNTPs and trace amounts of a single radiolabeled dNTP. After subtraction hybridization, the enriched single-stranded cDNA is radiolabeled to high specific activity in a second synthetic reaction by extension of random oligonucleotide primers using the Klenow fragment of *E. coli* DNA polymerase. Because the concentrations of dNTP in the first reaction are nonlimiting, both the amounts and size of cDNA generated are greater than those achieved in standard labeling protocols. For further information, please see page 9.89 in the print version of the manual. **IMPORTANT** Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- DTT (Dithiothreitol) (1 M)
- EDTA (0.5 M, pH 8.0)
- Ethanol
- ⚠ HCl (2.5 N)
- ⚠ NaOH (3 N)
- ⚠ Phenol:chloroform (1:1, v/v)
- Placental RNase inhibitor
  These inhibitors are sold by several manufacturers under various trade names (e.g., RNAsin, Promega; Prime Inhibitor, 5 Prime->3 Prime; RNaseOUT, Life Technologies).
- 5x Random primer buffer
- ⚠ SDS (20% w/v)
- Sodium acetate (3 M, pH 5.2)
- Tris-Cl (1 M, pH 7.4)

Enzymes and Buffers

Klenow fragment of *E. coli* DNA polymerase I

Reverse transcriptase
The cloned version of reverse transcriptase encoded by the Mo-MLV enzyme is the enzyme of choice in this protocol.

- 10x Reverse transcriptase buffer

Nucleic Acids and Oligonucleotides

- dNTP solution (complete) containing four dNTPs, each at 5 mM
- dNTP solution containing dCTP, dGTP, and dTTP, each at 5 mM

Oligo$(dT)_{12-18}$
Purchase and dissolve oligo$(dT)_{12-18}$ at 1 mg/ml in TE (pH 7.6). Store at -20°C.

Random deoxynucleotide primers six or seven bases in length
Because of their uniform length and lack of sequence bias, synthetic oligonucleotides of random sequence are the primers of choice. Oligonucleotides of optimal length (hexamers and heptamers; Suganuma and Gupta 1995) can be purchased from a commercial source (e.g., Pharmacia and Boehringer Mannheim) or synthesized locally on an automated DNA synthesizer. Store the solution of primers at 0.125 µg/µl in TE (pH 7.6) at -20°C in small aliquots.

Template RNAs
Prepare two sets of template RNAs: two-pass poly$(A)^+$-enriched mRNA prepared from cells or tissue that expresses the mRNA(s) of interest and two-pass poly$(A)^+$-enriched mRNA prepared from cells or tissue that does not express the mRNA(s) of interest. For mRNA preparation and oligo(dT) chromatography, please see Chapter 7. Both sets of RNAs should be dissolved in H_2O at a concentration of approx. 1 mg/ml.

Radioactive Compounds

- ⚠ [α-^{32}P]dATP (10 mCi/ml, sp. act. >3000 Ci/mmole)
- ⚠ [α-^{32}P]dCTP (10 mCi/ml, sp. act. 800-3000 Ci/mmole)

Additional Reagents

Steps 8 and 9 of this protocol require reagents listed in Chapter 9, Protocol 8.

METHOD

1. To synthesize first strand cDNA, mix the following ingredients at 4°C in a sterile microfuge tube:

template RNA (1 mg/ml)	10 µl
oligo$(dT)_{12-18}$ (1 mg/ml)	10 µl
5 mM dNTP solution (complete)	10 µl
50 mM dithiothreitol	1 µl
10x reverse transcriptase buffer	5 µl
10 mCi/ml [α-^{32}P]dCTP (sp. act. 800 or 3000 Ci/mmole)	5 µl
placental RNase inhibitor	25 units
RNase-free H_2O	to 46 µl
reverse transcriptase (approx. 800 units)	4 µl

   IMPORTANT *Add the reverse transcriptase last.*
   Mix the components by gently tapping the side of the tube. Collect the reaction mixture in the bottom of the tube by brief centrifugation in a microfuge. Incubate the reaction for 1 hour at 45°C.
   [α-^{32}P]dCTP is used as a tracer to measure the synthesis of the first strand of cDNA.
2. Measure the proportion of radiolabeled dNTPs that either are incorporated into material precipitated by TCA or adhere to a DE-81 filter. Calculate the yield of cDNA using the equation below.
   In a reaction containing 50 nmoles of each dNTP:
   $\frac{\text{cpm incorporated}}{\text{total cpm}} \times 200 \text{ nmoles dNTP} \times 330 \text{ ng/nmole} = \text{ng of cDNA synthesized}$

3. Stop the reaction by adding:

0.5 M EDTA (pH 8.0)	2 µl
20% (w/v) SDS	2 µl

Mix the reagents in the tube completely.
The single-stranded, radiolabeled cDNA is quite sticky and adheres nonspecifically to glass, filters, and some plastics. For this reason, it is important to maintain a minimum of 0.05% (w/v) SDS in Step 3 of the protocol, and 0.1-1.0% SDS in hybridization buffers.

4. Add 5 µl of 3 N NaOH to the reaction tube. Incubate the mixture for 30 minutes at 68°C to hydrolyze the RNA.
5. Cool the mixture to room temperature. Neutralize the solution by adding 10 µl of 1 M Tris-Cl (pH 7.4), mixing well, and then adding 5 µl of 2.5 N HCl. Check the pH of the solution by spotting <1 µl on pH paper.
6. Purify the cDNA by extraction with phenol:chloroform.
7. Separate the radiolabeled probe from the unincorporated dNTPs by chromatography through a spun column of Sephadex G-50.
IMPORTANT *Perform this step and all subsequent steps with siliconized tubes.*
8. Carry out two rounds of subtractive hybridization as described in Chapter 9, Protocol 8 , Steps 9-19.
9. Concentrate the final preparation of cDNA by sequential extractions with isobutanol, and remove salts by chromatography on Sephadex G-50 as described in Chapter 9, Protocol 8 , Steps 16 and 17.
10. Recover the cDNA by standard precipitation with ethanol. Dissolve the cDNA in H_2O at a concentration of 15 ng/µl.
IMPORTANT *Do not attempt to precipitate the cDNA with ethanol before removing the phosphate ions by spun-column chromatography.*
The subtracted cDNA prepared through Step 10 of this protocol can be converted into double-stranded DNA (please see Chapter 11, Protocol 2) and cloned into a bacteriophage or plasmid vector to produce a subtracted cDNA library.
11. To radiolabel the subtracted cDNA to high specific activity, mix the following in a 0.5-ml microfuge tube:

subtracted cDNA	5 µl
random deoxynucleotide primers (125 µg/ml)	5 µl

12. Heat the mixture to 60°C for 5 minutes, and then cool it to 4°C.
13. To the primer:cDNA template mixture, add:

5x random primer buffer	10 µl
5 mM dNTP solution of dCTP, dGTP, and dTTP	5 µl
10 mCi/ml [α-^{32}P]dATP (sp. act. >3000 Ci/mmole)	25 µl
Klenow fragment (12.5 units)	2.5 µl
H_2O	to 50 µl

Incubate the reaction for 4-6 hours at room temperature.
10-15 units of the Klenow fragment are required in each random priming reaction.
14. Stop the reaction by adding:

0.5 M EDTA (pH 8.0)	1 µl
20% (w/v) SDS	2.5 µl

15. Separate the radiolabeled cDNA from the unincorporated dNTPs by spun-column chromatography through Sephadex G-50.
The radiolabeled cDNA should be denatured by heating to 100°C for 5 minutes before it is used for hybridization. Use 5 x 10^7 dpm of radiolabeled cDNA for each 138-mm filter and 5 x 10^6 to 1 x 10^7 dpm for each 82-mm filter. Once radiolabeled, use the probe immediately to avoid damage by radiochemical decay.

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Protocol 10

Labeling 3´ Termini of Double-stranded DNA Using the Klenow Fragment of *E. coli* DNA Polymerase I

Templates for the end-filling reaction are produced by digestion of DNA with a restriction enzyme that creates a recessed 3´-hydroxyl terminus. The Klenow enzyme is then used to catalyze the incorporation of one or more [α-^{32}P]dNTPs into the recessed 3´ terminus in a template-dependent fashion.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
Optional, please see Step 4.

Ethanol

Enzymes and Buffers

Appropriate restriction enzyme(s)
Choose enzymes that produce 3´-recessed termini.

Klenow fragment of *E. coli* DNA polymerase I

Nucleic Acids and Oligonucleotides

dNTP solution containing the appropriate unlabeled dNTPs, each at 1 mM

Template DNA (0.1-5 µg)
Use linear, double-stranded DNA carrying appropriate recessed 3´ termini.

Radioactive Compounds

⚠ [α-^{32}P]dNTP (10 mCi/ml, sp. act. 800-3000 Ci/mmole)

METHOD

1. Digest up to 5 µg of template DNA with the desired restriction enzyme in 25-50 µl of the appropriate restriction enzyme buffer.
   The labeling reaction may be carried out immediately after digesting the DNA with a restriction enzyme.
2. To the completed restriction digest, add:

10 mCi/ml [α-^{32}P]dNTP (sp. act. 800-3000 Ci/mmole)	2-50 µCi
unlabeled dNTPs	to a final concentration of 100 µM
Klenow fragment	1-5 units

   Incubate the reaction for 15 minutes at room temperature.
   Approximately 0.5 unit of the Klenow enzyme is required for each µg of template DNA (1 µg of a 1000-bp fragment is equivalent to approx. 3.1 pmoles of termini of double-stranded DNA.
   Reverse transcriptase (1-2 units) can be used in place of the Klenow enzyme in this protocol. However, reverse transcriptase is not as forgiving of buffer conditions as the Klenow enzyme and is therefore used chiefly to label purified DNA fragments in reactions containing conventional reverse transcriptase buffer.
   When the labeled DNA is to be used for sequencing by the Maxam-Gilbert technique (please see Chapter 12, Protocol 7) or for mapping mRNA by the nuclease S1 method (please see Chapter 7, Protocol 10), the concentration of labeled dNTP in the reaction should be increased to the greatest level that is practicable. After the reaction has been allowed to proceed for 15 minutes at room temperature, add all four unlabeled dNTPs to a final concentration of 0.2 mM for each dNTP, and continue the incubation for a further 5 minutes at room temperature.
3. Stop the reaction by heating it for 10 minutes at 75°C.
4. Separate the radiolabeled DNA from unincorporated dNTP by spun-column chromatography through Sephadex G-50 or by two rounds of precipitation with ethanol in the presence of 2.5 M ammonium acetate.

REFERENCES

1. Cobianchi F. and Wilson S.H. 1987. Enzymes for modifying and labeling DNA and RNA. *Methods Enzymol.* 152:94-110.

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Protocol 11

Labeling 3´ Termini of Double-stranded DNA with Bacteriophage T4 DNA Polymerase

Bacteriophage T4 DNA polymerase enzyme, unlike the Klenow enzyme, rapidly digests 3´-protruding termini and then continues at a slower pace to remove 3´ nucleotides from the double-stranded portion of the DNA substrate. Consequently, in the absence of dNTPs, the enzyme will degrade double-stranded molecules to about half-length single strands. However, in the presence of high concentrations of dNTPs, recessed 3´-hydroxyl termini generated by exonucleolytic activity act as primers for template-directed addition of mononucleotides by the 5´->3´ polymerase. Because the synthetic capacity of bacteriophage T4 DNA polymerase exceeds its exonucleolytic abilities, protruding 3´ termini are converted to termini with flush ends. The reaction therefore consists of cycles of removal and replacement of the 3´-terminal nucleotides from recessed or blunt-ended DNA (O'Farrell et al. 1980). If one of the four dNTPs is radiolabeled, the resulting blunt-ended double-stranded molecules will be labeled at or near their 3´ termini.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
Ethanol
⚠ Phenol:chloroform (1:1, v/v)

Enzymes and Buffers

Appropriate restriction enzyme(s)
Bacteriophage T4 DNA polymerase
10x Bacteriophage T4 DNA polymerase buffer

Nucleic Acids and Oligonucleotides

dNTP solution containing three unlabeled dNTPs, each at 2 mM
The three dNTPs to be included in this solution depend on the DNA sequence of the restriction endonuclease site to be radiolabeled. None of them should carry the same base as the radiolabeled nucleotide.
dNTP solution containing one unlabeled dNTP at a concentration of 2 mM
IMPORTANT *The dNTP used here should carry the same base as the radiolabeled dNTP.*
Template DNA (0.1-5 µg)
Use linear, double-stranded DNA carrying appropriate blunt or protruding 3´ termini.

Radioactive Compounds

⚠ [α-^{32}P]dNTP (10 mCi/ml, sp. act. 800-3000 Ci/mmole)

METHOD

1. Digest 0.1-5.0 µg of the template DNA with a restriction enzyme(s) that generates a blunt or 3´-protruding end.
   In many cases, digestion can be carried out in T4 DNA polymerase buffer, allowing the digestion and labeling reactions to be carried out sequentially without an intermediate extraction with phenol:chloroform and precipitation with ethanol.
2. Purify the DNA by extraction with phenol:chloroform and precipitation with ethanol in the presence of 2.5 M ammonium acetate.
3. Dissolve the DNA pellet in:

10x bacteriophage T4 DNA polymerase buffer	2 µl
2 mM solution of three unlabeled dNTPs	1 µl
10 mCi/ml [α-^{32}P]dNTP (800-3000 Ci/mmole)	1 µl
bacteriophage T4 DNA polymerase (2.5 units/µl)	1 µl
H_2O	to 20 µl

   Incubate the reaction for 5 minutes at 37°C.
4. Add 1 µl of a 2 mM solution of the unlabeled fourth dNTP. Continue the incubation for a further 10 minutes.
5. Stop the reaction by heating it to 70°C for 5 minutes.
6. Separate the labeled DNA from unincorporated dNTPs by spun-column chromatography through Sephadex G-50 or by two rounds of precipitation with ethanol in the presence of 2.5 M ammonium acetate.

REFERENCES

1. Goulian M., Lucas Z.J., and Kornberg A. 1968. Enzymatic synthesis of deoxyribonucleic acid. XXV. Purification and properties of deoxyribonucleic acid polymerase induced by infection with phage T4. *J. Biol. Chem.* 243:627-638.
2. O'Farrell P.H., Kutter E., and Nakanishi M. 1980. A restriction map of the bacteriophage T4 genome. *Mol. Gen. Genet.* 179:421-435.

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Protocol 12

End Labeling Protruding 3´ Termini of Double-stranded DNA with [α-^{32}P]Cordycepin 5´-Triphosphate or [α-^{32}P]dideoxyATP

The 3´-protruding termini of DNA, generated by cleavage with restriction enzymes such as *Pst*I or *Sac*I, can be labeled using calf thymus terminal transferase to catalyze the transfer of [α-^{32}P]dideoxyATP or [α-^{32}P]cordycepin triphosphate. Because neither of these nucleotide analogs carries a 3´-hydroxyl group, no additional nucleotides can be added to the modified protruding 3´ terminus.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
Optional, please see Step 6.

Ethanol

⚠ Phenol:chloroform (1:1, v/v)

Enzymes and Buffers

Appropriate restriction enzyme(s)
Choose enzymes that produce 3´-protruding termini.

Calf thymus terminal transferase

5x Terminal transferase buffer

Nucleic Acids and Oligonucleotides

Template DNA (0.1-5 µg)
Use linear, double-stranded DNA carrying appropriate protruding 3´ termini.

Radioactive Compounds

⚠ [α-^{32}P]Cordycepin triphosphate (10 mCi/ml, sp. act. 5000 Ci/mmole)
or

⚠ [α-^{32}P]DideoxyATP (10 mCi/ml, sp. act. 3000 Ci/mmole)

METHOD

1. Digest 0.1-5 µg of template DNA with the appropriate restriction enzyme.
2. Purify the DNA by extraction with phenol:chloroform and standard precipitation with ethanol.
3. Dissolve the digested DNA in 10 µl of 5x terminal transferase buffer and 34 µl of H_2O.
4. Add 5 µl of 10 mCi/ml [α-^{32}P]cordycepin 5´-triphosphate (5000 Ci/mmole) or [α-^{32}P]dideoxyATP (3000 Ci/mmole) and 1 µl of calf thymus terminal transferase (approx. 20 units).
   20 units of calf thymus terminal transferase are required to catalyze the radiolabeling of approx. 10 pmoles of protruding 3´ termini.
5. Incubate the reaction for 1 hour at 37°C.
6. Separate the labeled DNA from unincorporated [α-^{32}P]cordycepin 5´-triphosphate (or [α-^{32}P]dideoxyATP) by spun-column chromatography through Sephadex G-50 or by two rounds of precipitation with ethanol in the presence of 2.5 M ammonium acetate (please see Appendix 8 in the print version of the manual).

REFERENCES

1. Tu C.P. and Cohen S.N. 1980. 3´-End labeling of DNA with [α-^{32}P]cordycepin-5´-triphosphate. *Gene* 10:177-183.
2. Yousaf S.I., Carroll A.R., and Clarke B.E. 1984. A new and improved method for 3´-end labelling DNA using [α-^{32}P]ddATP. *Gene* 27:309-313.

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Protocol 13

Dephosphorylation of DNA Fragments with Alkaline Phosphatase

Essentially any protein phosphatase (e.g., bacterial alkaline phosphatase [BAP], calf intestinal phosphatase [CIP], placental alkaline phosphatase, and shrimp alkaline phosphatase [SAP]) will catalyze the removal of 5´ phosphates from nucleic acid templates. Because CIP and SAP are readily inactivated, they are the most widely used phosphatases in molecular cloning. Although CIP is cheaper per unit of activity, SAP enzyme has the advantage of being readily inactivated in the absence of chelators.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform
EDTA (0.5 M, pH 8.0)
EGTA (0.5 M, pH 8.0), if using CIP
Ethanol
⚠ Phenol:chloroform (1:1, v/v)
⚠ SDS (10% w/v), if using CIP
Sodium acetate (3 M, pH 7.0 [if using CIP] and pH 5.2)
TE (pH 7.6)
Tris-Cl (1 M, pH 8.5)

Enzymes and Buffers

Alkaline phosphatase
Dephosphorylation buffer
Proteinase K
Restriction enzyme(s)

Nucleic Acids and Oligonucleotides

DNA sample (0.1-10 µg [1-100 pmoles])
Dephosphorylation reactions are usually carried out in a volume of 25-50 µl containing 1-100 pmoles of 5´-phosphorylated termini of DNA.

METHOD

1. Use the restriction enzyme of choice to digest to completion 1-10 µg (10-100 pmoles) of the DNA to be dephosphorylated.
   CIP and SAP will dephosphorylate DNA at a slightly reduced efficiency in restriction buffers that have been adjusted to pH 8.5 with 10x CIP or 10x SAP buffer, as is done in the next step. If this is unacceptable, the restricted DNA may be purified by extraction with phenol:chloroform and standard precipitation with ethanol and then dissolved in a minimal volume of 10 mM Tris-Cl (pH 8.5).
2. Dephosphorylate the 5´ ends of the restricted DNA with either CIP or SAP.

   To dephosphorylate DNA using CIP

   a. Add to the DNA:

10x CIP dephosphorylation buffer	5 µl
H_2O	to 48 µl

   b. Add the appropriate amount of CIP.

   1 unit of CIP will dephosphorylate approx. 1 pmole of 5´-phosphorylated termini (5´-recessed or blunt-ended DNA) or approx. 50 pmoles of 5´-protruding termini. These amounts may vary slightly from one manufacturer to the next.

   c. Incubate the reaction for 30 minutes at 37°C, add a second aliquot of CIP, and continue incubation for a further 30 minutes.

   d. To inactivate CIP at the end of the incubation period, add SDS and EDTA (pH 8.0) to final concentrations of 0.5% and 5 mM, respectively. Mix the reagents well, and add proteinase K to a final concentration of 100 µg/ml. Incubate for 30 minutes at 56°C.

   Alternatively, CIP can be inactivated by heating to 65°C for 30 minutes (or 75°C for 10 minutes) in the presence of 10 mM EGTA (pH 8.0).
   IMPORTANT *Use EGTA not EDTA.*

   e. Cool the reaction to room temperature and purify the DNA by extracting it twice with phenol:chloroform and once with chloroform alone.

   Proteinase K and SDS used to inactivate and digest CIP must be completely removed by extraction with phenol:chloroform prior to subsequent enzymatic treatments (phosphorylation by polynucleotide kinase, ligation, etc.). Glycogen or linear polyacrylamide can be added as a carrier before phenol:chloroform extraction if small amounts of DNA (<100 ng) were used in the reaction. Do not add carrier nucleic acid (tRNA, salmon sperm DNA, etc.), as it will compete with the dephosphorylated DNA for the radiolabeled ATP during the kinasing reaction.

   To dephosphorylate DNA using SAP

   a. Add to the DNA:

10x SAP dephosphorylation buffer	5 µl
H_2O	to 48 µl

   b. Add the appropriate amount of SAP.

   1 unit of SAP will dephosphorylate approx. 1 pmole of 5´-phosphorylated termini (3´-recessed or 5´- recessed) or approx. 0.2 pmole of blunt-ended DNA. These amounts may vary slightly from one enzyme manufacturer to the next.

   c. Incubate the reaction for 1 hour at 37°C.

   d. To inactivate SAP, transfer the reaction to 70°C, incubate for 20 minutes, and cool to room temperature.
3. Transfer the aqueous phase to a clean microfuge tube, and recover the DNA by standard ethanol precipitation in the presence of 0.1 volume of 3 M sodium acetate (pH 5.2) if SAP was used or 0.1 volume of 3 M sodium acetate (pH 7.0)

if CIP was used.

4. Allow the precipitate to dry at room temperature before dissolving it in TE (pH 7.6) at a DNA concentration of >2 nmoles/ml.

REFERENCES

1. Chaconas G. and van de Sande J.H. 1980. 5′-32P labeling of RNA and DNA restriction fragments. *Methods Enzymol.* 65:75-85.

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Protocol 14

Phosphorylation of DNA Molecules with Protruding 5'-Hydroxyl Termini

Bacteriophage T4 polynucleotide kinase catalyzes the transfer of γ-^{32}P of ATP to the terminal 5´-hydroxyl groups of single- or double-stranded nucleic acids. When [γ-^{32}P]ATP of high specific activity (3000-7000 Ci/mmole) is used as a substrate, approximately 40-50% of the protruding 5´ termini in the reaction become radiolabeled.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
Optional, please see Step 3.

EDTA (0.5 M, pH 8.0)

Ethanol

Enzymes and Buffers

Bacteriophage T4 polynucleotide kinase

10x Bacteriophage T4 polynucleotide kinase buffer

Nucleic Acids and Oligonucleotides

DNA (10-50 pmoles)
The DNA should be dephosphorylated as described in Chapter 9, Protocol 13 or synthesized with a 5´-hydroxyl moiety.

Radioactive Compounds

⚠ [γ-^{32}P]dATP (10 mCi/ml, sp. act. 3000-7000 Ci/mmole)

METHOD

1. In a microfuge tube, mix the following reagents:

dephosphorylated DNA	10-50 pmoles
10x bacteriophage T4 polynucleotide kinase buffer (10-20 units)	5 µl
10 mCi/ml [γ-^{32}P]ATP (sp. act. 3000-7000 Ci/mmole)	50 pmoles
bacteriophage T4 polynucleotide kinase	10 units
H_2O	to 50 µl

   Incubate the reaction for 1 hour at 37°C.
   Ideally, ATP should be in a fivefold molar excess over DNA 5´ ends, and the concentration of DNA termini should be ≥ 0.4 µM. The concentration of ATP in the reaction should therefore be >2 µM, but this is rarely achievable in practice. To increase the specific activity of the radiolabeled DNA product, increase the amount of [γ-^{32}P]ATP used in the phosphorylation reaction. Decrease the volume of H_2O to maintain a reaction volume of 50 µl.
2. Terminate the reaction by adding 2 µl of 0.5 M EDTA (pH 8.0). Measure the total radioactivity in the reaction mixture by Cerenkov counting in a liquid scintillation counter.
3. Separate the radiolabeled probe from unincorporated dNTPs by either:
   - spun-column chromatography through Sephadex G-50

     or
   - conventional size-exclusion chromatography through 1-ml columns of Sepahadex G-50 (equilibrated in TE)

     or
   - two rounds of selective precipitation of the radiolabeled DNA with ammonium acetate and ethanol
4. Measure the amount of radioactivity in the probe preparation by Cerenkov counting. Calculate the efficiency of transfer of the radiolabel to the 5´ termini by dividing the amount of radioactivity in the probe by the total amount in the reaction mixture (Step 2).

REFERENCES

1. Berkner K.L. and Folk W.R. 1977. Polynucleotide kinase exchange reaction: Quantitative assay for restriction endonuclease-generated 5´-phosphoryl termini in DNA. *J. Biol. Chem.* 252:3176-3184.
2. Wu G., Jay E., and Roychoudhury R. 1976. Nucleotide sequence analysis of DNA. *Methods Cancer Res.* 12:87-176.

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Protocol 15

Phosphorylation of DNA Molecules with Dephosphorylated Blunt Ends or Recessed 5' Termini

Blunt ends, recessed 5´ termini, or internal nicks in DNA are labeled less efficiently in reactions catalyzed by T4 DNA kinase than are protruding 5´ termini. However, the efficiency of phosphorylation of blunt-ended DNAs greater than 300 bp in length can be increased by including a condensing reagent such as polyethylene glycol in the reaction.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
Optional, please see Step 5.

EDTA (0.5 M, pH 8.0)

Ethanol

10x Imidazole buffer

⚠ PEG 8000 (24% w/v) in H_2O

Enzymes and Buffers

Bacteriophage T4 polynucleotide kinase

Nucleic Acids and Oligonucleotides

DNA (10-50 pmoles in a volume of ≤ 11 µl)
Dephosphorylate the DNA as described in Chapter 9, Protocol 13 or synthesize with a 5´-hydroxyl moiety.

Radioactive Compounds

⚠ [γ-^{32}P]dATP (10 mCi/ml, sp. act. 3000 Ci/mmole)

METHOD

1. In a microfuge tube, mix *in the order given:*

dephosphorylated DNA	10-50 pmoles
10x imidazole buffer	4 µl
H_2O	to 15 µl
24% (w/v) PEG	10 µl

2. Add 40 pmoles of [γ-^{32}P]ATP (10 mCi/ml; sp. act. 3000 Ci/mmole) to the tube and bring the final volume of the reaction to 40 µl with H_2O.
Ideally, ATP should be in a fivefold molar excess over DNA 5´ ends, and the concentration of DNA termini should be ≥ 0.4 µM. The concentration of ATP in the reaction should therefore be >2 µM, but this is rarely achievable in practice. To increase the specific activity of the radiolabeled DNA product, increase the amount of [γ-^{32}P]ATP used in the phosphorylation reaction. Decrease the volume of H_2O to maintain a reaction volume of 40 µl.
3. Add 40 units of bacteriophage T4 polynucleotide kinase to the reaction. Mix the reagents gently by tapping the side of tube, and incubate the reaction for 30 minutes at 37°C.
4. Terminate the reaction by adding 2 µl of 0.5 M EDTA (pH 8.0). Measure the total radioactivity in the reaction mixture by Cerenkov counting in a liquid scintillation counter.
5. Separate the radiolabeled probe from unincorporated dNTPs by either:
   - spun-column chromatography through Sephadex G-50

   or

   - conventional size-exclusion chromatography through 1-ml columns of Sepahadex G-50 (equilibrated in TE)

   or

   - two rounds of selective precipitation of the radiolabeled DNA with ammonium acetate and ethanol
6. Measure the amount of radioactivity in the probe preparation by Cerenkov counting. Calculate the efficiency of transfer of the radiolabel to the 5´ termini by dividing the amount of radioactivity in the probe by the total amount in the reaction mixture (Step 4).

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1. Harrison B. and Zimmerman S.B. 1986. T4 polynucleotide kinase: Macromolecular crowding increases the efficiency of reaction at DNA termini. *Anal. Biochem.* 158:307-315.
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Protocol 16

Phosphorylation of DNA Molecules with Protruding 5' Termini by the Exchange Reaction

The exchange reaction catalyzed by bacteriophage T4 polynucleotide kinase does not require that the 5´ termini of DNA substrates be dephosphorylated. However, the efficiency of the reaction is poor unless crowding reagents such as polyethylene glycol are included in the reaction mixture.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- ADP (1 mM)
- ⚠ Ammonium acetate (10 M)
  Optional, please see Step 3.
- ⚠ ATP (10 mM)
- EDTA (0.5 M, pH 8.0)
- Ethanol
- 10x Imidazole buffer
- ⚠ PEG 8000 (24% w/v\ ) in H_2O

Enzymes and Buffers

Bacteriophage T4 polynucleotide kinase

Nucleic Acids and Oligonucleotides

DNA (10-50 pmoles of 5'-phosphorylated termini)

Radioactive Compounds

⚠ [γ-^{32}P]dATP (10 mCi/ml, sp. act. 3000-7000 Ci/mmole)

METHOD

1. Mix the following reagents in a microfuge tube *in the order given:*

DNA with 5´ terminal phosphates	10-50 pmoles
10x imidazole buffer	5 µl
1 mM ADP	5 µl
50 nM ATP	1 µl
10 mCi/ml [γ-^{32}P]ATP (sp. act. 3000-7000 Ci/mmole)	20-100 pmoles
H_2O	to 40 µl
24% (w/v) PEG	10 µl
bacteriophage T4 polynucleotide kinase (20 units)	1 µl

   Mix reagents gently by tapping the side of tube, and incubate the reaction for 30 minutes at 37°C.
2. Terminate the reaction by adding 2 µl of 0.5 M EDTA (pH 8.0). Measure the total radioactivity in the reaction mixture by Cerenkov counting in a liquid scintillation counter.
3. Separate the radiolabeled probe from unincorporated dNTPs by either:
   - spun-column chromatography through Sephadex G-50

     or
   - conventional size-exclusion chromatography through 1-ml columns of Sepahadex G-50 (equilibrated in TE)

     or
   - two rounds of selective precipitation of the radiolabeled DNA with ammonium acetate and ethanol
4. Measure the amount of radioactivity in the probe preparation by Cerenkov counting. Calculate the efficiency of transfer of the radiolabel to the 5´ termini by dividing the amount of radioactivity in the probe by the total amount in the reaction mixture (Step 2).

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1. Berkner K.L. and Folk W.R. 1977. Polynucleotide kinase exchange reaction: Quantitative assay for restriction endonuclease-generated 5'-phosphoryl termini in DNA. *J. Biol. Chem.* 252:3176-3184.
2. Harrison B. and Zimmerman S.B. 1986. T4 polynucleotide kinase: Macromolecular crowding increases the efficiency of reaction at DNA termini. *Anal. Biochem.* 158:307-315.
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Chapter 10 Working with Synthetic Oligonucleotide Probes

Protocol 1: Purification of Synthetic Oligonucleotides by Polyacrylamide Gel Electrophoresis

As a rule of thumb, oligonucleotides >25 nucleotides should be purified by polyacrylamide gel electrophoresis, as should oligonucleotides of any length that yield anomalous results. After electrophoresis, the oligonucleotide is eluted from the gel and concentrated by reversed-phase chromatography on Sep-Pak C_{18} columns.

The method described here is a modification of a procedure that has been in use in Michael Smith's laboratory (University of British Columbia) for more than 20 years.

Protocol 2: Phosphorylating the 5' Termini of Oligonucleotides

Synthetic oligonucleotides lacking phosphate groups at their 5' termini are easily radiolabeled by transfer of the γ-^{32}P from [γ-^{32}P]ATP in a reaction catalyzed by bacteriophage T4 polynucleotide kinase. The reaction described below is designed to label 10 pmoles of an oligonucleotide to high specific activity. Labeling of different amounts of oligonucleotide can easily be achieved by increasing or decreasing the size of the reaction while keeping the concentrations of all components constant. When the reaction is carried out efficiently, >50% of the oligonucleotide molecules in the reaction become radiolabeled. Similar reaction conditions can be used when adding a nonradiolabeled phosphate to the 5´ end of a synthetic oligonucleotide prior to its use in site-directed mutagenesis.

Protocol 3: Purification of Radiolabeled Oligonucleotides by Precipitation with Ethanol

If radiolabeled oligonucleotides are to be used only as probes in hybridization experiments, then complete removal of unincorporated radiolabel is generally unnecessary. However, to reduce background, it is always advisable to separate the bulk of the unincorporated radioactivity from the oligonucleotide. Most of the residual radioactive precursors can be removed from the preparation by differential precipitation with ethanol if the oligonucleotide is more than 18 nucleotides in length (Chapter 10, Protocol 3) or with cetylpyridinium bromide, regardless of the length of the oligonucleotide (Chapter 10, Protocol 4). If complete removal of the unincorporated radiolabel is required (e.g., when the radiolabeled oligonucleotide will be used in primer extension reactions), then chromatographic methods (Chapter 10, Protocol 5 and Chapter 10, Protocol 6) or gel electrophoresis (essentially as described in Chapter 10, Protocol 1) should be used.

Protocol 4: Purification of Radiolabeled Oligonucleotides by Precipitation with Cetylpyridinium Bromide

Radiolabeled nucleic acids, including oligonucleotides, can be separated from unincorporated radiolabel by quantitative, differential precipitation with the cationic detergent cetylpyridinium bromide (CPB). The nucleic acids are first precipitated from aqueous solution with CPB. The detergent is then removed from the precipitate with ethanol (in which the nucleic acids are insoluble), and the nucleic acids are finally dissolved in the buffer of choice.

Protocol 5: Purification of Radiolabeled Oligonucleotides by Size-exclusion Chromatography

When radiolabeled oligonucleotides are to be used in enzymatic reactions such as primer extension, virtually all of the unincorporated radiolabel must be removed from the oligonucleotide. Chromatographic methods (Chapter 10, Protocol 5 and Chapter 10, Protocol 6) or gel electrophoresis (essentially as described in Chapter 10, Protocol 1) are superior in this respect than differential precipitation of the oligonucleotide with ethanol or CTAB.

Protocol 6: Purification of Radiolabeled Oligonucleotides by Chromatography on a Sep-Pak C_{18} Column

Radiolabeled oligonucleotides can be separated from unincorporated radiolabel by chromatography on silica gel resins. The protocol is suitable only for purifying oligonucleotides carrying 5´-phosphate groups. The method described in Chapter 10, Protocol 1 should be used to purify oligonucleotides with free 5´-hydroxyl groups.

Protocol 7: Labeling of Synthetic Oligonucleotides Using the Klenow Fragment of *E. coli* DNA Polymerase I

Probes of high specific activities can be obtained using the Klenow fragment of *E. coli* DNA polymerase I to catalyze synthesis of a strand of DNA complementary to a synthetic oligonucleotide. A short primer is hybridized to an oligonucleotide template whose sequence is the complement of the desired radiolabeled probe. The primer is then extended using the Klenow fragment of *E. coli* DNA polymerase I to incorporate [α-^{32}P]dNTPs in a template-directed manner. After the reaction, the template and product are separated by denaturation, followed by electrophoresis through a polyacrylamide gel under denaturing conditions. With this method, it is possible to generate oligonucleotide probes that contain several radioactive atoms per molecule of oligonucleotide and to achieve specific activities as high as 2 x 10^{10} cpm/µg of probe.

Protocol 8: Hybridization of Oligonucleotide Probes in Aqueous Solutions: Washing in Buffers Containing Quaternary Ammonium Salts

Quaternary ammonium salts greatly ameliorate the effects of G + C content on the melting temperature of oligonucleotides (Jacobs et al. 1988). In the following protocol, hybridization is carried out in conventional aqueous solvents at a temperature well below the predicted melting temperature. Nonspecific hybrids are then removed by washing at high stringency in buffers containing quaternary salts. Tetramethylammonium chloride (TMACl) is used with probes that are 14-50 nucleotides in length, whereas tetraethylammonium chloride (TEACl) is used with longer oligonucleotides.

The graph in Figure 10-2 on page 10.36 of the print version of the manual should be used to estimate a washing temperature in TMACl buffers for hybrids involving oligonucleotides of different lengths. When using TEACl buffers, subtract 33°C from the value obtained from Figure 10-2.

Protocol 9: Empirical Measurement of Melting Temperature

The melting temperature (T_m) of an oligonucleotide may be determined empirically by measuring the temperature (T_i) at which dissociation of the double-stranded DNA becomes irreversible. The procedure requires a cloned target sequence that is complementary (perfectly or imperfectly, depending on the experiment) to the oligonucleotide probe. If a target sequence is not available from "natural" sources, it can be synthesized chemically or by PCR. The experiment is carried out under nonequilibrium conditions that do not favor rehybridization of the released probe to the target.

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Protocol 1

Purification of Synthetic Oligonucleotides by Polyacrylamide Gel Electrophoresis

As a rule of thumb, oligonucleotides >25 nucleotides should be purified by polyacrylamide gel electrophoresis, as should oligonucleotides of any length that yield anomalous results. After electrophoresis, the oligonucleotide is eluted from the gel and concentrated by reversed-phase chromatography on Sep-Pak C_{18} columns.

The method described here is a modification of a procedure that has been in use in Michael Smith's laboratory (University of British Columbia) for more than 20 years.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Acetonitrile
Use 10 ml of high-performance liquid chromatography (HPLC)-grade acetonitrile for each Sep-Pak column.

⚠ Ammonium acetate (10 M)
Use 2 ml of 10 mM ammonium acetate solution for each Sep-Pak column.

⚠ *n*-Butanol

⚠ Formamide loading buffer without tracking dyes
This gel-loading buffer consists of undiluted formamide without the usual tracking dyes (bromophenol blue and/or xylene cyanol FF); the dyes or contaminants in them may migrate at the same rate as the oligonucleotide and interfere with its detection by absorption of UV light (please see Step 14 of protocol). If desired, 0.2% orange G can be included in the gel-loading buffer. This dye migrates with the buffer front and does not interfere with detection of the oligonucleotide.

Formamide-tracking dye mixture
This solution is a 50:50 mixture of formamide and an aqueous solution of tracking dyes (0.05% xylene cyanol FF and 0.05% bromophenol blue). It is used as a size standard in wells adjacent to those containing the oligonucleotide preparation.

⚠ Methanol:H_2O solution
Combine 6 ml of methanol with 4 ml of filter-sterilized Milli-Q H_2O. Use 3 ml of methanol:H_2O solution for each Sep-Pak column.

Oligonucleotide elution buffer

TE (pH 8.0)

Nucleic Acids and Oligonucleotides

Crude preparation of synthetic oligonucleotide

Additional Reagents

Steps 7-9 of this protocol require the reagents listed in Chapter 12, Protocol 8 and Chapter 12, Protocol 11.

METHOD

1. In a sterile microfuge tube, prepare a 10 µM solution of the crude oligonucleotide in sterile, filtered H_2O (Milli-Q or equivalent). Vortex the solution thoroughly.
   The solution is often slightly cloudy because of the presence of insoluble benzamides generated during the synthesis of the oligonucleotide.
   Synthetic oligonucleotides are usually supplied by the manufacturer as a lyophilized powder after removal of protecting groups used in the synthetic reactions (deprotection).
   Before purifying an oligonucleotide, confirm that the deprotection reaction has been carried out. If the oligonucleotide is supplied in NH_4OH, transfer 0.5-1.0-ml aliquots to 1.5-ml microfuge tubes and evaporate the NH_4OH to dryness on a centrifugal evaporator (Savant SpeedVac or its equivalent) at room temperature.
   When opening a tube of crude oligonucleotide for the first time, vent the tube by opening it slowly *to allow ammonia gas to escape (preferably into a chemical fume hood). This reduces the chance of spraying the oligonucleotide around the room.*
2. Centrifuge the tube at maximum speed for 5 minutes at room temperature in a microfuge. Transfer the supernatant to a fresh, sterile microfuge tube.
3. Extract the solution three times in succession with 400 µl of *n*-butanol. Discard the upper (organic) phase after each extraction.
4. Evaporate the solution to dryness in a centrifugal evaporator (Savant SpeedVac or its equivalent). The tube should contain a yellowish pellet and a creamy-white powder.
5. Dissolve the pellet and powder in 200 µl of sterile filtered H_2O (Milli-Q or equivalent).
6. Estimate the amount of oligonucleotide in the preparation as follows: Add 1 µl of the solution to 1 ml of H_2O. Mix the solution well and read the OD_{260}. Calculate the oligonucleotide concentration.
   Amounts of oligonucleotides are often described in OD units. One OD corresponds to the amount of oligonucleotide in a 1-ml volume that results in an optical density of 1 in a 1-cm path-length cuvette.
   Calculate the millimolar extinction coefficient of the oligonucleotide (ε) from the following equation:
   ε = A(*15.2*) + G(*12.01*) + C(*7.05*) + T(*8.4*)
   where A, G, C, *and* T *are the number of times each nucleotide is represented in the sequence of the oligonucleotide.*
   The numbers in parentheses are the molar extinction coefficients for each deoxynucleotide at pH 8.0.
   For example, a 19-mer containing 5 dA residues, 4 dG residues, 4 dC residues, and 6 dT residues would have a millimolar extinction coefficient of
   (5 x 15.2) + (4 x 12.01) + (4 x 7.05) + (6 x 8.4) = 202.64 $mM^{-1}cm^{-1}$
   Calculate the concentration (c) of the undiluted solution of oligonucleotide from the following equation:
   c = $(OD_{260})(1000)/\varepsilon$
7. Pour a denaturing polyacrylamide gel (as described in Chapter 12, Protocol 8) of the appropriate concentration (see table). The loading slots in the gel should be approx. 1 cm in length.

Range of Resolution of Gels Containing Different Concentrations of Acrylamide

Acrylamide (%)	Size of Oligonucleotides (in Bases)
20-30	2-8
15-20	8-25
13-15	15-35
10-13	35-45
8-10	45-70

6-8	70-300

8. Run the gel at constant wattage (50-70 W) for approx. 45 minutes or until the temperature of the gel reaches 45-50°C. Turn off the power supply and disconnect the electrodes.
   Prerunning the gel in this way causes ammonium persulfate to migrate from the wells and, more importantly, warms the gel to a temperature optimal for electrophoresis of DNA.
9. Without delay, load approx. 2 OD_{260} units of oligonucleotide (in a volume of 10 µl or less for maximum resolution) onto one or more slots of the gel as follows:
   a. Add an equal volume of formamide loading buffer lacking dyes to the oligonucleotide solution. Mix the reagents well by vortexing, and then heat the mixture to 55°C for 5 minutes to disrupt secondary structure.
   b. Flush out the urea from the wells with 1x TBE.
   c. Load the heated oligomer into the slots. Load 5 µl of formamide-tracking dye mixture into an unused slot.
      For further details on loading polyacrylamide gels, please see Chapter 12, Protocol 11.
10. Run the gel at 1500 V until the oligonucleotide has migrated approximately two thirds of the length of the gel.
    The position of the oligonucleotide may be estimated from the positions of the tracking dyes as detailed in the table. Note that a synthetic oligonucleotide carrying a hydroxyl residue at its 5´ terminus migrates more slowly through a denaturing polyacrylamide gel than does a phosphorylated oligonucleotide of equivalent length. Furthermore, the electrophoretic mobility of an oligonucleotide is dependent on its base composition and sequence. Thus, there may not be an exact correspondence between the predicted and observed positions of the oligonucleotide in the polyacrylamide gel.

Approximate lengths of Oligonucleotides Comigrating with Tracking Dyes

Polyacrylamide (%)	Xylene Cyanol FF	Bromophenol Blue
20	22	6
15	30	9-10
12	40	approx. 15

11. Lay the gel mold flat on plastic-backed protective bench paper with the smaller (notched) plate uppermost. Allow the gel to cool to <37°C before proceeding.
12. Remove any remaining pieces of electrical tape. Use a spacer or a plate-separating tool to slowly and gently pry apart the plates of the mold. The gel should remain attached to the longer (nonsiliconized) glass plate.
    If the gel adheres to both plates, replace the partially dislodged, smaller or notched plate back on the gel, invert the plates, and try again.
13. Place a piece of Saran Wrap on the gel, turn the glass plate over, and transfer the gel to the Saran Wrap. Place a piece of Parafilm or a fluorescent thin-layer chromatographic plate under the gel where the oligonucleotide is predicted to be.
14. Use a hand-held UV lamp to examine the gel by illumination from above at 260 nm.
    The DNA in the gel absorbs the UV radiation and appears as dark blue bands against a uniform fluorescent background contributed by the Parafilm or chromatographic plate. If the DNA is difficult to visualize, take the gel into a darkened room and illuminate it with the hand-held UV lamp.
15. Recover the desired oligonucleotide, which should be the slowest-migrating band (i.e., closest to the top of the gel), by excising each DNA band with a sharp, clean scalpel or razor blade. Avoid taking UV-absorbing material smaller in length than the desired oligonucleotide.
16. Transfer the gel slices to three or four microfuge tubes. Add 1 ml of oligonucleotide elution buffer to each tube. Crush the slices with a disposable pipette tip, using a circular motion and pressing the fragments of gel against the sides of the tubes. Seal the tubes well. Incubate the tubes for 12 hours at 37°C in a shaker incubator.
17. Centrifuge the tubes at maximum speed for 5 minutes at room temperature in a microfuge. Pool the supernatants, transfer them to a 5-cc disposable syringe, and pass them through a Millex HV filter. Collect the effluent in a 15-ml polypropylene tube.
18. Prepare a Sep-Pak C_{18} reversed-phase column as follows:
    a. Attach the barrel of a disposable 10-cc polypropylene syringe to the longer end of a Sep-Pak C_{18} classic column.
    b. Add 10 ml of acetonitrile to the barrel and slowly push it through the column with the plunger of the syringe.
    c. Remove the syringe from the Sep-Pak column and then take the plunger out of the barrel. This prevents air being pulled back into the column. Reattach the barrel to the column.
    d. Add 10 ml of sterile filtered H_2O (Milli-Q or equivalent) to the barrel and slowly push it through the column with the plunger. Repeat Step c.
    e. Add 2 ml of 10 mM ammonium acetate to the barrel and push it slowly through the column. Again remove the syringe, remove the barrel, and reattach the barrel to the column. The column is now ready for use.
19. Add the solution containing the gel-purified oligonucleotide (from Step 17) to the barrel and slowly push it through the column with the plunger. Collect the effluent in a sterile 50-ml polypropylene tube. Repeat Step 18c.
20. Add 10 ml of H_2O to the barrel and push it slowly through the column with the plunger. Repeat this wash step twice more.
21. Elute the bound oligonucleotide from the Sep-Pak column with three aliquots of 1 ml of methanol:H_2O solution. Repeat Step 18c after each elution. Collect each effluent in a separate microfuge tube. Read the OD_{260} of the solution in each of the three microfuge tubes, using the methanol:H_2O solution as a blank. More than 90% of the oligonucleotide applied to the column should elute in the first fraction.
22. Evaporate the solution containing the oligonucleotide to dryness in a centrifugal evaporator.
23. Dissolve the oligonucleotide in a total volume of 200 µl of H_2O or TE (pH 8.0).
24. Transfer 5 µl of the solution to a cuvette containing 995 µl of H_2O. Mix the contents of the cuvette, and read the OD_{260} of the diluted sample. Calculate the amount of oligonucleotide present in the total solution (Step 23) as described in Step 6 of this protocol.

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Protocol 2

Phosphorylating the 5' Termini of Oligonucleotides

Synthetic oligonucleotides lacking phosphate groups at their 5' termini are easily radiolabeled by transfer of the γ-^{32}P from [γ-^{32}P]ATP in a reaction catalyzed by bacteriophage T4 polynucleotide kinase. The reaction described below is designed to label 10 pmoles of an oligonucleotide to high specific activity. Labeling of different amounts of oligonucleotide can easily be achieved by increasing or decreasing the size of the reaction while keeping the concentrations of all components constant. When the reaction is carried out efficiently, >50% of the oligonucleotide molecules in the reaction become radiolabeled. Similar reaction conditions can be used when adding a nonradiolabeled phosphate to the 5´ end of a synthetic oligonucleotide prior to its use in site-directed mutagenesis.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Tris-Cl (1 M, pH 8.0)

Enzymes and Buffers

Bacteriophage T4 polynucleotide kinase

10x Bacteriophage T4 polynucleotide kinase buffer

Nucleic Acids and Oligonucleotides

Oligonucleotide

To achieve maximum efficiency of labeling, purify the oligonucleotide by reversed-phase chromatography as described in Protocol 1. Crude preparations of oligonucleotides are labeled with lower efficiency in reactions catalyzed by polynucleotide kinase. When using unpurified preparations of oligonucleotide, make sure that the last cycle of synthesis was programmed to be "trityl-off," i.e., that the dimethoxytrityl blocking group at the 5´ end of the oligonucleotide primer was removed before release of the DNA from the solid synthesis support. The dimethoxytrityl group efficiently protects the 5´-hydroxyl group of the oligonucleotide from 5´ modification.

Radioactive Compounds

⚠ [γ-^{32}P]ATP (10 mCi/ml, sp. act. >5000 Ci/mmole) in aqueous solution

10 pmoles of [γ-^{32}P]ATP is required to label 10 pmoles of dephosphorylated 5´ termini to high specific activity.

Additional Reagents

Step 4 of this protocol requires the reagents listed in Chapter 13, Protocol 7 or the reagents listed in Chapter 10, Protocol 5.

METHOD

1. Set up a reaction mixture in a 0.5-ml microfuge tube containing:

synthetic oligonucleotide (10 pmoles/µl)	1 µl
10x bacteriophage T4 polynucleotide kinase buffer	2 µl
[γ-^{32}P]ATP (10 pmoles, sp. act. >5000 Ci/mmole)	5 µl
H_2O	11.4 µl

   Mix the reagents well by gentle but persistent tapping on the outside of the tube. Place 0.5 µl of the reaction mixture in a tube containing 10 µl of 10 mM Tris-Cl (pH 8.0). Set aside the tube for use in Step 4.

   To label an oligonucleotide to the highest specific activity:

   - Increase the concentration of [γ-^{32}P]ATP in the reaction by a factor of 3 (i.e., use 15 µl of radiolabel and decrease the volume of H_2O to 1.4 µl).
   - Decrease the amount of oligonucleotide to 3 pmoles.

   Under these circumstances, only approx. 10% of the radiolabel is transferred, but a high proportion of the oligonucleotide becomes radiolabeled.
2. Add 10 units (approx. 1 µl) of bacteriophage T4 polynucleotide kinase to the remaining reaction mixture. Mix the reagents well, and incubate the reaction mixture for 1 hour at 37°C.
3. At the end of the incubation period, place 0.5 µl of the reaction in a second tube containing 10 µl of 10 mM Tris-Cl (pH 8.0). Heat the remainder of the reaction for 10 minutes at 68°C to inactivate the polynucleotide kinase. Store the tube containing the heated reaction mixture on ice.
4. Before proceeding, determine whether the labeling reaction has worked well by measuring the fraction of the radiolabel that has been transferred to the oligonucleotide substrate in a small sample of the reaction mixture. Transfer a sample of the reaction (exactly 0.5 µl) to a fresh tube containing 10 µl of 10 mM Tris-Cl (pH 8.0). Use this sample (along with the two aliquots set aside in Steps 1 and 3) to measure the efficiency of transfer of the γ-^{32}P from ATP by one of the following methods:
   - Measure the proportion of the radiolabel that binds to DE-81 filters. Oligonucleotides bind tightly to the positively charged filters, whereas [γ-^{32}P]ATP does not. For details of this method, please see Chapter 13, Protocol 7.

     or
   - Measure the efficiency of the labeling reaction by estimating the fraction of label that migrates with the oligonucleotide during size-exclusion chromatography through Sephadex G-15 or Bio-Rad P-60 columns. For details of this method, please see Protocol 5 of this chapter. In some ways, this is the easier of the two methods because the relative amounts of incorporated and unincorporated radioactivity can be estimated during chromatography on a hand-held minimonitor.
5. If the specific activity of the oligonucleotide is acceptable, purify the radiolabeled oligonucleotide as described in Chapter 10, Protocol 3 , Chapter 10, Protocol 4 , Chapter 10, Protocol 5 , or Chapter 10, Protocol 6 .

   If the specific activity is too low, add an additional 8 units of polynucleotide kinase, continue incubation for a further 30 minutes at 37°C (i.e., a total of 90 minutes), heat the reaction for 10 minutes at 68°C to inactivate the enzyme, and analyze the products of the reaction again, as described in Step 4.

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Protocol 3

Purification of Radiolabeled Oligonucleotides by Precipitation with Ethanol

If radiolabeled oligonucleotides are to be used only as probes in hybridization experiments, then complete removal of unincorporated radiolabel is generally unnecessary. However, to reduce background, it is always advisable to separate the bulk of the unincorporated radioactivity from the oligonucleotide. Most of the residual radioactive precursors can be removed from the preparation by differential precipitation with ethanol if the oligonucleotide is more than 18 nucleotides in length (Chapter 10, Protocol 3) or with cetylpyridinium bromide, regardless of the length of the oligonucleotide (Chapter 10, Protocol 4). If complete removal of the unincorporated radiolabel is required (e.g., when the radiolabeled oligonucleotide will be used in primer extension reactions), then chromatographic methods (Chapter 10, Protocol 5 and Chapter 10, Protocol 6) or gel electrophoresis (essentially as described in Chapter 10, Protocol 1) should be used.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)

Ethanol

TE (pH 7.6)

Nucleic Acids and Oligonucleotides

⚠ Radiolabeled oligonucleotide

The starting material for purification is the reaction mixture from Chapter 10, Protocol 2 (either Step 3 or Step 5), after it has been heated to 68°C to inactivate bacteriophage T4 polynucleotide kinase.

METHOD

1. Add 40 µl of H_2O to the tube containing the radiolabeled oligonucleotide. After mixing, add 240 µl of a 5 M solution of ammonium acetate. Mix the reagents again and then add 750 µl of ice-cold ethanol. Mix the reagents once more and store the ethanolic solution for 30 minutes at 0°C.
2. Recover the radiolabeled oligonucleotide by centrifugation at maximum speed for 20 minutes at 4°C in a microfuge.
3. Use a micropipettor equipped with a disposable tip to remove all of the supernatant carefully from the tube.
4. Add 500 µl of 80% ethanol to the tube, tap the side of the tube to rinse the nucleic acid pellet, and centrifuge the tube again at maximum speed for 5 minutes at 4°C in a microfuge.
5. Use a micropipettor equipped with a disposable tip to remove the supernatant (which will contain appreciable amounts of radioactivity) carefully from the tube. Stand the open tube behind a Plexiglas screen until the residual ethanol has evaporated.
6. Dissolve the radiolabeled oligonucleotide in 100 µl of TE (pH 7.6).
   The radiolabeled oligonucleotide may be stored for a few days at -20°C. However, during prolonged storage, decay of ^{32}P causes radiochemical damage that can impair the ability of the oligonucleotide to hybridize to its target sequence.

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Protocol 4

Purification of Radiolabeled Oligonucleotides by Precipitation with Cetylpyridinium Bromide

Radiolabeled nucleic acids, including oligonucleotides, can be separated from unincorporated radiolabel by quantitative, differential precipitation with the cationic detergent cetylpyridinium bromide (CPB). The nucleic acids are first precipitated from aqueous solution with CPB. The detergent is then removed from the precipitate with ethanol (in which the nucleic acids are insoluble), and the nucleic acids are finally dissolved in the buffer of choice.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ CPB (cetylpyridinium bromide) (1%, w/v)

EDTA-Tris (0.5 M, pH 6.0)

EDTA-Tris-DNA solution

Ethanol-sodium acetate solution
Alternatively, use an appropriate prehybridization solution. Please see Step 8.

TE (pH 7.6)

Nucleic Acids and Oligonucleotides

⚠ Radiolabeled oligonucleotide
The starting material for purification is the reaction mixture from Chapter 10, Protocol 2 (either Step 3 or Step 5), after it has been heated to 68°C to inactivate bacteriophage T4 polynucleotide kinase.

METHOD

1. Add 5-10 volumes of the EDTA-Tris-DNA solution to a microfuge tube containing the solution of radiolabeled oligonucleotide. Mix the reagents well.
2. Add sufficient 1% CPB to the tube to bring the concentration of the detergent in the mixture to 0.1%. Mix well.
3. Place the tube in a dry-ice/ethanol bath until the mixture is frozen. Remove the tube from the bath, and allow the mixture to thaw at room temperature.
4. Centrifuge the solution at maximum speed for 5 minutes at 4°C in a microfuge. Use a Pasteur pipette or a micropipettor equipped with a blue disposable tip to remove all of the supernatant carefully from the tube.
5. Add 500 µl of distilled H_2O to the tube, vortex the mixture for 20 seconds, and centrifuge the solution as in Step 4.
6. Remove the supernatant from the tube and add 500 µl of the ethanol-sodium acetate solution to the pellet. Vortex the mixture for 15 seconds and then centrifuge the solution at maximum speed for 2 minutes at room temperature in a microfuge.
7. Repeat Step 6.
8. Carefully remove the supernatant and stand the open tube on the bench behind a Plexiglas screen until the last visible traces of ethanol have evaporated. Dissolve the precipitated oligonucleotide in 20-50 µl of TE (pH 7.6) or in a small volume of prehybridization solution if the radiolabeled oligonucleotide is to be used as a probe.

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Protocol 5

Purification of Radiolabeled Oligonucleotides by Size-exclusion Chromatography

When radiolabeled oligonucleotides are to be used in enzymatic reactions such as primer extension, virtually all of the unincorporated radiolabel must be removed from the oligonucleotide. Chromatographic methods (Chapter 10, Protocol 5 and Chapter 10, Protocol 6) or gel electrophoresis (essentially as described in Chapter 10, Protocol 1) are superior in this respect than differential precipitation of the oligonucleotide with ethanol or CTAB.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform
Optional, please see Step 7.
EDTA (0.5 M, pH 8.0)
Ethanol
⚠ Phenol:chloroform
Optional, please see Step 7.
Sodium acetate (3 M, pH 5.2)
Optional, please see Step 7.
TE (pH 7.6)
Tris-Cl (1 M, pH 8.0)
Optional, please see Step 7.
Tris-SDS chromatography buffer

Nucleic Acids and Oligonucleotides

⚠ Radiolabeled oligonucleotide
The starting material for purification is the reaction mixture from Protocol 2 (either Step 3 or Step 5), after it has been heated to 68°C to inactivate bacteriophage T4 polynucleotide kinase.

Additional Reagents

Step 6 of this protocol may require the reagents listed in Chapter 13, Protocol 7.

METHOD

1. Add 30 µl of 20 mM EDTA (pH 8.0) to the tube containing the radiolabeled oligonucleotide. Store the solution at 0°C while preparing a column of size-exclusion chromatography resin.
   For convenience, Bio-Gel P-60 is used throughout this protocol as an example of a suitable resin; however, the method works equally well with Sephadex G-15.
2. Prepare a Bio-Gel P-60 column in a sterile Pasteur pipette.
   a. Equilibrate the slurry of Bio-Gel P-60 supplied by the manufacturer in 10 volumes of Tris-SDS chromatography buffer.
      If a centrifugal evaporator (Savant SpeedVac or its equivalent) is available, the Bio-Gel P-60 column may be poured and run in a solution of 0.1% ammonium bicarbonate. The pooled fractions containing the radiolabeled oligonucleotide (please see Step 6) can then be evaporated to dryness in a centrifugal evaporator, thereby eliminating the need for extraction of the oligonucleotide preparation with organic solvents and precipitation with ethanol.
   b. Tamp a sterile glass wool plug into the bottom of a sterile Pasteur pipette.
      A glass capillary tube works well as a tamping device.
   c. With the plug in place, pour a small amount of Tris-SDS chromatography buffer into the column and check that the buffer flows at a reasonable rate (one drop every few seconds).
   d. Fill the pipette with the Bio-Gel P-60 slurry. The column forms rapidly as the gel matrix settles under gravity and the buffer drips from the pipette. Add additional slurry until the packed column fills the pipette from the plug of glass wool to the constriction near the top of the pipette.
   e. Wash the column with 3 ml of Tris-SDS chromatography buffer.
      IMPORTANT *Do not allow the column to run dry. If necessary, seal the column by wrapping a piece of Parafilm around the bottom of the pipette.*
3. Use a pipette to remove excess buffer from the top of the column, and then rapidly load the radiolabeled oligonucleotide (in a volume of 100 µl or less) onto the column.
4. Immediately after the sample has entered the column, add 100 µl of buffer to the top of the column. As soon as the buffer has entered the column, fill the pipette with buffer. Replenish the buffer as necessary so that it continuously drips from the column. Do not allow the column to run dry.
5. Use a hand-held minimonitor to follow the progress of the radiolabeled oligonucleotide. When the radioactivity first starts to elute from the column, begin collecting two-drop fractions into microfuge tubes.
6. When nearly all of the radioactivity has eluted from the column, use a liquid scintillation counter to measure the radioactivity in each fraction by Cerenkov counting. If there is a clean separation of the faster-migrating peak (the radiolabeled oligonucleotide) from the slower peak of unincorporated [γ-^{32}P]ATP, pool the samples containing the radiolabeled oligonucleotide. If the peaks are not well separated, analyze approx. 0.5 µl of every other fraction either by adsorption to DE-81 filters (please see Chapter 13, Protocol 7, Step 3) or by thin-layer chromatography. Pool those fractions containing radiolabeled oligonucleotide that do not contain appreciable amounts of unincorporated [γ-^{32}P]ATP.
7. If the radiolabeled oligonucleotide is to be used in enzymatic reactions, then proceed as follows. Otherwise, proceed to Step 8.
   a. Extract the pooled fractions with an equal volume of phenol:chloroform.
   b. Back-extract the organic phase with 50 µl of 10 mM Tris-Cl (pH 8.0), and combine the two aqueous phases.
   c. Extract the combined aqueous phases with an equal volume of chloroform.
   d. Add 0.1 volume of 3 M sodium acetate (pH 5.2), mix well, and add 3 volumes of ethanol. Incubate the sample for 30 minutes at 0°C, and then centrifuge it at maximum speed for 20 minutes at 4°C in a microfuge. Use a micropipettor equipped with a disposable tip to remove the ethanol (which should contain very little radioactivity) from the tube.
8. Add 500 µl of 80% ethanol to the tube, vortex it briefly, and centrifuge the tube again at maximum speed for 5 minutes in a microfuge.
9. Use a micropipettor equipped with a disposable tip to remove the ethanol from the tube. Stand the open tube behind a Plexiglas screen until the residual ethanol has evaporated.
10. Dissolve the precipitated oligonucleotide in 20 µl of TE (pH 7.6) and store it at -20°C.

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Protocol 6

Purification of Radiolabeled Oligonucleotides by Chromatography on a Sep-Pak C_{18} Column

Radiolabeled oligonucleotides can be separated from unincorporated radiolabel by chromatography on silica gel resins. The protocol is suitable only for purifying oligonucleotides carrying 5´-phosphate groups. The method described in Chapter 10, Protocol 1 should be used to purify oligonucleotides with free 5´-hydroxyl groups.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Acetonitrile (5%, 30%, and 100%)
Use 10 ml HPLC-grade acetonitrile (100%) for each Sep-Pak column. Prepare the diluted solutions of acetonitrile in H_2O just before use.

Ammonium bicarbonate (25 mM, pH 8.0)

Ammonium bicarbonate (25 mM, pH 8.0) containing 5% (v/v) acetonitrile
Mix 5 ml of acetonitrile with 95 ml of 25 mM ammonium bicarbonate.

TE (pH 7.6)

Nucleic Acids and Oligonucleotides

⚠ Radiolabeled oligonucleotide
The starting material for purification is the reaction mixture from Chapter 10, Protocol 2 (either Step 3 or Step 5), after it has been heated to 68°C to inactivate bacteriophage T4 polynucleotide kinase.

METHOD

1. Prepare a Sep-Pak C_{18} reversed-phase column as follows:
   a. Attach a polypropylene syringe containing 10 ml of acetonitrile to a Sep-Pak C_{18} column.
   b. Slowly push the acetonitrile through the Sep-Pak column.
   c. Remove the syringe from the Sep-Pak column and then take the plunger out of the barrel. This prevents air being pulled back into the column. Reattach the barrel to the column.
   d. Flush out the organic solvent with two 10-ml aliquots of sterile H_2O. Repeat Step c after each wash.
2. Dilute the radiolabeled oligonucleotide preparation to 1.5 ml with sterile H_2O, and apply the entire sample to the column through the syringe.
3. Wash the Sep-Pak column with the following four solutions. Repeat Step 1c after each wash.
   10 ml of 25 mM ammonium bicarbonate (pH 8.0)
   10 ml of 25 mM ammonium bicarbonate/5% acetonitrile
   10 ml of 5% acetonitrile
   10 ml of 5% acetonitrile
4. Elute the radiolabeled oligonucleotide with three 1-ml aliquots of 30% acetonitrile. Collect each fraction in a separate 1.5-ml microfuge tube. Repeat Step 1c after each elution.
5. Recover the oligonucleotide by evaporating the eluate to dryness in a centrifugal evaporator (Savant SpeedVac or its equivalent).
6. Dissolve the radiolabeled oligonucleotide in a small volume (10 µl) of TE (pH 7.6).

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Protocol 7

Labeling of Synthetic Oligonucleotides Using the Klenow Fragment of *E. coli* DNA Polymerase I

Probes of high specific activities can be obtained using the Klenow fragment of *E. coli* DNA polymerase I to catalyze synthesis of a strand of DNA complementary to a synthetic oligonucleotide. A short primer is hybridized to an oligonucleotide template whose sequence is the complement of the desired radiolabeled probe. The primer is then extended using the Klenow fragment of *E. coli* DNA polymerase I to incorporate [α-^{32}P]dNTPs in a template-directed manner. After the reaction, the template and product are separated by denaturation, followed by electrophoresis through a polyacrylamide gel under denaturing conditions. With this method, it is possible to generate oligonucleotide probes that contain several radioactive atoms per molecule of oligonucleotide and to achieve specific activities as high as 2 x 10^{10} cpm/µg of probe.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Formamide loading buffer

10x Klenow buffer

Enzymes and Buffers

Klenow fragment of *E. coli* DNA polymerase I

Nucleic Acids and Oligonucleotides

Oligonucleotide primer
Purify the primer as described in Chapter 10, Protocol 1 . To ensure efficient radiolabeling, the primer should be in three- to tenfold molar excess over the template DNA in the reaction mixture.

Template oligonucleotide
Purify the template as described in Chapter 10, Protocol 1 . The sequence of the oligonucleotide template should be the complement of the desired radiolabeled probe.

Radioactive Compounds

⚠ [α-^{32}P]dNTPs
To keep the substrate concentration high, perform the extension reaction in as small a volume as possible. Thus, it is best to use radiolabeled dNTPs supplied in ethanol/H_2O rather than those supplied in buffered aqueous solvents. Appropriate volumes of the ethanolic [α-^{32}P]dNTPs can be mixed and evaporated to dryness in the microfuge tube that will be used to carry out the reaction.

Additional Reagents

Step 9 of this protocol requires reagents listed in Chapter 5, Protocol 12 .

METHOD

1. Transfer to a microfuge tube the calculated amounts of [α-^{32}P]dNTPs necessary to achieve the desired specific activity and sufficient to allow complete synthesis of all template strands.
   The concentration of dNTPs should not drop below 1 µM at any stage during the reaction. To keep the substrate concentration high, the extension reaction should be carried out in as small a volume as possible.
2. Add to the tube the appropriate amounts of oligonucleotide primer and template oligonucleotide.
   To ensure efficient radiolabeling, the primer should be in three- to tenfold molar excess over the template DNA in the reaction mixture.
3. Add 0.1 volume of 10x Klenow buffer to the tube. Mix the reagents well.
4. Add 2-4 units of the Klenow fragment per 5 µl of reaction volume. Mix well. Incubate the reaction for 2-3 hours at 14°C.
   If desired, the progress of the reaction may be monitored by removing small (0.1 µl) aliquots and measuring the proportion of radioactivity that has become precipitable with 10% trichloroacetic acid (TCA).
5. Prepare a denaturing polyacrylamide gel (as described in Chapter 12, Protocol 8) of the appropriate concentration (see table). Dilute the reaction mixture with an equal volume of formamide-loading buffer, heat the mixture to 80°C for 3 minutes, and load the entire sample on the gel.

Percent Polyacrylamide Required to Resolve Oligonucleotides

Length of Oligonucleotide	Polyacrylamide (%)
12-15 nucleotides	20
25-35 nucleotides	15
35-45 nucleotides	12
45-70 nucleotides	10

6. Following electrophoresis, disassemble the electrophoresis apparatus, leaving the polyacrylamide gel attached to one of the glass plates (for details, please see Chapter 12, Protocol 11).
7. Wrap the gel and its backing plate in Saran Wrap. Note the position of the tracking dyes and use a hand-held minimonitor to check the amount of radioactivity in the region of the gel that should contain the oligonucleotide. Attach a set of adhesive dot labels, marked with either very hot radioactive ink or phosphorescent spots, around the edge of the sample on the Saran Wrap. Cover the radioactive dots with Scotch Tape to prevent contaminating the film holder or intensifying screen with the radioactive ink.
8. Expose the gel to autoradiographic film.
   Usually, the amount of radioactivity incorporated into the probe is so great that the time needed to obtain an image on film is no more than a few seconds.
9. After developing the film, align the images of the radioactive ink with the radioactive marks on the labels, and locate the position of the probe in the gel. Excise the band and recover the radioactive oligonucleotide as described in Chapter 5, Protocol 12 .

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Protocol 8

Hybridization of Oligonucleotide Probes in Aqueous Solutions: Washing in Buffers Containing Quaternary Ammonium Salts

Quaternary ammonium salts greatly ameliorate the effects of G + C content on the melting temperature of oligonucleotides (Jacobs et al. 1988). In the following protocol, hybridization is carried out in conventional aqueous solvents at a temperature well below the predicted melting temperature. Nonspecific hybrids are then removed by washing at high stringency in buffers containing quaternary salts. Tetramethylammonium chloride (TMACl) is used with probes that are 14-50 nucleotides in length, whereas tetraethylammonium chloride (TEACl) is used with longer oligonucleotides.
The graph in Figure 10-2 on page 10.36 of the print version of the manual should be used to estimate a washing temperature in TMACl buffers for hybrids involving oligonucleotides of different lengths. When using TEACl buffers, subtract 33°C from the value obtained from Figure 10-2.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Oligonucleotide hybridization solution
- Oligonucleotide prehybridization solution
- 6x SSC
  Alternatively, 6x SSPE may be used. Place these solutions on ice before using in Steps 4 and 5 of the protocol.
- TEACl wash solution
- TMACl (5 M)/TEACl (3 M)
- TMACl wash solution

Nucleic Acids and Oligonucleotides

Nitrocellulose or nylon filters or membranes containing the immobilized target nucleic acids of interest (e.g., Southern or northern blots, lysed bacterial colonies filters, or bacteriophage plaques)

Probes

- ⚠ Radiolabeled oligonucleotide probe
  Prepared as described in Chapter 10, Protocol 2 or Chapter 10, Protocol 7 . We recommend that phosphorylated probes be purified by precipitation with CPB as described in Chapter 10, Protocol 4 before use in hybridization.

METHOD

1. Prehybridize the filters or membranes for 4-16 hours in oligonucleotide prehybridization solution at 37°C.
   Prehybridization, hybridization, and washing of circular filters are best carried out in Sears Seal-A-Meal bags or plastic boxes with tight-fitting lids. For Southern and northern blots, a hybridization device equipped with sealable glass tubes may be used.
2. Discard the prehybridization solution and replace it with oligonucleotide hybridization solution containing a radiolabeled oligonucleotide probe at a concentration of 180 pM.
   When hybridizing with several oligonucleotides simultaneously, each probe should be present at a concentration of 180 pM and the specific activity of the radiolabeled probe should be 5 x 10^5 to 1.5 x 10^6 cpm/pmole.
3. Incubate the filters for 12-16 hours at 37°C.
4. Discard the radiolabeled hybridization solution into an appropriate disposable container. Rinse the filters three times at 4°C with ice-cold 6x SSC or 6x SSPE to remove most of the dextran sulfate.
5. Wash the filters twice for 30 minutes at 4°C in ice-cold 6x SSC or 6x SSPE.
6. Rinse the filters at 37°C in two changes of the TMACl or TEACl wash solution.
   The aim of this step is to replace the SSPE and SSC with the solution of quaternary alkylammonium salts. Unless this step is carried out diligently, the full benefits of using TEACl or TMACl will not be realized.
7. Wash the filters twice for 20 minutes each in TMACl or TEACl wash solution at a temperature that is 2-4°C below the T_m indicated in Figure 10-2 of the print version of the manual.
   Note that the T_m of a hybrid is 33°C lower in a buffer containing TEACl than in a buffer containing TMACl. Make sure that the buffers are prewarmed to the desired temperature and that fluctuations in temperature are less then ±1°C.
8. Remove the filters from the washing solution. Blot them dry at room temperature and autoradiograph them.

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Protocol 9

Empirical Measurement of Melting Temperature

The melting temperature (T_m) of an oligonucleotide may be determined empirically by measuring the temperature (T_i) at which dissociation of the double-stranded DNA becomes irreversible. The procedure requires a cloned target sequence that is complementary (perfectly or imperfectly, depending on the experiment) to the oligonucleotide probe. If a target sequence is not available from "natural" sources, it can be synthesized chemically or by PCR. The experiment is carried out under nonequilibrium conditions that do not favor rehybridization of the released probe to the target.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Denaturation solution (for double-stranded DNA targets only)
- Neutralizing solution for neutral transfer (for double-stranded DNA targets only)
- Oligonucleotide prehybridization solution (10-9)
- ⚠ Phenol:chloroform (1:1, v/v) (for double-stranded target DNA only)
- Sodium acetate (3 M, pH 5.2) (for double-stranded target DNA only)
- 2x SSC

Enzymes and Buffers

Appropriate restriction enzyme for double-stranded target DNA
Please see Step 3.

Nucleic Acids and Oligonucleotides

Control DNA
Single-stranded or double-stranded DNA vector or sequence unrelated to the target DNA. Please see Step 3.

Target DNA
Ideally, this sequence should be cloned into a bacteriophage M13 vector and isolated as a single-stranded DNA (please see Chapter 3). Double-stranded plasmids or PCR products can also be used after denaturation as described in Step 3 of the protocol.

Probes

Oligonucleotide probe
Crude oligonucleotides are acceptable for this procedure provided the preparation can be labeled by phosphorylation to a specific activity >10^6 cpm/µg.
Before synthesizing the probe, check for potential homology and/or complementarity between its sequence and that of the vector used to propagate the target. Most of the commercially available programs to analyze DNA can be used to search common vector sequences for matches that might cause problems during hybridization.

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 10, Protocol 2 and Chapter 10, Protocol 4 .

METHOD

1. Radiolabel 1-10 pmoles of the oligonucleotide to be used as a probe by phosphorylation (Chapter 10, Protocol 2), and remove excess unincorporated [γ-^{32}P]ATP by precipitation with CPB (Chapter 10, Protocol 4).
2. Use a paper-hole punch to cut four small circles (diameter 3-4 mm) out of a nitrocellulose or nylon membrane for hybridization. Arrange the circles on a piece of Parafilm. Mark two of the membranes with a soft-lead pencil.
3. Apply target and control DNAs to the membrane circles as follows.

   Single-stranded target DNA

   a. Apply approx. 100 ng of target DNA in a volume of 1-3 µl of 2x SSC to each of the marked membranes.
   b. Apply an equal amount of vector DNA (e.g., single-stranded bacteriophage M13 DNA without insert) to the unmarked membranes.
   c. After the fluid has dried, use blunt-ended forceps (e.g., Millipore forceps) to remove the two sets of membranes from the Parafilm, and place them between sheets of thick blotting paper.
   d. Fix the DNAs to the membranes by baking for 1-2 hours at 80°C in a vacuum oven.
   Alternatively, place the membranes on a sheet of blotting paper and fix the DNA by cross-linking using UV light.

   Double-stranded target DNA

   a. If the target DNA has been cloned into a plasmid, linearize both the recombinant plasmid and the vector by digestion with a restriction enzyme that does not cleave within the target sequence.
   b. Purify the resulting double-stranded DNA or the PCR product by extraction with phenol:chloroform and standard precipitation with ethanol. Dissolve the DNA in 2x SSC at a concentration of 50-100 ng/µl.
   c. Apply the solution of target DNA, as well as a control DNA, to the membranes prepared as described above. Use blunt-ended forceps to transfer the membranes to a sheet of thick blotting paper saturated with denaturation solution. Incubate the membranes for 5-10 minutes at room temperature.
   d. Transfer the membranes to a fresh sheet of thick blotting paper saturated with neutralizing solution. Incubate the membranes for 10 minutes at room temperature.
   e. Transfer the membranes to a dry sheet of thick blotting paper, and leave them at room temperature until all of the fluid has evaporated. Immobilize the DNA to the membranes either by baking for 1-2 hours at 80°C in a vacuum oven or by cross-linking using UV light.
4. Use blunt-ended forceps to transfer all of the membranes to a polyethylene tube containing 2 ml of oligonucleotide prehybridization solution (10-9). Seal the tube and incubate, with occasional shaking, at a temperature estimated to be 25°C below the T_m for the solvent being used.
   Although the above protocol uses sodium salts in the hybridization solution, other solutes such as TMACl or TEACl can be substituted if desired to determine the T_i *in these solvents.*
5. After 2 hours, add the radiolabeled oligonucleotide to the prehybridization solution. The final concentration of oligonucleotide should be approx. 1 pmole/ml. Continue incubating at 25°C below the T_m for a further 2-4 hours, with occasional shaking.
6. Remove the membranes from the hybridization solution, and immediately immerse them in 2x SSC at room temperature. Agitate the fluid continuously. Replace the fluid every 5 minutes until the amount of radioactivity on the membranes remains constant (as measured with a hand-held minimonitor).
7. Adjust the temperature of a circulating water bath to 25°C below the T_m. Dispense 5 ml of 2x SSC into each of 20 glass

test tubes (17 x 100 mm). Monitor the temperature of the fluid in one of the tubes with a thermometer. Incubate the tubes in the water bath until the temperature of the 2x SSC is 25°C below the T_m.

8. Transfer the membranes individually to four empty glass tubes, and add 1 ml of 2x SSC (from one of the tubes prepared in Step 7 and prewarmed to 25°C below the T_m) to each membrane. Place the tubes in the water bath for 5 minutes.
9. Remove the tubes containing membranes from the bath, transfer the liquid to scintillation vials, and wash the tubes and membranes with 1 ml of 2x SSC at room temperature. Add the wash solutions to the appropriate scintillation vials.
10. Increase the temperature of the water bath by 3°C, and wait for the temperature of the 2x SSC in the tubes prepared in Step 7 to equilibrate.
11. Add 1 ml of 2x SSC at the higher temperature to each of the four tubes containing the membranes. Place the tubes in the water bath for 5 minutes.
12. Repeat Steps 9, 10, and 11 at successively higher temperatures until a temperature of 30°C above the T_m is achieved.
13. Place the membranes in separate glass tubes (17 x 100 mm) containing 1 ml of 2x SSC, and heat them to boiling for 5 minutes to remove any remaining radioactivity. Cool the solutions in ice, and transfer them to scintillation vials. Wash the membranes and tubes used for boiling with 1 ml of 2x SSC, and add the washing solutions to the appropriate scintillation vials.
14. Use a liquid scintillation counter to measure the radioactivity (by Cerenkov counting) in all of the vials. Calculate the proportion of the total radioactivity that has eluted at each temperature.
    For details on calculations, please see Step 14 on page 10.41 of the print version of the manual.
    If the experiment has worked well, very little radioactivity should be associated with the membranes containing vector DNA alone. Furthermore, this radioactivity should be completely released from the membranes at temperatures much lower than the estimated T_m. *On the other hand, considerable radioactivity should be associated with the membranes containing the target DNA; the elution of this radioactivity should show a sharp temperature dependence. Very little radioactivity should be released from the membranes until a critical temperature is reached, and then approx. 90% of the radioactivity should be released during the succeeding 6-9°C rise in temperature.*

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Chapter 11 Preparation of cDNA Libraries and Gene Identification

Protocol 1: Construction of cDNA Libraries
Stage 1: Synthesis of First-strand cDNA Catalyzed by Reverse Transcriptase

This traditional method is based on the Gubler-Hoffman method (Gubler and Hoffman 1983) and is divided into six stages:

- Stage 1: Synthesis of First-strand cDNA Catalyzed by Reverse Transcriptase
- Stage 2: Second-strand Synthesis
- Stage 3: Methylation of cDNA
- Stage 4: Attachment of Linkers or Adaptors
- Stage 5: Fractionation of cDNA by Gel Filtration through Sepharose CL-4B
- Stage 6: Ligation of cDNA to Bacteriophage λ Arms

This protocol describes the conversion of poly(A)$^+$ mRNA into first-strand cDNA in a reaction catalyzed by a murine RNase H$^-$ reverse transcriptase and primed by oligo(dT), random hexamers, or prime-adapters. The reaction conditions for first-strand synthesis using other enzymes are given in Table 11-3 on p. 11.38 in the print version of the manual.

Protocol 2: Construction of cDNA Libraries
Stage 2: Second-strand Synthesis

Here, the DNA-RNA hybrids synthesized in Stage 1 are converted into full-length double-stranded cDNAs. The primers for synthesis of second-strand cDNA are created by RNase H, which introduces nicks into the RNA moiety of the cDNA-mRNA hybrids. *E. coli* DNA polymerase I extends the newly created 3'-hydroxyl termini, using the first-strand cDNA as a template. In this way, the remaining segments of mRNA in the cDNA-mRNA hybrid are replaced with the newly synthesized second strand of DNA. Residual nicks are then repaired by *E. coli* DNA ligase, and the frayed termini of the double-stranded cDNA are polished by a DNA polymerase such as bacteriophage T4 DNA polymerase or *Pfu*. Finally, bacteriophage T4 polynucleotide kinase is used to catalyze the phosphorylation of 5'-hydroxyl groups on the ends of the cDNAs in preparation for ligation of linkers or adaptors (Chapter 11, Protocol 4).

Protocol 3: Construction of cDNA Libraries
Stage 3: Methylation of cDNA

The goal of this stage is to introduce methyl groups that will modify and protect naturally occurring *Eco*RI sites in the double-stranded cDNA. Note that Stage 3 is obligatory if synthetic linkers are used to attach the cDNA to a vector. However, the entire stage should be omitted if adaptors are used.

Protocol 4: Construction of cDNA Libraries
Stage 4: Attachment of Linkers or Adaptors

This stage achieves four goals: polishing the ends of double-stranded DNA, ligation of synthetic linkers or adaptors, digestion of the attached linkers to create cohesive termini, and preparing the cDNA for cloning.

Protocol 5: Construction of cDNA Libraries
Stage 5: Fractionation of cDNA by Gel Filtration through Sepharose CL-4B

Unused linkers, adaptor-linkers, and low-molecular-weight products created by digestion of linkers in Step 4 are removed by size-exclusion chromatography.

Protocol 6: Construction of cDNA Libraries
Stage 6: Ligation of cDNA to Bacteriophage λ Arms

The final stage of cDNA library construction involves optimizing ligation of the size-fractionated cDNA to bacteriophage λ arms, followed by packaging and plating of the recombinant bacteriophages.

Protocol 7: Construction and Screening of Eukaryotic Expression Libraries
Stage 1: Construction of cDNA Libraries in Eukaryotic Expression Vectors

This stage summarizes how to construct a cDNA library in a mammalian vector, using commercial kits. These kits are available from several manufacturers and generally include all the required reagents.

Protocol 8: Construction and Screening of Eukaryotic Expression Libraries
Stage 2: Screening cDNA Libraries Constructed in Eukaryotic Expression Vectors

Two methods of screening pools of cloned cDNAs are discussed: transfection into mammalian cells and injection into *Xenopus* oocytes.

Protocol 9: Exon Trapping and Amplification
Stage 1: Construction of the Library

This protocol describes how to construct and amplify a genomic DNA library in a plasmid vector (pSPL3) equipped to express cloned exons in transfected mammalian cells. This protocol was provided by Deanna M. Church (Church and Buckler 1999).

Protocol 10: Exon Trapping and Amplification
Stage 2: Electroporation of the Library into COS-7 Cells

In this stage, COS-7 cells are transfected with a library of plasmids containing the genomic DNA of interest. This protocol was provided by Deanna M. Church (Church and Buckler 1999).

Protocol 11: Exon Trapping and Amplification
Stage 3: Harvesting the mRNA

Standard techniques are used here to isolate cytoplasmic RNA from COS cells transfected with a library of recombinant plasmids containing the genomic DNA of interest. This protocol was provided by Deanna M. Church (Church and Buckler 1999). Prepare all reagents used in this protocol with DEPC-treated H_2O.

Protocol 12: Exon Trapping and Amplification
Stage 4: Reverse Transcriptase-PCR

In this stage, cDNA molecules generated from the RNA isolated in Chapter 11, Protocol 11 are amplified by PCR. Amplified cDNAs containing functional 3´ and 5´ splice sites are selected by digestion with *Bst*XI and cloned into a Bluescript vector. This protocol was provided by Deanna M. Church (Church and Buckler 1999).

Protocol 13: Exon Trapping and Amplification

Stage 5: Analysis of Clones

The final stage of this protocol involves amplification and sequencing of the exon-trapped products. This protocol was provided by Deanna M. Church (Church and Buckler 1999).

Protocol 14: Direct Selection of cDNAs with Large Genomic DNA Clones

The goal of this method is to identify transcriptionally active genes in cloned segments of genomic DNA. The protocol uses hybridization and affinity purification to recover biotin-labeled cDNAs that bind to a 500-kb segment of human DNA cloned in a BAC vector. However, the method can be easily adapted to other clones of genomic DNAs cloned in high-capacity vectors. This protocol was provided by Michael Lovett (Morgan et al. 1992; Simmons and Lovett 1999).

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Protocol 1

Construction of cDNA Libraries
Stage 1: Synthesis of First-strand cDNA Catalyzed by Reverse Transcriptase

This traditional method is based on the Gubler-Hoffman method (Gubler and Hoffman 1983) and is divided into six stages:

- Stage 1: Synthesis of First-strand cDNA Catalyzed by Reverse Transcriptase
- Stage 2: Second-strand Synthesis
- Stage 3: Methylation of cDNA
- Stage 4: Attachment of Linkers or Adaptors
- Stage 5: Fractionation of cDNA by Gel Filtration through Sepharose CL-4B
- Stage 6: Ligation of cDNA to Bacteriophage λ Arms

This protocol describes the conversion of poly(A)$^+$ mRNA into first-strand cDNA in a reaction catalyzed by a murine RNase H$^-$ reverse transcriptase and primed by oligo(dT), random hexamers, or prime-adapters. The reaction conditions for first-strand synthesis using other enzymes are given in Table 11-3 on p. 11.38 in the print version of the manual.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Actinomycin D
Actinomycin D is needed only when a wild-type version of Mo-MLV RT is used that possesses RNase H activity.

DTT (dithiothreitol) (1 M)

EDTA (0.5 M, pH 8.0)

KCl (1 M)

$MgCl_2$ (1 M)

Tris-Cl (1 M, pH 8.3 at room temperature)

Enzymes and Buffers

Reverse transcriptase
Mo-MLV RT is temperature-sensitive and should be stored at -20°C until needed at the end of Step 1.

RNase inhibitor (optional)

Nucleic Acids and Oligonucleotides

dNTP solution containing all four dNTPs, each at 5 mM

Oligonucleotide primers for cDNA synthesis (1 mg/ml)
Synthesis of first-strand cDNA is generally primed by random hexamers, oligo(dT)$_{12-18}$, or a mixture of the two. For directional cloning, primer-adaptors of the general structure $^{5'}$p(dX)-(dR)-(dT)$_x$-OH$^{3'}$ are used, where X = a clamp composed of four nucleotides (usually GAGA), R = a recognition site for a restriction enzyme, and (dT)$_x$ = a homopolymeric run of 15 dT residues used for directional cloning.

Poly(A)$^+$ RNA for cDNA synthesis (1 mg/ml)
Approximately 5-10 µg of poly(A)$^+$ RNA is required to synthesize enough double-stranded cDNA to construct a large library. (One confluent 90-mm plate of cultured mammalian cells yields 1-2 µg of poly(A)$^+$ RNA.) The synthetic reactions will still work if less template is available, but the losses of cDNA at each stage in the protocol will be proportionately greater.

The integrity of the poly(A)$^+$ RNA to be used should ideally be checked by (1) agarose gel electrophoresis and northern hybridization with a control probe (please see Chapter 7, Protocol 5 and Chapter 7, Protocol 8), (2) translation in a cell-free system followed by SDS-polyacrylamide gel electrophoresis of the resulting polypeptides, and (3) analysis of the size of the first strand of cDNA synthesized in a pilot reaction.

Radioactive Compounds

⚠ [α-^{32}P]dCTP (10 mCi/ml, 400 Ci/mmole)
[α-^{32}P]dATP and [α-^{32}P]dTTP should not be used as radioactive tracers to monitor synthesis of the first and second strands of cDNA, since they may be incorporated preferentially into cDNA derived from the poly(A)$^+$ tract at the 3' terminus of the mRNA. Batches of [α-^{32}P]dCTP >2 weeks old should not be used in this protocol.
Thaw the [α-^{32}P]dCTP just before starting the protocol. Store the thawed radiolabeled dCTP on ice until needed in Step 2. Return the radiolabeled dCTP to the freezer immediately after use.
IMPORTANT *The Stratagene cDNA Synthesis Kit recommends the use of any label other than dCTP with its protocol. This kit includes 5-methyl dCTP in the dNTP mixes to protect the cDNA against cleavage at internal sites by* XhoI*, which is used to release* XhoI *ends for ligation. The use of radiolabeled dCTP with the Stratagene protocol could create unprotected* XhoI *sites within the cDNA sequence.*

Additional Reagents

Step 5 of this protocol requires the reagents listed in Chapter 5, Protocol 8.

METHOD

1. To synthesize first-strand cDNA, mix the following in a sterile microfuge tube on ice:

1 µg/µl poly(A)$^+$ RNA	10 µl
1 µg/µl oligonucleotide primer(s)	1 µl
1 M Tris-Cl (pH 8.0 at 37°C)	2.5 µl
1 M KCl	3.5 µl
250 mM $MgCl_2$	2 µl
solution of all four dNTPs, each at 5 mM	10 µl
0.1 M dithiothreitol	2 µl
RNase inhibitor (optional)	25 units
H_2O	to 48 µl

Add the manufacturer's recommended amount of Mo-MLV H$^-$ RT to the reaction. Mix the reagents well by gentle vortexing.
Mo-MLV RT is temperature-sensitive and should be stored at -20°C until needed.
IMPORTANT *If a preparation of AMV RT is used, please see Table 11-3 on p. 11.38 in the print version of the manual*

for reaction conditions.

2. After all of the components of the reaction have been mixed at 0°C, transfer 2.5 µl of the reaction to a fresh 0.5-ml microfuge tube. Add 0.1 µl of [α-^{32}P]dCTP (400 Ci/mmole, 10 mCi/ml) to the small-scale reaction.
3. Incubate the large- and small-scale reactions for 1 hour at 37°C.
   Higher temperatures (up to 55°C) can be used with some mutant forms of Mo-MLV RT.
4. At the end of the incubation period, add 1 µl of 0.25 M EDTA to the small-scale reaction containing the radioisotope. Transfer the small-scale reaction to ice. Heat the large-scale reaction to 70°C for 10 minutes and then transfer it to ice.
5. Measure both the total amount of radioactivity and the amount of trichloroacetic acid (TCA)-precipitable radioactivity in 0.5 µl of the small-scale reaction, as described in Appendix 8 in the print version of the manual. In addition, it is worthwhile analyzing the products of the small-scale reaction through an alkaline agarose gel using appropriate DNA markers (please see Chapter 5, Protocol 8).
   The remainder of the small-scale reaction can be stored at -20°C.
6. Calculate the amount of first-strand cDNA synthesized as follows:
   a. Since 10 µl of a solution containing all four dNTPs at a concentration of 5 mM each was used (i.e., 10 µl of 20 mmoles/liter of total dNTP), the large-scale reaction must contain
      20 nmoles/µl dNTP x 10 µl = 200 nmoles of dNTP
   b. Because the molecular weight of each dNMP incorporated into DNA is approx. 330 g/mole, the reaction is capable of generating a total of
      200 nmoles x 330 ng/nmole = 66 µg of DNA
   c. Therefore, from the results of the small-scale reaction,
      $\frac{\text{cpm incorporated}}{\text{total cpm}}$ x 66 µg = µg of first strand of cDNA synthesized
7. Proceed as soon as it is feasible to the next stage in the synthesis of the cDNA.

REFERENCES

1. Gerard G.F. and D'Alessio J.M. 1993. Reverse transcriptase (EC2.7.7.49): The use of cloned Moloney murine leukemia virus reverse transcriptase to synthesize DNA from RNA. *Methods Mol. Biol.* 16:73-94.
2. Gubler U. and Hoffman B.J. 1983. A simple and very efficient method for generating cDNA libraries. *Gene* 25:263-269.

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Protocol 2

Construction of cDNA Libraries
Stage 2: Second-strand Synthesis

Here, the DNA-RNA hybrids synthesized in Stage 1 are converted into full-length double-stranded cDNAs. The primers for synthesis of second-strand cDNA are created by RNase H, which introduces nicks into the RNA moiety of the cDNA-mRNA hybrids. *E. coli* DNA polymerase I extends the newly created 3'-hydroxyl termini, using the first-strand cDNA as a template. In this way, the remaining segments of mRNA in the cDNA-mRNA hybrid are replaced with the newly synthesized second strand of DNA. Residual nicks are then repaired by *E. coli* DNA ligase, and the frayed termini of the double-stranded cDNA are polished by a DNA polymerase such as bacteriophage T4 DNA polymerase or *Pfu*. Finally, bacteriophage T4 polynucleotide kinase is used to catalyze the phosphorylation of 5'-hydroxyl groups on the ends of the cDNAs in preparation for ligation of linkers or adaptors (Chapter 11, Protocol 4).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Ammonium sulfate $(NH_4)_2SO_4$ (1 M)
- 10x Bacteriophage T4 polynucleotide kinase buffer
- ⚠ Chloroform
- EDTA (0.5 M, pH 8.0)

Ethanol

$MgCl_2$ (1 M)

- β-Nicotinamide adenine dinucleotide (β-NAD) (50 mM)
  β-NAD is a cofactor for E. coli *DNA ligase, as well as the electron acceptor; do not confuse it with α-NAD.*
- ⚠ Phenol:chloroform (1:1, v/v)
- RNase H buffer (optional)
  Use this buffer only if carrying out the alternative to Step 1.
- Sodium acetate (3 M, pH 5.2)
- TE (pH 7.6)
- Tris-Cl (2 M, pH 7.4)

Enzymes and Buffers

Bacteriophage T4 DNA polymerase (2.5 units/µl)

Bacteriophage T4 polynucleotide kinase (30 units/µl)

E. coli DNA ligase

E. coli DNA polymerase I

RNase H
The RNase H purified from E. coli *is sold by several manufacturers and is required at a specific activity of approx. 1000 units/ml.*

Nucleic Acids and Oligonucleotides

- dNTP solution containing all four dNTPs, each at 10 mM

First-strand cDNA
Use the large-scale, first-strand reaction mixture prepared in Chapter 11, Protocol 1.

Marker DNA

Radioactive Compounds

⚠ [α-^{32}P]dCTP (10 mCi/ml, 400 Ci/mmole)
Do not use [α-^{32}P]dTTP as a radioactive tracer to monitor synthesis of the second strands of cDNA; it may be incorporated preferentially into the region of the second strand corresponding to the poly(A)$^+$ tract at the 3' terminus of the mRNA.
Thaw the [α-^{32}P]dCTP just before starting the protocol. Store the thawed radiolabeled dCTP on ice until needed in Step 1. Return the radiolabeled dCTP to the freezer immediately after use.

METHOD

1. Add the following reagents directly to the large-scale first-strand reaction mixture (Chapter 11, Protocol 1):

10 mM $MgCl_2$	70 µl
2 M Tris-Cl (pH 7.4)	5 µl
10 mCi/ml [α-^{32}P]dCTP (400 Ci/mmole)	10 µl
1 M $(NH_4)_2SO_4$	1.5 µl
RNase H (1000 units/ml)	1 µl
E. coli DNA polymerase I (10,000 units/ml)	4.5 µl

   Mix the reagents by gently vortexing, and centrifuge the reaction mixture briefly in a microfuge to eliminate any bubbles. Incubate the reaction for 2-4 hours at 16°C.
   Second-strand synthesis catalyzed by RNase H and DNA polymerase I results in loss of sequences (approx. 20 nucleotides) from the extreme 5´ end of the mRNA template. The alternative to Step 1 reduces the risk of losing 5´-terminal regions of cDNA clones. Unfortunately, the alternative method results in a reduced yield of double-stranded cDNA, and most investigators will therefore opt for the standard Step 1 method.

   Incubate the second-strand synthetic reaction (above) for 2 hours at 16°C in the absence of RNase H*. Purify the resulting double-stranded cDNA by extraction with phenol:chloroform and precipitation with ethanol in the presence of 0.3 M sodium acetate (pH 5.2). Dissolve the precipitated DNA in 20 µl of 20 mM Tris-Cl (pH 7.6), 20 mM KCl, 0.1 mM EDTA (pH 8.0), 0.1 mM dithiothreitol. Digest the DNA for 20 minutes at 37°C with RNase H (0.5 unit). Proceed to Step 2.*
2. At the end of the incubation, add the following reagents to the reaction mixture:

β-NAD (50 mM)	1 µl
E. coli DNA ligase (1000-4000 units/ml)	1 µl

   Incubate the reaction for 15 minutes at room temperature.
3. At the end of the incubation, add 1 µl of a mixture containing all four dNTPs each at a concentration of 10 mM and 2 µl

(5 units) of bacteriophage T4 DNA polymerase. Incubate the reaction mixture for 15 minutes at room temperature.

4. Remove a small aliquot (3 µl) of the reaction. Measure the mass of second-strand DNA in the aliquot as described in Steps 7 and 8.
5. To the remainder of the reaction, add 5 µl of 0.5 M EDTA (pH 8.0). Extract the mixture once with phenol:chloroform and once with chloroform. Recover the DNA by precipitation with ethanol in the presence of 0.3 M sodium acetate (pH 5.2). Dissolve the DNA in 90 µl of TE (pH 7.6).
6. To the DNA, add:

10x T4 polynucleotide kinase buffer	10 µl
T4 polynucleotide kinase (3000 units/ml)	1 µl

   Incubate the reaction at room temperature for 15 minutes.
7. Use the small aliquot from Step 4 to determine the total amount of radioactivity and the TCA-precipitable counts in 1 µl of the second-strand synthesis reaction as described in Appendix 8 in the print version of the manual.
8. Use the following equation to calculate the weight of the cDNA synthesized in the second-strand reaction, taking into account the amount of dNTPs already incorporated into the first strand of cDNA:
   $$\frac{\text{cpm incorporated in the second-strand reaction}}{\text{total cpm}} \times (66\ \mu g - x\ \mu g) = \mu g \text{ of second-strand cDNA synthesized}$$
   where *x* is the weight of the first strand of cDNA. The amount of second-strand cDNA synthesized is usually 70-80% of the weight of the first strand.
9. Extract the reaction containing phosphorylated cDNA (from Step 6) with an equal volume of phenol:chloroform.
10. Separate the unincorporated dNTPs from the cDNA by spun-column chromatography through Sephadex G-50 equilibrated in TE (pH 7.6) containing 10 mM NaCl (please see Appendix 8 in the print version of the manual).
11. Precipitate the eluted cDNA by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. Store the sample on ice for at least 15 minutes. Recover the precipitated DNA by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge. Use a hand-held minimonitor to check that all of the radioactivity has been precipitated.
12. Wash the pellet with 70% ethanol and centrifuge again.
13. Gently aspirate all of the fluid (check to see that none of the radioactivity is in the aspirated fluid), and allow the pellet to dry in the air.
14. Dissolve the cDNA in 80 µl of TE (pH 7.6) if it is to be methylated by *Eco*RI methylase (please see Chapter 11, Protocol 3). Alternatively, if the cDNA is to be ligated directly to *Not*I or *Sal*I linkers, or an adaptor oligonucleotide (please see Chapter 11, Protocol 4), resuspend the cDNA in 29 µl of TE (pH 7.6).

REFERENCES

1. Gubler U. 1987. Second-strand cDNA synthesis: mRNA fragments as primers. *Methods Enzymol.* 152:330-335.
2. Gubler U. and Hoffman B.J. 1983. A simple and very efficient method for generating cDNA libraries. *Gene* 25:263-269.

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Protocol 3

Construction of cDNA Libraries
Stage 3: Methylation of cDNA

The goal of this stage is to introduce methyl groups that will modify and protect naturally occurring *Eco*RI sites in the double-stranded cDNA. Note that Stage 3 is obligatory if synthetic linkers are used to attach the cDNA to a vector. However, the entire stage should be omitted if adaptors are used.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

10x *Eco*RI buffer

10x *Eco*RI methylase buffer (Optional)

EDTA (0.5 M, pH 8.0)

Ethanol

$MgCl_2$ (1 M)

NaCl (5 M)

⚠ Phenol:chloroform

⚠ *S*-Adenosylmethionine
New England Biolabs provides a solution of S*-adenosylmethionine when* Eco*RI methylase is purchased.*

Sodium acetate (3 M, pH 5.2)

TE (pH 8.0)

Tris-Cl (2 M, pH 8.0)

Enzymes and Buffers

*Eco*RI

*Eco*RI methylase
New England Biolabs sells an enzyme isolated from a strain of E. coli *that expresses the methylase gene from a multicopy plasmid.*

Nucleic Acids and Oligonucleotides

Double-stranded cDNA
Use the cDNA prepared in Chapter 11, Protocol 2, Step 14.

Test DNA
Either linearized plasmid DNA or bacteriophage λ DNA is used to check the efficiency of methylation of Eco*RI sites. The linearized plasmid DNA should contain at least one* Eco*RI site located some distance from the termini. Use a bacteriophage λ DNA such as that used as a size standard for agarose gel electrophoresis (e.g.,* Hind*III-digested λ DNA). For plasmid DNA, digest 1 µg to completion with* Pst*I. Purify the DNA by extraction with phenol:chloroform and precipitation with ethanol. Dissolve the DNA in 5 µl of TE (pH 7.6) and store it at -20°C.*

METHOD

1. Add to the cDNA (from Protocol 11, Stage 2, Step 14):

2 M Tris-Cl (pH 8.0)	5 µl
5 M NaCl	2 µl
0.5 M EDTA (pH 8.0)	2 µl
20 mM *S*-adenosylmethionine	1 µl
H_2O	to 96 µl

2. Remove two 2-µl aliquots and place each in a separate 0.5-ml microfuge tube. Number the tubes 1 and 2, and store the numbered tubes on ice.
3. Add 2 µl of *Eco*RI methylase (80,000 units/ml) to the remainder of the reaction mixture and then store the reaction mixture at 0°C until Step 4 is completed.
4. Remove two additional aliquots (2 µl each) from the large-scale reaction and place each in a separate 0.5-ml microfuge tube. Number these tubes 3 and 4.
5. To each of the four small aliquots (Steps 2 and 4), add test DNA (100 ng of plasmid DNA *or* 500 ng of bacteriophage λ DNA), prepared as described in Materials. These unmethylated DNAs are used as substrates in pilot reactions to assay the efficiency of methylation.
   IMPORTANT *Do not add any DNA to the large-scale reaction!*
6. Incubate all four pilot reactions and the large-scale reaction for 1 hour at 37°C.
7. Heat the five reactions to 68°C for 15 minutes. Extract the large-scale reaction once with phenol:chloroform and once with chloroform.
8. To the large-scale reaction add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. Mix the reagents well and store the ethanolic solution at -20°C until the results of the pilot reactions are available.
9. Analyze the four pilot reactions as follows:
   a. To each control reaction, add:

0.1 M $MgCl_2$	2 µl
10x *Eco*RI buffer	2 µl
H_2O	to 20 µl

   b. Add 20 units of *Eco*RI to reactions 2 and 4.
   c. Incubate all four samples for 1 hour at 37°C, and analyze them by electrophoresis through a 1% agarose gel.
10. Recover the precipitated cDNA (Step 8) by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge. Remove the supernatant, add 200 µl of 70% ethanol to the pellet, and centrifuge again.
11. Use a hand-held minimonitor to check that all of the radioactivity is recovered in the pellet. Remove the ethanol by gentle aspiration, dry the pellet in the air, and then dissolve the DNA in 29 µl of TE (pH 8.0).
12. Proceed as soon as is feasible to the next stage in the synthesis of the cDNA.

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Protocol 4

Construction of cDNA Libraries
Stage 4: Attachment of Linkers or Adaptors

This stage achieves four goals: polishing the ends of double-stranded DNA, ligation of synthetic linkers or adaptors, digestion of the attached linkers to create cohesive termini, and preparing the cDNA for cloning.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)

5x Bacteriophage T4 DNA polymerase repair buffer

Bromophenol blue (0.25% w/v in 50% glycerol)

EDTA (0.5 M, pH 8.0)

Ethanol

⚠ Phenol:chloroform

Sodium acetate (3 M, pH 5.2)

TE (pH 8.0)

Tris-Cl (1 M, pH 8.0)

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Bacteriophage T4 DNA polymerase

Restriction enzymes

Nucleic Acids and Oligonucleotides

cDNA, either unmethylated or methylated
Use the cDNA prepared in either Chapter 11, Protocol 2, Step 14 or Chapter 11, Protocol 3, Step 11.

Control DNA
Please see note to Step 10.

dNTP solution containing all four dNTPs, each at 5 mM

Test DNA
Either linearized plasmid DNA or bacteriophage λ DNA is used to check the efficiency of digestion following linker addition. A plasmid DNA should contain at least one EcoRI *site located some distance from the ends of the linear DNA (e.g., Xf3 or pBR322 linearized by digestion with* PstI*). Use a bacteriophage λ DNA such as that used as a size standard for agarose gel electrophoresis (e.g.,* HindIII*-digested λ DNA). For plasmid DNA, digest 1 µg to completion with* PstI*. Purify the DNA by extraction with phenol:chloroform and precipitation with ethanol. Dissolve the DNA in 5 µl of TE (pH 7.6). Store at -20°C. Predigested λ DNA can be used as is.*

Synthetic linkers/adaptors
Phosphorylated linkers and adaptors are sold by several manufacturers. If possible, obtain linkers that are at least 4-8 nucleotides longer than the site recognized by the restriction enzyme. Many restriction enzymes inefficiently cleave recognition sites located very close to the ends of DNA molecules.

METHOD

1. Heat the cDNA (Chapter 11, Protocol 3) to 68°C for 5 minutes.
2. Cool the cDNA to 37°C and add the following to the tube:

5x bacteriophage T4 DNA polymerase repair buffer	10 µl
5 mM dNTP solution	5 µl
H_2O	to 50 µl

3. Add 1-2 units of bacteriophage T4 DNA polymerase (500 units/ml) and incubate the reaction for 15 minutes at 37°C.
4. Stop the reaction by adding 1 µl of 0.5 M EDTA (pH 8.0).
5. Extract the sample with phenol:chloroform, and remove the unincorporated dNTPs by spun-column chromatography through Sephadex G-50 (please see Appendix 8 in the print version of the manual).
6. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol to the column flow-through. Store the sample for at least 15 minutes at 4°C.
7. Recover the precipitated cDNA by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge. Dry the pellet in the air, and then dissolve it in 13 µl of 10 mM Tris-Cl (pH 7.6).
8. Add the following to the repaired DNA:

10x T4 DNA ligase buffer	2 µl
800-1000 ng of phosphorylated linkers or adaptors	2 µl
10^5 Weiss units/ml bacteriophage T4 DNA ligase	1 µl
10 mM ATP	2 µl

   Mix and incubate for 8-12 hours at 16°C.
   It is essential to use a vast molar excess of linkers (>100-fold) to ensure that the ends of the cDNA become ligated to a linker and not to each other.
9. Withdraw 0.5 µl from the reaction and store the aliquot at 4°C. Inactivate the ligase in the remainder of the reaction by heating for 15 minutes at 68°C.
   If endonuclease digestion is necessary to permit ligation of the cDNA to the vector, then proceed with Steps 10-12. However, when using certain adaptor molecules, endonuclease digestion is not required. In these cases, skip to Step 13.
10. To the heated ligase reaction, add:

10x restriction enzyme buffer	20 µl
H_2O	150 µl
restriction enzyme	200 units

    Mix the reagents at 0°C.
    As a control, transfer 2 µl of the reaction to a 0.5-ml microfuge tube. To the 2-µl aliquot, add 100 ng (in a volume of no more than 0.5 µl) of control DNA, i.e., either a linearized plasmid or a preparation of cleaved bacteriophage λ DNA that contains an internal site for the particular restriction enzyme used.
11. Incubate the large-scale reaction and the control reaction for 2 hours at 37°C.
12. Analyze the DNA in the control sample and the 0.5-µl aliquot withdrawn at Step 9 by electrophoresis through a 1%

agarose gel. Load one or two lanes of the gel with plasmid or bacteriophage λ marker DNA.

13. Purify the cDNA by extraction with phenol:chloroform and standard precipitation with ethanol. Dissolve the cDNA in 20 µl of TE (pH 8.0).
14. Add 2.5 µl of a solution of 0.25% bromophenol blue in 50% glycerol. This addition simplifies the next stage in the cDNA library — size-fractionation of the cDNA by chromatography through Sepharose CL-4B (Chapter 11, Protocol 5).

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1. Helfman D.M., Fiddes J.C., and Hanahan D. 1987. Directional cDNA cloning in plasmid vectors by sequential addition of oligonucleotide linkers. *Methods Enzymol.* 152:349-359.
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Protocol 5

Construction of cDNA Libraries
Stage 5: Fractionation of cDNA by Gel Filtration through Sepharose CL-4B

Unused linkers, adaptor-linkers, and low-molecular-weight products created by digestion of linkers in Step 4 are removed by size-exclusion chromatography.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol

- Sodium acetate (3 M, pH 5.2)
- TE (pH 7.6)
- TE (pH 7.6) containing 0.1 M NaCl
- Tris-Cl (1 M, pH 8.0)

Nucleic Acids and Oligonucleotides

cDNA
Use the cDNA prepared in Chapter 11, Protocol 4, Steps 13 and 14.

Marker DNA
The marker DNA should be end-labeled fragments ranging in size from 200 bp to 5 kb.

METHOD

1. Use a hypodermic needle with a bent end to pull the cotton wool pledget halfway out of the end of a sterile, disposable 1-ml pipette. Cut the pledget in half with sterile scissors. Discard the loose piece of cotton wool. Use filtered compressed air to blow the remainder of the pledget to the narrow end of the pipette.
2. Attach a piece of sterile polyvinyl chloride tubing (of the type normally used in peristaltic pumps) to the narrow end of the pipette. Dip the wide end of the pipette into a solution of TE (pH 7.6) containing 0.1 M NaCl in a beaker. Attach the tubing to an Erlenmeyer flask connected to a vacuum line. Apply gentle suction until the pipette is filled with buffer. Close the tubing with a hemostat.
3. Attach a piece of vinyl bubble tubing to the wide end of the pipette. Fill the bubble tubing with Sepharose CL-4B equilibrated with TE (pH 7.6) containing 0.1 M NaCl. Allow the slurry to settle for a few minutes and then release the hemostat. The column will form as the buffer drips from the pipette. If necessary, add more Sepharose CL-4B until the packed matrix almost fills the pipette.
   The dimension of the packed column should be about 27 x 0.3 cm.
4. Wash the column with several column volumes of TE (pH 7.6) containing 0.1 M NaCl. After washing is completed, use a hemostat to close the tubing at the bottom of the column.
5. Use a Pasteur pipette to remove the fluid above the Sepharose CL-4B. Apply the cDNA (in a volume of 50 µl or less) to the column. Release the hemostat and allow the cDNA to enter the gel matrix. Wash the microfuge tube used to store the cDNA with 50 µl of TE (pH 7.6), and apply this to the column. Fill the bubble tubing with TE (pH 7.6) containing 0.1 M NaCl.
   IMPORTANT *Do not allow the column to run dry at any stage!*
6. Monitor the progress of the cDNA through the column using a hand-held minimonitor. Begin collecting 2-drop fractions (approx. 60 µl) in microfuge tubes when the radioactive cDNA has traveled two thirds the length of the column. Continue collecting fractions until all of the radioactivity has eluted from the column.
7. Measure the radioactivity in each fraction by Cerenkov counting (please see Appendix 8 in the print version of the manual).
8. Analyze small aliquots of each fraction (approx. 5 µl) by electrophoresis through a 1% agarose gel, using as markers end-labeled fragments of DNA of known size (200 bp to 5 kb). Store the remainder of the fractions at -20°C until the autoradiograph of the agarose gel is available.
9. After electrophoresis, transfer the gel to a piece of Whatman 3MM paper. Cover the gel with Saran Wrap, and dry it on a commercial gel dryer. Heat the gel to 50°C for the first 20-30 minutes of drying and then turn off the heat. Continue drying the gel under vacuum for a further 1-2 hours.
10. Expose the dried gel to X-ray film at -70°C with an intensifying screen (please see Appendix 9 in the print version of the manual).
11. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol to fractions containing cDNA molecules that are $\geq$ 500 bp in length. Allow the cDNA to precipitate for at least 15 minutes at 4°C. Recover the DNA by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge.
12. Dissolve the DNA in a total volume of 20 µl of 10 mM Tris-Cl (pH 7.6).
13. Determine the amount of radioactivity present in a small aliquot, and calculate the total amount of cpm available in the selected fractions. Calculate the total quantity of cDNA available for ligation to bacteriophage λ arms (for calculation of second-strand cDNA data, please see Chapter 11, Protocol 2, Step 8):

    $$\frac{\text{cpm available}}{\text{cpm incorporated into second strand}} \times 2x \text{ µg second-strand cDNA synthesized} = \text{µg of cDNA available for ligation}$$

    If everything has gone well, 10 µg of poly(A)$^+$ RNA should yield at least 250-400 ng, and possibly as much as 3 µg, of cDNA whose size is larger than 500 bp in length.

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Protocol 6

Construction of cDNA Libraries
Stage 6: Ligation of cDNA to Bacteriophage λ Arms

The final stage of cDNA library construction involves optimizing ligation of the size-fractionated cDNA to bacteriophage λ arms, followed by packaging and plating of the recombinant bacteriophages.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)

SM

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Bacteriophage T4 DNA ligase buffer

Nucleic Acids and Oligonucleotides

cDNA
Use the cDNA prepared in Chapter 11, Protocol 5, Step 12.

Control bacteriophage λ DNA
This DNA is usually provided with the commercial packaging extract and is used to determine the packaging efficiency of the extract.

Marker DNA
The marker DNA should be fragments ranging in size from 500 bp to 5 kb.

Additional Reagents

Step 4 of this protocol requires the reagents listed in Chapter 2, Protocol 1.

Step 7 of this protocol requires the reagents listed in Chapter 2, Protocol 23.

Vectors and Bacterial Strains

Bacteriophage λ arms
If making a large number of libraries, it is much less expensive to prepare arms by agarose gel electrophoresis or by sucrose density gradients (please see Chapter 2, Protocol 16) than to purchase them. For occasional users, dephosphorylated arms for some vectors (e.g., λgt10, λFix, and λZipLox) are available from Stratagene, Life Technologies, and other commercial suppliers. It is important to carry out a series of pilot reactions to check that the arms can be ligated to foreign DNA and packaged efficiently into infectious bacteriophage λ particles. DNAs for these controls are usually included with commercial preparations of vector arms.

E. coli strain, freshly prepared overnight cultures
*Use strains C600 (BNN93) for growth and BNN102 (C600*hflA*) for screening of cDNA libraries constructed in bacteriophage λgt10. Use strain Y1090*hsdR *for growth and screening of cDNA libraries constructed in bacteriophage λgt11. Use strain BB4 for growth and screening of cDNA libraries constructed in λZAP and λZAPII or strain XL1-Blue for λZAPII. (Strain XL1-Blue supports vigorous growth of λZAPII but not of λZAP.) For λZipLox, use strain Y1090(ZL).*

Packaging extracts for bacteriophage λ
Packaging extracts are difficult to make and should therefore be purchased commercially. Several commercial suppliers provide packaging kits with unique features.

METHOD

1. Set up four test ligation/packaging reactions as follows:

Ligation	A	B	C	D
λ vector DNA (0.5 µg/µl)	1.0 µl	1.0 µl	1.0 µl	1.0 µl
10x T4 DNA ligase buffer	1.0 µl	1.0 µl	1.0 µl	1.0 µl
cDNA	0 ng	5 ng	10 ng	50 ng
Bacteriophage T4 DNA ligase (100 Weiss units/µl)	0.1 µl	0.1 µl	0.1 µl	0.1 µl
10 mM ATP	1.0 µl	1.0 µl	1.0 µl	1.0 µl
H_2O to a final volume of	10 µl	10 µl	10 µl	10 µl

   Incubate the ligation mixtures for 4-16 hours at 16°C. Store the unused portion of the cDNA at -20°C.
2. Package 5 µl of each ligation into bacteriophage λ particles following the directions provided by the manufacturer of the packaging extract.
3. After the packaging reaction is complete, add 0.5 ml of SM to each mixture.
4. Use the fresh overnight cultures of the appropriate strain(s) of *E. coli* to plate 10 µl and 100 µl of a 10^{-2} dilution of each packaging mixture on each strain (please see Chapter 2, Protocol 1). Incubate the plates for 8-12 hours at 37°C or 42°C.
5. Count the number of recombinant and nonrecombinant plaques. Ligation A should yield no recombinant plaques, whereas ligations B, C, and D should yield increasing numbers of recombinant plaques.
6. From the number of recombinant plaques, calculate the efficiency of cloning of cDNA (pfu/ng cDNA). If all has gone well, the efficiency should be at least 2 x 10^4 pfu/ng cDNA. The total yield of recombinants from 5 µg of poly(A)$^+$ RNA should be in excess of 5 x 10^6.
7. Pick 12 recombinant bacteriophage λ plaques, grow small-scale lysates, and prepare DNAs for digestion with the appropriate restriction enzyme.
8. Analyze the size of the cDNA inserts by electrophoresis through a 1% agarose gel, using as markers fragments of DNA 500 bp to 5 kb in length.

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Protocol 7

Construction and Screening of Eukaryotic Expression Libraries
Stage 1: Construction of cDNA Libraries in Eukaryotic Expression Vectors

This stage summarizes how to construct a cDNA library in a mammalian vector, using commercial kits. These kits are available from several manufacturers and generally include all the required reagents.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

10x Universal KGB buffer (restriction endonuclease buffer[s])

Enzymes and Buffers

Restriction enzymes
Please see Step 3.

Nucleic Acids and Oligonucleotides

Poly(A)$^+$-enriched mRNA
This mRNA should be isolated from a cell line or tissue that expresses the activity of interest and purified by two-pass chromatography on oligo(dT) cellulose (please see Chapter 7, Protocol 3). Identification of the highest expressing tissue or cell line must be accomplished empirically using the biological assay that will be employed in the expression cloning screen.

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 11, Protocol 1 to Chapter 11, Protocol 4.

Step 2 of this protocol requires the reagents listed in Chapter 11, Protocol 5.

Steps 4 and 5 of this protocol require the reagents listed in Chapter 11, Protocol 6.

Step 8 of this protocol may require the reagents listed in Chapter 1, Protocol 26.

Vectors and Bacterial Strains

Electrocompetent *E. coli* cells
If a plasmid expression vector is chosen, prepare (please see Chapter 1, Protocol 26) or purchase electrocompetent E. coli *as hosts for the cDNA library. The titer of the electrocompetent cells should be at least 5 x 10^8 colonies/µg plasmid DNA. Ideally, use a single lot or preparation of cells throughout the expression cloning and screening procedure.*

Expression vector
The choice of (plasmid or bacteriophage λ expression) vector is dictated by the host system used for expression. If Xenopus *oocytes are used, the expression vector should be a bacteriophage λ DNA or a plasmid vector DNA carrying promoters for the bacteriophage SP6, T3, or T7 DNA-dependent RNA polymerases flanking the polylinker (e.g., λZAP Express, λExCell, and pSPORT). If cultured mammalian cells are used, then choose a plasmid-based expression vector that contains a powerful promoter and a strong transcription terminator sequence. Examples include the pCMV series (Stratagene) of plasmids and pcDNA 4 (Invitrogen).*

Packaging extracts
If a bacteriophage λ vector is chosen, then purchase (or prepare) a very high titer packaging extract with which to insert the recombinant cDNAs into the virus. The titer of the extract should be at least 10^9 pfu/µg viral DNA.

METHOD

1. Use a commercial kit or follow the instructions in Chapter 11, Protocol 1 to Chapter 11, Protocol 4 to synthesize blunt-ended, double-stranded cDNA and equip the termini with the appropriate linkers or adaptors.
2. Fractionate the double-stranded cDNA according to size, using gel-filtration chromatography. For details, please see Chapter 11, Protocol 5.
   If the size of the target mRNA is known, pool the column fractions that contain double-stranded cDNAs ranging in size from 1 kb smaller than the target mRNA to 1 kb larger than the target mRNA. If the size of the target mRNA is unknown, fractionate the preparation of double-stranded cDNA into three pools containing molecules of different sizes: 500-1500 bp, 1500-3000 bp, and >3000 bp.
3. Digest 10-25 µg of the plasmid or bacteriophage λ expression vector with two restriction enzymes whose recognition sequences occur in the linker-adaptors placed at the 5' and 3' ends of the cDNA.
   IMPORTANT *Take care to ensure that both restriction enzymes digest the vector DNA to completion. Carry out the digestions in sequence rather than simultaneously; purify the DNA by extraction with phenol:chloroform and precipitation with ethanol between digests, and where possible, use gel electrophoresis to check that both of the digests have gone to completion.*
   To ensure complete cleavage of the expression vector before the cloning of cDNA, some investigators insert a short "stuffer" fragment of 200-300 bp between the SalI *and* NotI *(or other enzyme combination) sites of the polylinker.*
4. Set up trial ligations using different ratios of cDNA to bacteriophage λ arms or plasmid DNA.
5. Package an aliquot of the products of each of the ligation reactions into bacteriophage λ particles. Determine the titer of infectious particles generated in each packaging reaction. Alternatively, use electroporation to transform *E. coli* with aliquots of each ligation reaction (please see Chapter 1, Protocol 26).
6. Test six bacteriophage λ or plasmid recombinants for the presence of cDNA inserts of the appropriate size and determine the ratio of cDNA to vector DNA that generates the largest number of recombinant clones. Calculate the size of the library that can be generated from the ligation reactions containing the optimum ratio of cDNA to vector.
7. Using the optimum ratio of cDNA insert to vector, ligate as much of the cDNA as possible to the bacteriophage λ or plasmid DNA.
   It is usually better to set up many small ligation reactions, rather than one large reaction.
8. Prepare and analyze the recombinants using one of the methods below:
   If a bacteriophage λ vector is used: Package the ligated cDNA into bacteriophage λ particles following the directions provided by the manufacturer of the packaging extract, measure the titer of the virus stock, and store the stock at 4°C.
   If a plasmid vector is used: Measure the number of potential recombinants in the ligation reaction by electroporating small aliquots of the ligation mixture into *E. coli* cells (please see Chapter 1, Protocol 26).
9. Proceed as soon as is feasible to the screening of the eukaryotic expression library.

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Protocol 8

Construction and Screening of Eukaryotic Expression Libraries
Stage 2: Screening cDNA Libraries Constructed in Eukaryotic Expression Vectors

Two methods of screening pools of cloned cDNAs are discussed: transfection into mammalian cells and injection into *Xenopus* oocytes.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- SM

Enzymes and Buffers

Restriction endonucleases
Restriction endonucleases will be required for certain procedures. Please see Steps 1 and 3.

Nucleic Acids and Oligonucleotides

cDNA library, prepared as described in Chapter 11, Protocol 7

Transfection/Injection controls
Whenever possible, choose a positive control cDNA that encodes a biological activity similar to that of the desired target cDNA. The control cDNA is used to establish the optimum pool size and to check that a given transfection/injection experiment worked. The positive control cDNA should be transferred into the appropriate strain of E. coli *(if using a plasmid expression vector) or packaged into bacteriophage λ before carrying out Step 1.*
Two types of negative controls should be included in screening experiments using Xenopus *oocytes: The plasmid vector alone and the transcription reaction without added template DNA.*
In addition, Step 1 requires either an empty bacteriophage λ expression vector packaged into bacteriophage λ particles or E. coli *transformed with the empty plasmid expression vector.*

Media

- TB agar plates containing the appropriate antibiotic
- Terrific broth (or other rich medium) containing the appropriate antibiotic

Additional Reagents

Steps 1 and 2 of this protocol may require a commercial plasmid purification kit (see Chapter 1, Protocol 9).

Steps 1 and 3 of this protocol may require the reagents from one of the transfection protocols in Chapter 16.

Steps 1 and 3 of this protocol may require the reagents listed in Chapter 9, Protocol 6.

Steps 1 and 3 of this protocol may require the reagents necessary to inject mRNA molecules into *Xenopus* oocytes.

Step 1 of this protocol may require the reagents listed in Chapter 2, Protocol 1 or Chapter 2, Protocol 5 and Chapter 2, Protocol 23 or Chapter 2, Protocol 24.

Step 1 of this protocol requires the reagents necessary to assay for the biological activity encoded by the positive control cDNA.

Step 2 of this protocol may require the reagents listed in Chapter 1, Protocol 26, and Chapter 2, Protocol 1 and Chapter 2, Protocol 23 or Chapter 2, Protocol 24.

Step 4 of this protocol requires the reagents necessary to assay for the biological activity encoded by the target cDNA.

Step 6 of this protocol requires the reagents listed in Chapter 7, Chapter 12, Chapter 15, and Chapter 16.

Cells and Tissues

Host cells
Xenopus *oocytes are taken from female South African clawed frogs, which are available from several biological supply houses (such as Carolina Biological Supply or Kons Scientific). Difficulties in expressing mRNAs can sometimes be overcome by switching suppliers.*
Simian COS cells or derivatives of the 293 line of human embryonic kidney cells are the mammalian hosts of choice for transient expression of cloned cDNAs because of their superior efficiencies of transfection. However, many other cell lines have been used successfully (e.g., Chinese hamster ovary and NIH-3T3).
Before embarking on expression cloning, test lysates of mock-injected oocytes or lysates of mock-transfected cells for the activity of target protein. It is essential to start with an expression host that has low to undetectable levels of the activity to be cloned.

METHOD

1. Set up a series of trial experiments to optimize the transfection and expression systems used to screen the library for cDNA clones of interest. This is best done by using a previously cloned cDNA encoding a biological activity for which reliable assays are available.

   If a eukaryotic plasmid expression vector and cultured cell host are used

   a. Inoculate a single colony of *E. coli* harboring a plasmid carrying the cDNA used as a positive control into 10 ml of rich medium (e.g., Terrific Broth containing a selective antibiotic) together with 10, 100, 1000, 10,000, or 100,000 colonies derived from electroporation of *E. coli* with an empty vector. Grow the cells to saturation in an overnight culture incubated with agitation at 37°C.

   b. Use a commercial kit to prepare plasmid DNA of a purity sufficient for efficient transfection of cultured mammalian cells (please see Chapter 1, Protocol 9).

   c. Use one or more of the methods described in Chapter 16 to transfect the various plasmid preparations into cultures of eukaryotic cells and assay for the biological activity encoded by the positive control cDNA.
   When screening the cDNA library, use the transfection method that generates the maximum signal and an acceptably low level of background.

   If a bacteriophage RNA polymerase (e.g., T3, T7, or Sp6) is used to transcribe cDNAs from a plasmid expression vector followed by *Xenopus* oocyte injection

   a. Follow Steps a and b above.

b. Linearize the pooled, purified plasmid DNAs at the rare restriction site placed at the 3' end of the cDNAs during library construction, and transcribe the templates in vitro into mRNA (please see Chapter 9, Protocol 6).

c. Inject the mRNA prepared from the pooled cDNAs into *Xenopus* oocytes and assay the appropriate biological activity or transfect the mRNA into the appropriate cell line.

If a bacteriophage λ vector containing bacteriophage-encoded RNA polymerase promoters was chosen as an expression vector

a. Generate a set of bacteriophage suspensions containing different ratios (10:1, 100:1, 1000:1, etc.) of an empty bacteriophage λ vector to a recombinant bacteriophage λ harboring the control cDNA. Infect an appropriate strain of *E. coli* with a multiplicity of bacteriophage particles that yields near-confluent lysis of bacterial lawns or complete lysis of infected cells grown in liquid culture (please see Chapter 2, Protocol 1 or Chapter 2, Protocol 5).

b. Prepare bacteriophage λ DNA from the plates (please see Chapter 2, Protocol 23) or from the liquid cultures (please see Chapter 2, Protocol 24).

c. Linearize the bacteriophage λ DNA at the rare restriction site placed at the 3' end of the cDNAs during library construction, and transcribe the cDNAs into mRNA as described in Chapter 9, Protocol 6.

d. Inject the prepared mRNA into *Xenopus* oocytes, and assay the mRNAs for their ability to encode the biological activity of the cDNA used as a positive control.

2. Using the results obtained in Step 1 as a rough guide, divide the cDNA library into pools of a suitable size for screening, and transform or transfect *E. coli* with the expression library pools. Prepare plasmid or bacteriophage λ DNA that will be used to screen the pools for the presence of the target cDNA.

If the expression library is constructed in a plasmid vector and pools are grown on solid medium

a. Withdraw a sufficient number of aliquots (e.g., 50-100 aliquots, each capable of generating approx. 1000 recombinants) from the ligation mixture (Chapter 11, Protocol 7, Step 8), and introduce each aliquot individually into *E. coli* by electroporation. Plate the entire contents of each electroporation cuvette onto a single dish of Terrific agar medium containing the appropriate antibiotic. Incubate the plates overnight at 37°C.

b. Estimate the number of colonies on each plate. Scrape the colonies from each plate into 5 ml of rich medium (e.g., Terrific broth containing a selectable antibiotic). Grow the bacterial suspensions to saturation, and then prepare purified plasmid DNA for screening.

If the expression library is constructed in a plasmid vector and pools are grown in liquid medium

The advantages of this procedure are that the plating step is omitted, thus saving time and effort, and there is less chance that any individual cDNA will be lost because of poor growth on agar, or because of mechanical damage.

a. Withdraw a sufficient number of aliquots (e.g., 50-100 aliquots, each capable of generating approx. 1000 recombinants) from the ligation mixture (Chapter 11, Protocol 1, Step 8), and introduce them individually into separate cultures of *E. coli* by electroporation. The number of transformants should equal the desired pool size in each culture.

b. Add 1 ml of rich medium (e.g., SOC or Terrific broth) without antibiotics to each bacterial culture immediately after electroporation. Incubate the culture for 1 hour at 37°C with very gentle agitation.

c. Use an aliquot of each bacterial culture to inoculate separate 5-ml cultures. After growing the bacterial cultures to saturation, prepare purified plasmid DNA for screening.

If the expression library is constructed in a bacteriophage λ vector and pools are to contain 10,000 to 100,000 cDNAs

a. Plate an appropriate volume of packaging mixture to produce a semiconfluent lawn of recombinant bacteriophage plaques. Alternatively, use a sufficient amount of packaging mixture to obtain complete lysis of *E. coli* cells grown in liquid culture.

b. Isolate bacteriophage λ DNA by a plate lysis procedure or a liquid culture procedure (please see Chapter 2, Protocol 23 or Chapter 2, Protocol 24).

If the expression library is constructed in a bacteriophage λ vector and pools are to contain 10,000 or fewer cDNAs

a. Plate recombinant bacteriophages, equal in number to the pool size, at a density of 1000 pfu per 100-mm dish.

b. When plaque formation is complete, overlay each dish with 2-3 ml of SM to produce a low-titer plate lysate. Calculate the titer of the lysate.

c. From the low-titer plate lysate, produce a high-titer stock in liquid culture.

d. Purify bacteriophage λ DNA by a liquid culture procedure (please see Chapter 2, Protocol 24).

3. Transfer the purified clone into the appropriate background to analyze for expression:
If the clone is purified plasmid DNA: Either transfect into cultured mammalian cells or use as a template for in vitro synthesis of RNA by a bacteriophage-encoded RNA polymerase, followed by injection into *Xenopus* oocytes.
If the clone is purified bacteriophage λ DNA: Linearize the DNA and use as a template for in vitro synthesis of RNA, followed by injection into *Xenopus* oocytes.
4. Assay for the biological activity encoded by the target cDNA.
5. After a positive pool(s) of cDNAs has been identified, subdivide and rescreen it in an iterative fashion until a single cDNA encoding the target activity has been identified. Carry out this goal, which is most easily accomplished using the preparation of purified plasmid or bacteriophage λ DNA corresponding to the positive pool(s) of recombinants, as follows:

a. Divide the positive (primary) pool of plasmid or bacteriophage λ DNA into aliquots that generate approximately one tenth of the original number of transformed colonies or plaques.

b. Repeat Steps 3 and 4 above. Include the original positive pool as a positive control in the rescreening assay.

c. Repeat the process of subdivision until a single recombinant is obtained that encodes the desired activity. Make sure that only single well-isolated plaques or bacterial colonies are used to prepare bacteriophage or plasmid DNA for the ultimate screen.

6. Characterize the single isolate that encodes the target activity by DNA sequencing (Chapter 12), expression (Chapter 15 or Chapter 16), and RNA blotting (Chapter 7).

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Protocol 9

Exon Trapping and Amplification
Stage 1: Construction of the Library

This protocol describes how to construct and amplify a genomic DNA library in a plasmid vector (pSPL3) equipped to express cloned exons in transfected mammalian cells. This protocol was provided by Deanna M. Church (Church and Buckler 1999).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)

⚠ Phenol:chloroform (1:1, v/v)

TE (pH 8.0)

Enzymes and Buffers

*Pvu*II

Restriction endonucleases

T4 DNA ligase

Nucleic Acids and Oligonucleotides

Cosmid, BAC, or PAC recombinant DNA, encompassing the target genomic region
Purify the recombinant to be analyzed as described in Chapter 4.
or

Genomic DNA
Prepare the DNA as described in one of the methods presented in Chapter 6.

Media

LB agar plates containing 50 µg/ml ampicillin
Please see Step 8 to determine the appropriate size plates to use.

LB broth containing 50 µg/ml ampicillin

SOC medium

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 1, Protocol 20.

Step 6 of this protocol requires the reagents required for transformation listed in Chapter 1, Protocol 23 to Chapter 1, Protocol 25.

Steps 10 and 12 of this protocol require the reagents listed in Chapter 1, Protocol 1 or Chapter 1, Protocol 9.

Vectors and Bacterial Strains

E. coli strain HB101, competent for transformation
Prepare as described in Chapter 1, Protocol 23 to Chapter 1, Protocol 25.

pSPL3
The vector pSPL3 is available separately or as part of a kit from Life Technologies.

METHOD

1. Digest pSPL3 with a restriction enzyme(s) that will permit insertion of the genomic DNA. Dephosphorylate the ends of the linearized vector, and purify the vector by gel electrophoresis.
2. Digest 1-2 µg of genomic DNA or recombinant vector carrying genomic target DNA with a restriction enzyme(s) that is compatible with the enzyme used to prepare pSPL3 in Step 1.
3. Analyze 10% of the digested genomic DNA on a 0.9% agarose gel cast and run in TAE.
4. Inactivate the restriction enzyme from the digested genomic DNA by heating the reaction mixture to 65°C for 15 minutes. Extract the reaction mixture with phenol:chloroform and recover the DNA by standard ethanol precipitation. Resuspend the DNA at a concentration of 100 µg/ml in TE (pH 8.0).
5. Ligate the genomic DNA to the vector by combining the following:

digested genomic DNA	150 ng
digested, dephosphorylated pSPL3 vector	50 ng
10x ligation buffer	1 µl
bacteriophage T4 DNA ligase	10 Weiss units
H_2O	to 10 µl

   Incubate the reaction mixture for 2-3 hours at room temperature or overnight at 15°C.
   Be sure to include a control containing only the vector DNA. This control is important for assessing the quality of the library.
6. Transform 40 µl of competent HB101 cells with the ligation reaction.
   Be sure to include positive (vector alone DNA) and negative (no DNA) transformation controls to assess efficiency.
   IMPORTANT *It is imperative that HB101 be used to propagate the library and the vector, because pSPL3 is unstable in other bacterial strains (Church and Buckler 1999).*
7. After transformation, add 800 µl of SOC medium to the cells and incubate the culture for 30-45 minutes at 37°C to allow expression of the antibiotic resistance marker encoded on the plasmid.
8. Amplify the library by growing the bacteria overnight at 37°C in the presence of ampicillin.
   If using a single cosmid: Plate 100 µl of the transformation mixture onto an LB agar plate containing 50 µg/ml ampicillin. Grow the remainder of the transformation in a 2-ml liquid culture of LB broth containing 50 µg/ml ampicillin.
   If using a BAC, PAC, or pools of cosmids: Plate the entire contents of the transformation onto separate 150-mm LB agar plates containing 50 µg/ml ampicillin.
9. Estimate the efficiency of ligation by comparing the number of recombinant and nonrecombinant clones (i.e., colonies arising from the transformation of HB101 with the ligation mixture containing only pSPL3).
10. If the library was amplified on a 150-mm LB agar plate, proceed to Step 11. If the genomic library was amplified in liquid culture, purify plasmid DNA from the bacteria using a standard alkaline lysis procedure (please see Chapter 1, Protocol 1) or use a commercial plasmid purification kit. Proceed to Step 13.
11. If the entire library was plated onto a large LB agar plate, flood the plate with 10 ml of LB broth, and gently scrape the colonies from the surface of the agar. Minimize the amount of agar that is scraped into the LB broth.
12. Purify the plasmid DNA using a modification of the standard alkaline lysis minipreparation: triple the amounts of

Alkaline lysis solutions I (300 µl), II (600 µl), and III (450 µl) added to the bacterial pellet. To simplify the procedure, after addition of Alkaline lysis solution I, transfer the bacterial slush to a 2-ml microfuge tube. The rest of the procedure can be carried out in 2-ml tubes. After adding Alkaline lysis solution III and centrifuging, divide the recovered, crude nucleic acid supernatant into two 1.5-ml tubes. Extract the resulting supernatant with phenol:chloroform and with chloroform. Precipitate the supernatant with isopropanol and combine the two dissolved DNA pellets. Alternatively, use a commercial plasmid purification kit (Chapter 1, Protocol 9).

13. Digest an aliquot of the purified DNA with *Pvu*II to determine the quality of the library.

REFERENCES

1. Church D.M. and Buckler A.J. 1999. Gene identification by exon amplification. *Methods Enzymol.* 303:83-99.

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Protocol 10

Exon Trapping and Amplification
Stage 2: Electroporation of the Library into COS-7 Cells

In this stage, COS-7 cells are transfected with a library of plasmids containing the genomic DNA of interest. This protocol was provided by Deanna M. Church (Church and Buckler 1999).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- PBS without divalent cations

Enzymes and Buffers

- Trypsin-EDTA solution

Nucleic Acids and Oligonucleotides

DNA
The plasmid DNA to be transfected was prepared in Chapter 11, Protocol 9, Steps 10-12.

Plasmid vector for use as control (Step 3)

Media

Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum

Cells and Tissues

COS-7 cells
COS-7 cells are grown to a confluency of 75-85% in DMEM containing 10% heat-inactivated fetal calf serum.

METHOD

1. Wash the monolayer of COS-7 cells with phosphate-buffered saline lacking divalent cations. Remove the cells from the surface of the dishes by treatment with trypsin-EDTA. Collect the cells by centrifugation at 250*g* (1100 rpm in a Sorvall H1000B rotor) for 10 minutes at 4°C.
   IMPORTANT *Keep the COS-7 cells cold throughout the procedure.*
2. Adjust the volume of the DNA sample to be used for transfection to a total volume of 100 µl in PBS without divalent cations.
3. Resuspend 4 x 10^6 COS-7 cells in a volume of 700 µl of PBS without divalent cations. Mix the cells with the DNA in a cooled electroporation cuvette (0.4-cm chamber). Incubate the mixture for 10 minutes on ice.
4. Gently resuspend the cells and introduce the DNA into the cells by electroporation at 1.2 kV (3 kV/cm) and 25 µF. Immediately return the cuvettes to ice, and store them for 10 minutes.
5. Use a wide-bore pipette to transfer 1 x 10^6 of the electroporated cells into a series of 100-mm tissue culture dishes that contain 10 ml of DMEM containing 10% heat-inactivated fetal calf serum that has been warmed to 37°C. Incubate the cultures for 48-72 hours at 37°C in a humidified incubator with an atmosphere of 5-7% CO_2.

REFERENCES

1. Church D.M. and Buckler A.J. 1999. Gene identification by exon amplification. *Methods Enzymol.* 303:83-99.

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Protocol 11

Exon Trapping and Amplification
Stage 3: Harvesting the mRNA

Standard techniques are used here to isolate cytoplasmic RNA from COS cells transfected with a library of recombinant plasmids containing the genomic DNA of interest. This protocol was provided by Deanna M. Church (Church and Buckler 1999). Prepare all reagents used in this protocol with DEPC-treated H_2O.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ DEPC-treated H_2O

Ethanol

NaCl (5 M)

PBS without divalent cations

⚠ Phenol

⚠ Phenol:chloroform

⚠ SDS (5% w/v)

TKM buffer

Triton X-100 (10% v/v) or Nonidet P-40 (4% v/v)

Cells and Tissues

COS-7 cells transfected with the recombinant and nonrecombinant forms of pSPL3

METHOD

1. Rinse the tissue culture plates of transfected COS-7 cells three times with ice-cold PBS lacking calcium and magnesium ions. Keep plates on a bed of ice between rinses.
2. Add 10 ml of ice-cold PBS to each plate. Place the plate on the bed of ice, and gently scrape the cells off the plate.
3. Transfer the cell suspensions to chilled 15-ml polystyrene tubes.
4. Recover the cells by centrifugation at 300*g* (1200 rpm in a Sorvall H1000B rotor) for 8 minutes at 4°C.
5. Decant as much of the supernatant as possible. Remove the residual supernatant with a pipette.
6. Resuspend the cell pellet from 1 x 10^6 to 2 x 10^6 COS cells in 300 µl of TKM buffer and store the suspension on ice for 5 minutes.
7. Add 15 µl of 10% Triton X-100 or 4%-Nonidet P-40 and store the cell suspension on ice for a further 5 minutes.
8. Recover the nuclei by centrifugation at 500*g* (1500 rpm in a Sorvall H1000B rotor) for 5 minutes at 4°C.
9. Transfer the supernatant to a chilled 1.5-ml microfuge tube.
   IMPORTANT *Use extreme care when removing the supernatant. Do not touch the nuclear pellet. If the nuclear membranes burst, the sample will become too viscous to pipette due to the contamination with genomic DNA.*
10. Add 20 µl of 5% SDS and 300 µl of phenol. Vortex the mixture vigorously and separate the organic and aqueous phases by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.
11. Transfer the aqueous layer to a 1.5-ml microfuge tube containing 300 µl of phenol:chloroform. Vortex the suspension vigorously and then separate the organic and aqueous phases by centrifugation at maximum speed for 3 minutes at room temperature in a microfuge.
12. Transfer the aqueous (upper) layer to a chilled 1.5-ml microfuge tube containing 12 µl of 5 M NaCl and 750 µl of ethanol. Allow the RNA to precipitate for 2-3 hours at -20°C or for 30 minutes at -80°C.
13. Recover the RNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge.
14. Discard the supernatant and wash the pellet with 70% ethanol. Dry the pellet in air and redissolve it in 20 µl of DEPC-treated H_2O.

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1. Church D.M. and Buckler A.J. 1999. Gene identification by exon amplification. *Methods Enzymol.* 303:83-99.

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Protocol 12

Exon Trapping and Amplification
Stage 4: Reverse Transcriptase-PCR

In this stage, cDNA molecules generated from the RNA isolated in Chapter 11, Protocol 11 are amplified by PCR. Amplified cDNAs containing functional 3´ and 5´ splice sites are selected by digestion with *Bst*XI and cloned into a Bluescript vector. This protocol was provided by Deanna M. Church (Church and Buckler 1999).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- ⚠ Chloroform
- ⚠ DEPC-treated H_2O
- dNTP solution containing all four dNTPs, each at a concentration of 1.25 mM
- DTT (dithiothreitol) (1 M)
- ⚠ Phenol
- RNase inhibitor

Enzymes and Buffers

*Bst*XI

*Eco*RV

Mo-MLV reverse transcriptase

Taq DNA polymerase

Uracil DNA glycosylase (1 unit/µl)

Nucleic Acids and Oligonucleotides

Oligonucleotides primers (20 mM in TE [pH 8.0]):

SA2	*5′ ATC TCA GTG GTA TTT GTG AGC 3′*
SD6	*5′ TCT GAG TCA CCT GGA CCA CC 3′*
SDDU	*5′ AUA AGC UUG AUC UCA CAA GCT GCA CGC TCT AG 3′*
SADU	*5′ UUC GAG UAG UAC UTT CTA TTC CTT CGG GCC TGT 3′*
BSD-U	*5′ GAU CAA GCU UAU CGA TAC CGT CGA CCT 3′*
BSA-U	*5′ AGU ACU ACU CGA AUT CCT GCA GCC 3′*

Please note that the 5′ ends of oligonucleotides SDDU, SADU, BSD-U, and BSA-U contain uracil residues to facilitate ligation-independent cloning. SADU and SDDU are nested primers.

RNA isolated from COS-7 cells transfected with control and recombinant vectors (Chapter 11, Protocol 11)

Media

- LB agar plates containing 50 µg/ml ampicillin

Additional Reagents

Step 14 of this protocol requires the reagents listed in Chapter 1, Protocol 25.

Vectors and Bacterial Strains

E. coli strain DH5α (or another strain that permits α-complementation)

pBluescript II (KS or SK) (Stratagene)

METHOD

1. Generate the first-strand cDNA from the cytoplasmic RNAs isolated in the previous stage (Stage 3), using the SA2 oligonucleotide as a primer. In an RNase-free 0.5-ml tube combine:

RNA	3 µl
10x amplification buffer	2.5 µl
dNTP solution at 1.25 mM	4 µl
0.1 M dithiothreitol	1 µl
3' oligo (SA2, 20 µM)	1.25 µl
DEPC-treated H_2O	11.25 µl

Heat the reaction mixture to 65°C for 5 minutes.
IMPORTANT *To reduce formation of secondary structures in the RNAs, do not place the reaction mixture on ice!*

Then add:

RNase inhibitor	1 µl
Mo-MLV reverse transcriptase (200 units)	1 µl

Incubate the reactions for 90 minutes at 42°C.

2. Use both the SA2 oligonucleotide primer and the SD6 oligonucleotide primer to carry out a limited second-strand synthesis. Combine the following in a sterile thin-walled amplification tube:

reverse transcriptase reaction (Step 1)	12.5 µl
10x amplification buffer	4 µl
dNTP solution at 1.25 mM	6 µl
SA2 oligonucleotide primer (20 µM)	2 µl
SD6 oligonucleotide primer (20 µM)	2.5 µl
Taq DNA polymerase	1-2 units
DEPC-treated H_2O	to 40 µl

Process the vector-only control in the same way. This will serve as an important control during the PCR analysis.

3. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Alternatively, place a bead of paraffin wax into the tube if using a hot start PCR. Place the tubes in the thermal cycler.

4. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
1	5 min at 94°C		
5-6	30 sec at 94°C	30 sec at 62°C	3 min at 72°C
Last cycle			5 min at 72°C

Times and temperatures may need to be adapted to suit the particular reaction conditions.
The purpose of this PCR is to generate double-stranded material for Bst*XI digestion.*
Three-minute extensions are used in this initial PCR to increase the efficiency of capture of longer cDNA products.
Limited cycling time is used to minimize the possibility of generating PCR artifacts.
Continue to process the material amplified from COS cells transfected with vector DNA alone (Chapter 11, Protocol 10).

5. Add 30 units of *Bst*XI to the PCRs. Overlay the reaction mixtures with light mineral oil (if not already on the reaction) and incubate the mixtures overnight at 55°C.
6. Add an additional 20 units of *Bst*XI to the PCR and incubate the reaction for an additional 2-3 hours at 55°C.
7. Meanwhile, digest 1 µg of pBSII(KS or SK) to completion with *Eco*RV. Purify the DNA by extraction with phenol and precipitation with ethanol. Dissolve the digested DNA in H_2O at a concentration of 2 ng/µl.
8. In a 0.5-ml amplification tube, mix the following:

*Bst*XI-digested RT-PCR (Step 6)	5-10 µl
10x amplification buffer	4.5 µl
dNTP solution at 1.25 mM	7.5 µl
SADU oligonucleotide primer (20 µM)	2.5 µl
SDDU oligonucleotide primer (20 µM)	2.5 µl
Taq DNA polymerase	10-20 units
H_2O	to 45 µl

9. In another 0.5-ml amplification tube, mix the following:

pBSII(KS or SK) digested with *Eco*RV (2 ng/ml)	10 µl
10x amplification buffer	10 µl
dNTP solution at 1.25 mM	16 µl
BSD-U oligonucleotide primer (20 µM)	5 µl
BSA-U oligonucleotide primer (20 µM)	5 µl
Taq DNA polymerase	2-4 units
H_2O	to 100 µl

10. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures (Steps 8 and 9) with 1 drop (approx. 50 µl) of light mineral oil. Alternatively, place a bead of paraffin wax into the tubes if using a hot start PCR. Place the tubes in the thermal cycler.
11. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
1	5 min at 94°C		
25-30	30 sec at 94°C	30 sec at 62°C	3 min at 72°C
Last cycle			5 min at 72°C

Times and temperatures may need to be adapted to suit the particular reaction conditions.
The 5' ends of the primers used in the second round of PCR amplification contain dUTP rather than dTTP. This facilitates ligation-independent cloning. The 5' ends of BSD-U and BSA-U are complementary to the 5' ends of SADU and SDDU.
It is best to perform the uracil DNA glycosylase reaction (Step 13) the same day as the amplification. If this is not possible, purify the amplification reactions by extraction with phenol:chloroform, followed by ethanol precipitation, and store them at -20°C until ready for use. The dU-amplified vector (Step 9) can be stored for several weeks at -20°C.
12. Run an aliquot of the PCR on a 1.5% agarose gel in TBE to assess the quality of the reaction. There should be a smear representing several amplification products.
13. In a 0.5-ml microfuge tube, mix the following:

*Bst*XI-digested cDNA (Step 8) amplified in Step 11	3 µl
*Eco*RV-digested pBSII DNA (Step 9) amplified in Step 11	1 µl
10x amplification buffer	1 µl
uracil DNA glycosylase (UDG; 1 unit/µl)	1 µl
H_2O	to 10 µl

Incubate the reaction mixture for at least 30 minutes at 37°C.
IMPORTANT *During this reaction, DNA containing dU residues is digested by uracil DNA glycosylase. In addition, the complementary termini of the plasmid and the amplifed cDNA anneal to form recombinant molecules. To prevent nonspecific annealing of the vector and PCR product, do not place the reaction on ice after the 37°C incubation. If Steps 14 and 15 cannot be carried out immediately, leave the reaction at 37°C or store it at -20°C.*
14. Use the entire UDG reaction mixture to transform 30-50 µl of DH5α *E. coli* cells using a $CaCl_2$ transformation procedure (please see Chapter 1, Protocol 25).
15. Plate 100-500 µl of the transformation reaction onto LB agar plates containing 50 µg/ml ampicillin, and incubate them overnight at 37°C.

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1. Church D.M. and Buckler A.J. 1999. Gene identification by exon amplification. *Methods Enzymol.* 303:83-99.

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Protocol 13

Exon Trapping and Amplification
Stage 5: Analysis of Clones

The final stage of this protocol involves amplification and sequencing of the exon-trapped products. This protocol was provided by Deanna M. Church (Church and Buckler 1999).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer

Enzymes and Buffers

Taq DNA polymerase

Nucleic Acids and Oligonucleotides

- dNTP solution containing all four dNTPs, each at 1.25 mM

Exon-trapped products cloned into a pBSII vector
Clones are in the form of recombinant DH5α colonies prepared in Chapter 11, Protocol 12.

Oligonucleotide primers (20 mM in TE [pH 8.0])

T3	*5′ AAT TAA CCC TCA CTA AAG GG 3′*
T7	*5′ GTA ATA CGA CTC ACT ATA GGG C 3′*
-20	*5′ GTA AAA CGA CGG CCA GT 3′*
REV	*5′ AGC GGA TAA CAA TTT CAC ACA GG 3′*

Media

- LB broth containing 50 µg/ml ampicillin
- LB broth containing 30% (v/v) glycerol

Additional Reagents

Step 8 of this protocol requires the reagents listed in Chapter 12, Protocol 3 to Chapter 12, Protocol 5 .

METHOD

1. Dispense 100 µl of LB broth containing 50 µg/ml ampicillin into each well of a 96-well microtiter plate. Transfer one transformed bacterial colony (from Step 15 of Chapter 11, Protocol 12) at a time into individual wells. Cover the plate with Parafilm, and grow the colonies for 3-4 hours at 37°C. It is not necessary to agitate the plate.
2. Prepare a master mix (sufficient for 100 wells) of PCRs by combining the following components together:

10x amplification buffer	250 µl
dNTP solution at 1.25 mM	400 µl
-20 oligonucleotide primer (20 µM)	125 µl
REV oligonucleotide primer (20 µM)	125 µl
H_2O	1475 µl
Taq DNA polymerase	75 units

   Aliquot 24 µl of the master mix into each well of fresh 96-well PCR plates.
3. Use a 96-prong replicating device to transfer bacterial cultures from the plate in Step 1 to the plate containing the PCR master mix.
4. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Alternatively, place a bead of paraffin wax into the wells if using a hot start PCR. Place the 96-well plates in the thermal cycler.
5. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
1	5 min at 94°C		
25-30	30 sec at 94°C	30 sec at 62°C	30 sec at 72°C
Last cycle	30 sec at 94°C	30 sec at 62°C	5 min at 72°C

   Times and temperatures may need to be adapted to suit the particular reaction conditions.
6. Use a multichannel pipettor to make a replica of the 96-well plate (from Step 1) containing the bacterial colonies and allow them to grow overnight at 37°C. The next day, add to each well 100 µl of LB broth containing 30% glycerol. Seal the plate with Parafilm, and store it at -80°C.
7. Analyze the amplification products from Step 5 by gel electrophoresis on a 1.5% agarose gel.
8. Determine the sequence of each of the ETPs (please see Chapter 12, Protocol 3 to Chapter 12, Protocol 5).

   If pBSIISK$^-$ was used for cloning, sequencing with T7 will produce sequence from the 5′ end of the ETP. The ETP will be flanked by the following pSPL3-derived sequence:
   5′ GTCGACCCAGCA ETP sequence ACCTGGAGATCC 3′

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1. Church D.M. and Buckler A.J. 1999. Gene identification by exon amplification. *Methods Enzymol.* 303:83-99.

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Protocol 14

Direct Selection of cDNAs with Large Genomic DNA Clones

The goal of this method is to identify transcriptionally active genes in cloned segments of genomic DNA. The protocol uses hybridization and affinity purification to recover biotin-labeled cDNAs that bind to a 500-kb segment of human DNA cloned in a BAC vector. However, the method can be easily adapted to other clones of genomic DNAs cloned in high-capacity vectors. This protocol was provided by Michael Lovett (Morgan et al. 1992; Simmons and Lovett 1999).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
  Include 0.01% (w/v) gelatin in the buffer.
- ⚠ ATP (10 mM)
- 2x Hybridization solution
- ⚠ NaOH (0.1 M)
- 10x Nick translation buffer
- ⚠ SDS (10%)
- 20x SSC
- Streptavidin bead-binding buffer
- Tris-HCl (1 M, pH 7.5)

Enzymes and Buffers

DNA polymerase/DNase I (5 units/µl; Boehringer Mannheim)

Restriction endonucleases

T4 DNA ligase

Thermostable DNA polymerase

Nucleic Acids and Oligonucleotides

BAC DNA encompassing the target genomic region
Purify as described in Chapter 4, Protocol 8 or Chapter 4, Protocol 9.

Blunt-ended, random-primed cDNAs
Please see Chapter 11, Protocol 1.

C_0t1 genomic DNA
Purchased from Life Technologies.

Cytoplasmic poly(A)$^+$ RNA, purified from tissue or cell line of interest
Please see Chapter 11, Protocol 1.

DNA size markers

- dNTP solution for nick translation containing all four dNTPs, each at 0.4 mM (pH 8.0)
- dNTP solution for PCR containing all four dNTPs, each at 2.5 mM (pH 8.0)

Oligonucleotides (10 mM in TE [pH 8.0]):

Oligo3 *5'-CTCGAGAATTCTGGATCCTC-3'*

Oligo4 *5'-GAGGATCCAGAATTCTCGAGTT-3'*

Positive-control DNA
The positive-control DNA should be a part of a gene or expressed sequence tag (EST) known to be present in the starting segment of DNA. If no genes are known, then a single-copy control DNA can be seeded into the genomic target before labeling (at a 1:1 molar ratio) and diluted into the cDNA (at a 1:10^6 molar ratio). Label the DNA sample using one of the protocols given in Chapter 9; the choice of labeling protocol is determined by the experimental goal.

Labeled Compounds

⚠ [α-^{32}P]dCTP (3000Ci/mmole)

Biotin-16-dUTP (0.4 mmole)

Additional Reagents

Step 1 of this protocol requires reagents listed in Chapter 10, Protocol 2.

Step 2 of this protocol requires reagents for the synthesis of random primed double-stranded cDNAs or PCR-amplified inserts from a cDNA library listed in Chapter 11, Protocol 1 and Chapter 11, Protocol 1.

Step 2 of this protocol requires reagents for labeling listed in Chapter 9, Protocol 3, Chapter 9, Protocol 5, and Chapter 9, Protocol 6.

Steps 19 and 20 require reagents listed in Chapter 6, Protocol 8 and Chapter 6, Protocol 10.

METHOD

1. Label oligonucleotides 3 and 4 separately by phosphorylation at their 5' termini using polynucleotide kinase, as described in Chapter 10, Protocol 2. Mix the labeled complementary oligos in equal molar ratios (approx. 2 µg of each), denature them for 10 minutes at 100°C, and slowly cool them to room temperature. During this process, the oligonucleotides anneal to form an adaptor. Adjust the concentration of the adaptor to 1 µg/ml.
2. Prepare at least 2 µg of double-stranded cDNA from cytoplasmic polyadenylated RNA by random priming (please see Chapter 11, Protocol 1).
3. Prepare the following ligation reaction in a sterile 0.5-ml microfuge tube:

double-stranded blunt-ended cDNA (Step 2)	2 µg
oligonucleotide amplification cassette mixture at 1 µg/µl, from Step 1	3 µl
10x T4 DNA ligase buffer	3 µl
10 mM ATP	3 µl
T4 DNA ligase at 1 unit/µl	3 µl
H_2O	to 30 µl

Incubate the ligation for 16 hours at 14°C. Inactivate the T4 DNA ligase by a 10-minute incubation at 65°C.

4. Purify the products of the ligation reaction by phenol:chloroform extraction, by spun-column chromatography through Sephadex G-50, and by standard ethanol precipitation. Dry the pellet and resuspend it in 10 µl TE (pH 7.6).
5. Incorporate biotinylated residues into the BAC genomic DNA clone using nick translation. Prepare the following nick translation reaction in a sterile 0.5-ml microfuge tube (label each BAC DNA separately):

purified BAC DNA (0.1 mg/ml)	1 µl
biotin-16-dUTP (0.04 mM)	1 µl
10x nick translation buffer	2 µl
dNTP mix for nick translation (0.4 mM)	1 µl
[α-^{32}P]dCTP (3000 Ci/mmole)	1 µl
DNA polymerase/DNase I (5 units/µl)	1 µl
H_2O	to 20 µl

Incubate the reaction for 2 hours at 4°C.

6. Purify the radiolabeled and biotinylated products of the nick translation reaction by spun-column chromatography through Sephadex G-50 and standard ethanol precipitation. Resuspend the pellet in 10 µl of TE and store it at -20°C.
7. In a 1.5-ml sterile microfuge tube, wash 3 mg (300 µl) of beads with 500 µl of streptavidin bead-binding buffer three times. Following each wash, remove the beads from the binding buffer using a magnetic separator. Resuspend the beads at a concentration of 10 mg/ml in streptavidin bead-binding buffer.
8. Test an aliquot of each labeling reaction (from Step 6) for the ability to bind to streptavidin beads. Prepare the following binding reaction in a sterile 0.5-ml microfuge tube:

washed streptavidin-coated beads (Step 7)	20 µl
labeled genomic DNA contig* (Step 6)	1 µl
streptavidin bead-binding buffer	29 µl

**Mix equimolar amounts of each separately labeled BAC in the contig to yield a mixture at a concentration of 10 ng/µl.*
Incubate the reaction for 15 minutes at room temperature with occasional gentle mixing. Remove the beads from the binding buffer using a magnetic separator, and transfer the supernatant to a fresh sterile 0.5-ml microfuge tube. Use a Geiger counter or Cerenkov counter to measure the radioactivity present on the beads and in the supernatant. If the ratio of bound to free cpm is >8:1, then proceed with the selection.

If the ratio of bound to free cpm is <8:1, it is likely that the DNA was resistant to proper labeling in Step 5. Before labeling, try purifying the BAC DNA further by several rounds of extraction with phenol:chloroform and passing it through a Sephadex G-50 spun column.

9. Block or "repeat suppress" repetitive sequences in the pool of cDNA (from Step 4) using C_0t1 DNA as follows:
   a. Prepare the following annealing reaction in a sterile 0.5-ml microfuge tube:

cDNA carrying linkers (Step 4)	5 µl (1 µg)
human genomic C_0t1 DNA	5 µl (1 µg)

   b. Overlay the reaction mixture with light mineral oil (approx. 50 µl) to prevent evaporation, and denature the DNA by heating for 10 minutes at 100°C. Cool the reaction mixture to 65°C, deliver 10 µl of 2x hybridization solution under the oil. Mix the components gently. Incubate the reaction mixture for 4 hours at 65°C.
10. After hybridization of the cDNA pools to the C_0t1 DNA is complete, set up the primary direct selection. Deliver 5 µl (50 ng) of biotinylated BAC DNAs from Step 6 into a fresh microfuge tube, and overlay the solution with light mineral oil (approx. 50 µl) to prevent evaporation. Denature the BAC DNA by heating to 100°C for 10 minutes. Cool the reaction to 65°C.
11. Prepare the following annealing reaction in a sterile 0.5-ml microfuge tube:

biotinylated BAC DNA from Step 10	5 µl (50 ng)
blocked cDNA from Step 9 (1 µg cDNA plus 1 µg C_0t1 DNA)	20 µl
2x hybridization solution	5 µl

Mix the reagents gently, and incubate the reaction for >54 hours at 65°C in a rotating hybridization oven or shaking water bath.

12. To capture and wash the genomic DNA and hybridized cDNAs, prepare the following in a sterile 1.5-ml microfuge tube:

washed streptavidin-coated beads	100 µl
annealing reaction from Step 11	30 µl
streptavidin bead-binding buffer	100 µl

Incubate the mixture for 15 minutes at room temperature with occasional gentle mixing. Remove the beads from the binding buffer using a magnetic separator, and then remove and discard the supernatant. Wash the beads twice, for 15 minutes each time, in 1 ml of 1x SSC/0.1% SDS at room temperature followed by three washes, 15 minutes each, in 1 ml of 0.1x SSC/0.1% SDS at 65°C. After the final wash, transfer the beads to a fresh microfuge tube.

13. To elute the hybridizing cDNAs from the beads in Step 12:
   a. Add 100 µl of 0.1 M NaOH, and incubate the reaction mixture for 10 minutes at room temperature.
   b. Add 100 µl of 1 M Tris-Cl (pH 7.5).
   c. Desalt the mixture by spun-column chromatography through Sephadex G-50 (please see Appendix 8 in the print version of the manual).
14. Transfer three aliquots (1 µl, 5 µl, and 10 µl, respectively) from the 200 µl of eluted cDNA (Step 13) to 0.5-ml sterile amplification tubes.
15. To each tube, add the following:

primer oligo3 (10 mM)	5 µl
10x amplification buffer	2.5 µl
dNTP mixture for PCR (2.5 mM)	2.5 µl
Taq DNA polymerase (Perkin Elmer 5 units/µl)	0.2 µl
H_2O	to 25 µl

Set up two control reactions at the same time as the above test reactions. For the negative control, include all of the components listed above, but omit the template cDNA. For the second control, include 10 ng of the starting cDNA (Step 4).

16. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil to prevent evaporation. Alternatively, place a bead of wax into the tube if using a hot start PCR. Place the tubes in the thermocycler.
17. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed below.

Cycle Number	Denaturation	Annealing	Polymerization
30 cycles	30 sec at 94°C	30 sec at 55°C	1 min at 72°C

Times and temperatures may need to be adapted to suit the particular reaction conditions.

18. Analyze 10% of each amplification reaction on a 1% agarose gel cast and run in 0.5x TBE, including DNA markers of an appropriate size. Stain the gel with ethidium bromide or SYBR Gold to visualize the DNAs, estimate the concentration of the amplified cDNA, and determine which input cDNA concentration produces the highest yield of cDNA.
19. Onto a second 1% agarose gel, load the same amounts (approx. 0.5 µg per lane) of the amplifiied products of each PCR, as well as the appropriate size markers. Also load 0.5 µg of randomly primed cDNA from Step 4.
20. Transfer the separated DNA species to a membrane by Southern blotting (Chapter 6, Protocol 8) and hybridize with the radiolabeled, positive control cDNA.
21. Once the positive control enrichment is confirmed, scale up the optimal amplification reaction to yield at least 1.5 µg of selected cDNAs. Extract the pooled reactions with phenol:chloroform and recover the DNA by standard ethanol precipitation. Resuspend the dried cDNA pellet in 7.5 µl of TE (200 µg/ml).
    Proceed to carry out the secondary selection under the same conditions as the primary selection, using 1 µg of the primary selected cDNA and 50 ng of the target DNA (total length 500 kb in this example).
22. Block repetitive sequences in the primary selected cDNA as described in Step 9 (with 1 µg of cDNA being used and 0.5 µg of cDNA being held in reserve for later analysis).
23. Set up the secondary selection as described in Steps 9 through 13.

24. Analyze the products of the second amplification by electrophoresis through a 1% agarose gel as in Step 18, including a lane with 0.5 µg of primary cDNA (held in reserve) and a lane with 0.5 µg of the starting cDNA.
25. Analyze the gel by Southern blotting and hybridization with the radiolabeled reporter probe as described in Step 20.
26. Once enrichment of the control is confirmed, clone the selected cDNAs into the appropriate vector(s). The restriction enzyme sites in the amplification cassette facilitate the cloning of the secondary selected cDNAs into bacteriophage or plasmid vectors.

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Chapter 12 DNA Sequencing

Protocol 1: Generation of a Library of Randomly Overlapping DNA Inserts

Shotgun sequencing of a large segment of DNA involves random fragmentation of the target region into smaller segments that are subsequently cloned into a bacteriophage M13 vector. The goal is to create a library of overlapping clones that provide at least fivefold coverage over the entire length of the target fragment.
In this protocol, the target segment is sheared by sonication or nebulization. Various sonication methods are described in Table 12-1 on page 12.11 of the print version of the manual. The termini of the resulting DNA fragments are repaired, phosphorylated, and fractionated according to size by gel electrophoresis. Molecules 0.8 to 1.5 kb in size are then recovered and cloned into the linearized, dephosphorylated DNA of a bacteriophage M13 vector.

Protocol 2: Preparing Denatured Templates for Sequencing by Dideoxy-mediated Chain Termination

In this protocol, double-stranded plasmid DNA is denatured by alkali and annealed to an appropriate oligonucleotide primer, in preparation for dideoxy-sequencing reactions catalyzed by Sequenase (described in Chapter 12, Protocol 3).

Protocol 3: Dideoxy-mediated Sequencing Reactions Using Bacteriophage T7 DNA Polymerase (Sequenase)

This protocol describes DNA-sequencing of single-stranded DNA templates in reactions catalyzed by Sequenase (version 2.0). All the materials required for sequencing with Sequenase version 2.0 are contained in kits, sold by USB/Amersham Life Science, which include a detailed protocol describing the reagents and steps used in the sequencing reaction. This kit is invaluable for first-time or occasional sequencers, but it is expensive. After gaining experience, it may turn out to be more economical to purchase Sequenase version 2.0 in bulk and then assemble the remaining reagents locally.

Protocol 4: Dideoxy-mediated Sequencing Reactions Using the Klenow Fragment of *E. coli* DNA Polymerase I and Single-stranded DNA Templates

The following protocol has its roots in methods devised in the Sanger laboratory in the 1970s, in the early days of dideoxy-DNA sequencing.

Protocol 5: Dideoxy-mediated Sequencing of DNA Using Taq DNA Polymerase

Because sequencing reactions catalyzed by thermostable DNA polymerases such as *Taq* are carried out at elevated temperatures, problems caused by mismatched annealing of primers or templates rich in secondary structure are greatly alleviated.

Protocol 6: Cycle Sequencing: Dideoxy-mediated Sequencing Reactions Using PCR and End-labeled Primers

In this protocol, asymmetric PCR is used to generate single-stranded DNA templates for dideoxy-sequencing.

Protocol 7: Chemical Sequencing

This protocol is based on the classic base-specific cleavage method of DNA sequencing devised in 1977 by Maxam and Gilbert.

Protocol 8: Preparation of Denaturing Polyacrylamide Gels

In 1978, Fred Sanger and Alan Coulson devised a method to pour and run thin polyacrylamide gels, which are now used ubiquitously to resolve the products of DNA sequencing reactions.

Protocol 9: Preparation of Denaturing Polyacrylamide Gels Containing Formamide

The inclusion of formamide in sequencing gels eliminates secondary structure in the DNA during electrophoresis. Formamide gels are particularly useful and almost a necessity when sequencing DNA templates with a G/C content >55%. This protocol requires all of the reagents listed in Chapter 12, Protocol 8.

Protocol 10: Preparation of Electrolyte Gradient Gels

Electrolyte gradients are formed when buffers of different concentrations are used in the upper (low electrolyte concentration) and lower (high electrolyte concentration) chambers of the electrophoresis device. Fragments of DNA migrate more slowly as they travel anodically into regions of progressively higher ionic strength. The spacing between bands of DNA is therefore reduced at the bottom of the gel and increased at the top. In consequence, the number of bases that can be read is increased by approx. 30%. This protocol requires all of the reagents listed in Chapter 12, Protocol 8.

Protocol 11: Loading and Running DNA-sequencing Gels

The sets of nested DNA fragments generated by DNA sequencing methods are resolved by electrophoresis through thin denaturing polyacrylamide gels. **WARNING** Large voltages are passed through DNA-sequencing gels at substantial amperages. More than enough current is used in these gels to cause severe burns, ventricular fibrillation, central respiratory arrest, and asphyxia due to paralysis of the respiratory muscles. Make sure that the gel boxes used for electrophoresis are well insulated, that all buffer chambers are covered, and that the box is used on a stable bench top that is dry. Always turn off the power to the box before sample loading or dismantling the gel.

Protocol 12: Autoradiography and Reading of Sequencing Gels

For information on autoradiography, please see Appendix 9 of the print version of the manual (Volume 3, pages A9.9-9.15). For information on reading of sequencing gels, please see the information panel on Reading an Autoradiograph in the print version on page 12.113.

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Protocol 1

Generation of a Library of Randomly Overlapping DNA Inserts

Shotgun sequencing of a large segment of DNA involves random fragmentation of the target region into smaller segments that are subsequently cloned into a bacteriophage M13 vector. The goal is to create a library of overlapping clones that provide at least fivefold coverage over the entire length of the target fragment.
In this protocol, the target segment is sheared by sonication or nebulization. Various sonication methods are described in Table 12-1 on page 12.11 of the print version of the manual. The termini of the resulting DNA fragments are repaired, phosphorylated, and fractionated according to size by gel electrophoresis. Molecules 0.8 to 1.5 kb in size are then recovered and cloned into the linearized, dephosphorylated DNA of a bacteriophage M13 vector.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (10 M)
⚠ ATP
Optional, please see Step 1.
Deionized distilled H_2O
Ethanol
Glycerol (sterile, 100%)
IPTG
$MgCl_2$
NaCl
⚠ Phenol (saturated with Tris at pH 7.6)
⚠ Phenol:chloroform (1:1, v/v)
⚠ PEG 8000 (30% w/v)
Optional, please see note to Step 1.
⚠ PEG 8000 (20% w/v) in 2.5 M NaCl
TE (pH 7.6)
10x Tris-Mg buffer
TTE
⚠ X-gal

Enzymes and Buffers

Bacteriophage T4 DNA ligase
10x Bacteriophage T4 DNA ligase buffer
Bacteriophage T4 DNA polymerase
Bacteriophage T4 polynucleotide kinase
10x Bacteriophage T4 polynucleotide kinase buffer
Klenow fragment of *E. coli* DNA polymerase I

Nucleic Acids and Oligonucleotides

Bacteriophage λ or ΦX174 DNA, cleaved to completion with *Alu*I (1 µg in a volume of 20 µl of TE (pH 7.6)
After digestion, remove the restriction enzyme from the DNA by extraction with phenol:chloroform. Recover the DNA by precipitation with ethanol and dissolve in TE (pH 7.6). The digested DNA will be used to check the efficiency of ligation of the dephosphorylated, linearized vector as described in Step 13. The digested DNA is not required if commercial preparations of vector are used.
DNA size markers
Appropriate size markers are, for example, fragments generated by digestion of pUC18 or pUC19 DNA with Sau*3AI, or the 123-bp ladder sold by Life Technologies, or ΦX174 DNA digested with* Hae*III (New England Biolabs).*
dNTP solution containing all four dNTPs, each at 2.0 mM
Target DNA
Target DNA is usually prepared by digesting a recombinant constructed in a high-capacity vector (e.g., cosmid, BAC, PAC, or P1) with a restriction enzyme that does not cleave within the cloned sequences. Wherever possible, use a restriction enzyme that generates cohesive termini. This simplifies ligation of the purified target DNA into concatemers (Step 1). After release from the vector, purify the target DNA by electrophoresis through a gel cast with low-melting-temperature agarose (please see Chapter 5, Protocol 6 or Chapter 5, Protocol 7). Dissolve the purified DNA in TE (pH 7.6) at a concentration of 1 mg/ml. Check the integrity and recovery of the purified DNA by analyzing an aliquot (50 ng) by agarose gel electrophoresis.

Media

Rich M13 medium containing 5 mM $MgCl_2$
Top agarose
YT agar plates

Vectors and Bacterial Strains

Bacteriophage M13 vector DNA, equipped for blue-white screening, linearized, blunt-ended, and dephosphorylated.
Vector DNAs of this type may be purchased ready-made from several commercial vendors.
E. coli competent cells of an appropriate strain (e.g., XL1F´-Blue, DH5αF´)
To obtain a library that covers the target DNA to the greatest depth, it is important that the efficiency of transfection of E. coli *be as high as possible (>10^9 transfectants/µg of closed circular vector DNA). Preparations of competent* E. coli *that can be transformed with high efficiency may be purchased ready-made from several commercial vendors or may be prepared as described in Chapter 1, Protocols 23, Chapter 1, Protocol 24, Chapter 1, Protocol 25, and Chapter 1, Protocol 26. If an electroporation apparatus is available, we recommend using preparations of electrocompetent* E. coli, *because of their higher efficiencies of transformation.*

METHOD

1. Self-ligation is required to ensure that sequences at the ends of the target DNA are adequately represented in the

population of fragments used to construct the library.

Transfer 5-10 µg of the purified target DNA (see Materials) to a fresh microfuge tube and add:

10x bacteriophage T4 DNA ligase buffer	2.5 µl
5 mM ATP	2.5 µl
30% w/v PEG 8000 (*optional*)	5.0 µl
bacteriophage T4 DNA ligase	0.5-2.0 Weiss units
H_2O	to 25 µl

Incubate the mixture for 4 hours at 16°C, and then inactivate the ligase by heating the mixture for 15 minutes at 68°C.
If the target DNA is blunt-ended, include PEG in the reaction to increase the efficiency of ligation.
Use 0.5 Weiss unit for cohesive termini, and 2.0 Weiss units for blunt termini.
Omit ATP if included in the ligase buffer.

2. Add 175 µl of TE (pH 7.6), and purify the ligated DNA by extraction with phenol:chloroform. Recover the ligated DNA by precipitation with 3 volumes of ethanol in the presence of 2.0 M ammonium acetate. After centrifugation at maximum speed for 5 minutes in a microfuge, wash the pellet with 0.5 ml of 70% ethanol at room temperature and centrifuge again.
3. Remove as much of the supernatant as possible and allow the last traces of ethanol to evaporate at room temperature. Dissolve the DNA in 25 µl of TE (pH 7.6) in a microfuge tube.
4. Fragment the target DNA into segments 0.8-1.5 kb in length by sonication or nebulization.

To fragment the DNA by sonication

a. Place ice water in the cup horn of the sonicator. Set the sonicator power switch to on, the timer to hold, and the power setting to 10. Apply two 40-second pulses, and allow the sonicator to warm up.
b. Place the tube containing the DNA in the ice water such that the bottom of the tube is 1-2 mm above the hole in the center of the cup horn probe (please see Figure 12-3 on page 12.16 in the print version of the manual), and sonicate the DNA.
c. Centrifuge the tube briefly to collect the sonicated DNA sample at the bottom of the tube and place it on ice.
d. Analyze 1 µl of the sonicated DNA sample with the appropriate molecular-weight markers by electrophoresis through a 0.7% agarose gel. Keep the remainder of the DNA on ice while the gel is running.

To fragment the DNA by nebulization

a. Prepare the following DNA solution and place it in the nebulizer cup.

DNA sample (from Step 3)	5-10 µg
10x Tris-Mg buffer (pH 8.0)	200 µl
sterile 100% glycerol	1 ml
sterile H_2O	to a final volume of 2 ml

b. Place the DNA sample in an ice-water bath and nebulize using the empirically determined optimal conditions (please see Figure 12-4 on page 12.17 in the print version of the manual).
c. Place the nebulizer in a suitable centrifuge rotor and cushion it with pieces of styrofoam. Centrifuge at 2000*g* (1000 rpm in a centrifuge equipped with a microplate adaptor) briefly at 4°C to collect the DNA sample at the bottom of the nebulizer cup.
d. Divide the DNA sample into four equal aliquots in 1.5-ml microfuge tubes, carry out standard ethanol precipitation of the DNA, and dry the DNA pellets under vacuum.
e. Dissolve each DNA pellet in 35 µl of TE (pH 7.6), and analyze 1 µl of the sheared DNA with appropriate molecular-weight markers by electrophoresis through a 0.7% agarose gel. Keep the remainder of the DNA at 4°C while the gel is running.

5. To the fragmented DNA (approx. 25 µl) add:

10x bacteriophage T4 DNA polymerase buffer	4.0 µl
2.0 mM solution of four dNTPs	4.0 µl
bacteriophage T4 DNA polymerase	5 units
H_2O	to 40 µl

Incubate the reaction for 15 minutes at room temperature, and then add approx. 5 units of the Klenow fragment. Continue incubation for a further 15 minutes at room temperature.
The termini produced by nebulization and sonication are highly heterogeneous, consisting of blunt-ended and frayed ends, with and without phosphate residues. Because only a fraction of these molecules can be repaired by DNA polymerases, the efficiency with which hydrostatically sheared DNA can be cloned in bacteriophage M13 vectors is generally low. However, 5-10 µg of sonicated, repaired, and size-selected target DNA generally yields several thousand recombinant clones.

6. Purify the DNA by extraction with phenol:chloroform. Transfer the aqueous (upper) phase to a fresh tube, and adjust the solution to 0.1 M NaCl. Recover the DNA by precipitation with 2 volumes of ethanol. Wash the pellet with 70% ethanol.
7. Redissolve the precipitated DNA in 25 µl of TE (pH 7.6).
8. Combine the following in a microfuge tube:

fragmented DNA	23 µl
10x polynucleotide kinase buffer	3 µl
20 mM ATP	3 µl
bacteriophage T4 polynucleotide kinase	1 unit

Bacteriophage T4 polynucleotide kinase catalyzes the phosphorylation of the 5´ termini of the blunt-ended DNA fragments. This step is not mandatory, but, in most cases, leads to more efficient ligation of the fragments to the vector.

9. Incubate the reaction for 30 minutes at 37°C.
10. Purify the fragments of DNA of the desired size (0.8-1.5 kb) by electrophoresis through a low-melting-temperature agarose gel (0.8%) or 5% neutral polyacrylamide gel (see Chapter 5).
11. Recover the target DNA from the gel by one of the methods described in Chapter 5. Dissolve the purified DNA in 25 µl of TE (pH 7.6).
12. Check the integrity and recovery of the purified DNA by analyzing an aliquot (1.0 µl) by electrophoresis through a 1% agarose gel.
13. Set up a series of test ligations containing 50 ng (approx. 0.01 pmole) of linearized and dephosphorylated vector DNA and increasing concentrations of fragmented, blunt-ended, phosphorylated target DNA (please see the table below).
14. Introduce aliquots of the test ligations into competent *E. coli* of the appropriate strain by electroporation or transformation (please see Chapter 1, Protocols 23-26). Plate the bacteria on media containing IPTG and X-gal. Incubate the plates overnight at 37°C.
15. The following day, count the number of blue and colorless plaques.
16. Set up a large-scale ligation reaction using the minimum amount of fragmented, blunt-ended target that will yield sufficient recombinant clones to complete the sequencing project and transform *E. coli* with the ligated DNA. Incubate the plates overnight at 37°C.
17. The following day, collect the plates and store the resulting transformants under the appropriate conditions until required. Prepare template DNAs from a series of individual colorless plaques as described in Chapter 3, Protocol 4.

Test Ligation Reactions of Dephosphorylated Vector DNA

TUBE	TYPE OF DNA[a] A	B	C	H_2O (µl)	10X LIGATION BUFFER (µl)	ATP (5 mM) (µl)[b]	T4 DNA LIGASE (WEISS UNITS)	30% PEG (OPTIONAL)[c] (µl)
1	+			5.0	1.0	1.0	2.0	1.6
2	+			6.0	1.0	1.0	-	1.6
3	+	+		3.0	1.0	1.0	2.0	1.6
4	+		+	3.0	1.0	1.0	2.0	1.6
5	+		+	3.0	1.0	1.0	2.0	1.6
6	+		+	3.0	1.0	1.0	2.0	1.6
7	+		+	3.0	1.0	1.0	2.0	1.6

A = 50 ng of linearized, dephosphorylated vector (in a volume of 2 µl); B = 20 ng of bacteriophage λ DNA or ΦX174 RF DNA cleaved to completion with AluI *(in a volume of 2 µl); C = fragmented, blunt-ended, phosphorylated target DNA. Tubes 4, 5, 6, and 7 should contain 10, 20, 50, and 100 ng of size-selected, fragmented target DNA, respectively (100 ng of size-selected fragments = approx. 0.1 pmole).*

[b]*Some commercial ligase buffers contain ATP. When using such buffers, omit the ATP.*

[c]*The efficiency of ligation can be increased by adding PEG 8000 (30% w/v) to a concentration of 5% in the final ligation mixture (see Chapter 1, Protocol 19). It is important to warm the stock solution of PEG 8000 to room temperature before adding it as the last component of the ligation reactions. DNA can precipitate at cold temperatures from solutions containing PEG 8000.*

18. For each set of 96 clones, inoculate 100 ml of LB medium or 2x YT medium in a 500-ml flask with a single colony of a suitable F´ strain of *E. coli* (e.g. XL1-Blue, XL1-Blue MRF´, or DH5αF´). Incubate the culture for 6-8 hours at 37°C with agitation at 300 rpm or until it enters the early log phase of growth.
19. Add $MgSO_4$ to the culture to a final concentration of 5 mM.
20. Use a multichannel pipettor to transfer 0.8-ml aliquots of the cells to individual tubes in a 96-tube box.
21. Wearing gloves, use sterile toothpicks to transfer individual well-separated, colorless bacteriophage M13 plaques into each tube of the 96-tube arrays. Stab the toothpick into the middle of a plaque and then drop the toothpick into the culture tube.
22. To avoid confusion, leave the toothpicks in the tubes until all 96 tubes in the box have been inoculated.
23. When the last plaque has been picked, remove the toothpicks, and seal the box. Label the box and place it in an orbital shaker set at 250-300 rpm and 37°C. Repeat Steps 20 and 21 as needed. Incubate the infected cultures for 8-12 hours.
24. Remove the boxes from the incubator and pellet the bacterial cells by centrifugation at 2400*g* (3000-3250 rpm in a centrifuge equipped with a microplate adaptor) for 20 minutes.
25. Use a multichannel pipettor to transfer 120 µl of 20% PEG 8000 in 2.5 M NaCl to individual tubes in a fresh 96-tube box.
26. Carefully remove the tubes from the centrifuge; use the multichannel pipettor to transfer 0.6 ml of each supernatant to a tube containing the PEG/NaCl solution.
    IMPORTANT *Do not disturb the pellet of bacterial cells during this step. Inclusion of bacteria will drastically reduce the quality of DNA sequence obtained.*
27. Place a 96-tube cap over the tubes containing the bacteriophage suspensions and PEG/NaCl solution. Make sure that a liquid-tight seal has formed, and then mix the solutions by inverting the box several times. Incubate the box for 30 minutes at room temperature, followed by a 30-60-minute incubation on ice.
28. Collect the precipitated bacteriophage by centrifugation of the boxes at 2400*g* (3000-3250 rpm in a centrifuge equipped with a microplate adaptor) for 30 minutes. Remove a row of tubes and drain the supernatant by inversion over a sink. A small white pellet should be visible on the bottom of each tube. Return the row of tubes to the box.
29. When all of the tubes have been emptied, invert the boxes on a paper towel and allow the last traces of supernatants to drain for a few minutes. Keeping the box in an upside-down position, replace the wet paper towel with a fresh dry towel. Transfer the inverted box-towel combinations to the centrifuge. Remove the last traces of PEG/NaCl solution from the bacteriophage M13 pellets by centrifugation at 300 rpm for 3-5 minutes.
30. Remove the boxes from the centrifuge, check that the pellets have remained in place, and add 20 µl of TTE to each tube.
31. Seal the tubes with 3M silver foil tape and shake the boxes vigorously on a multitube vortexer for 15-30 minutes.
32. Centrifuge the boxes briefly to bring the solution to the bottom of the tubes. Pry the base from each of the 96-tube boxes and place the tubes in an 80°C water bath for 10 minutes.
33. Remove the tubes from the water bath and allow them to cool to room temperature. Replace the bottoms of the boxes and briefly centrifuge each unit to bring the solutions to the bottom of the tubes.
34. Use a multichannel pipettor to transfer 70 µl of sterile H_2O to the individual wells of 96-well microtiter plates. Transfer the bacteriophage lysates from the tubes in Step 33 to the microtiter plate wells. Mix the two solutions by pipetting up and down. Seal the plates with a strip of 3M silver foil tape or with the plate lid if sequencing is to be carried out within the next 24-48 hours. Label each plate and store it at -20°C.
    The yield of single-stranded M13 DNA should be 2.5-5 µg per starting culture.
35. Examine aliquots (5 µl) of DNA selected at random from a few wells by electrophoresis through a 1% agarose gel.
    Use between 2 µl and 7.5 µl of each DNA preparation in cycle sequencing reactions (see Chapter 12, Protocol 6).

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Protocol 2

Preparing Denatured Templates for Sequencing by Dideoxy-mediated Chain Termination

In this protocol, double-stranded plasmid DNA is denatured by alkali and annealed to an appropriate oligonucleotide primer, in preparation for dideoxy-sequencing reactions catalyzed by Sequenase (described in Chapter 12, Protocol 3).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium acetate (5 M, unbuffered, pH approx. 7.4)

⚠ Chloroform:isoamyl alcohol (24:1, v/v)

⚠ DMSO
Optional, please see Step 10.

Ethanol

⚠ NaOH (2 N)/EDTA (2 mM)
Prepare by appropriate dilution of a 10 N concentrated stock solution of NaOH just before use.

⚠ Phenol, equilibrated with H_2O or TE (pH 7.6)

Sequenase reaction buffer

Nucleic Acids and Oligonucleotides

Oligonucleotide primers
Oligonucleotide primers used to sequence denatured double-stranded DNA templates are frequently longer (20-30 nucleotides) than primers normally used for sequencing single-stranded templates. Longer primers give rise to fewer artifactual bands when used with denatured double-stranded DNA templates.

Plasmid DNA, closed circular, double-stranded
Prepare plasmid DNA from small- or large-scale cultures of bacteria (see Chapter 1); 5 µg of DNA is required for each set of four sequencing reactions (ddA, ddG, ddC, and ddT).

METHOD

1. Transfer approx. 5 µg of purified plasmid DNA to a 1.5-ml microfuge tube. Adjust the volume to 50 µl with H_2O. Add 10 µl of freshly prepared 2 N NaOH/2 mM EDTA solution. Incubate the mixture for 5 minutes at room temperature.
2. Add 30 µl of 5 M ammonium acetate and mix the contents of the tube by vortexing.
3. Add 45 µl of equilibrated phenol and mix the contents of the tube by vortexing.
4. Add 135 µl of chloroform:isoamyl alcohol, mix the contents of the tube by vortexing, and then separate the phases by centrifugation at maximum speed for 15 minutes at room temperature in a microfuge.
5. Carefully remove the upper aqueous phase to a fresh microfuge tube and precipitate the DNA by the addition of 330 µl of ice-cold ethanol. Mix the contents of the tube and place it in a dry ice-ethanol bath for 15 minutes.
6. Recover the denatured DNA by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge.
7. Very carefully decant the supernatant without disturbing the DNA pellet, which may or may not be visible on the side of the tube.
8. Recentrifuge the tube for 2 seconds and remove the last traces of ethanol with a drawn-out pipette tip without disturbing the DNA precipitate.
9. Allow the precipitate to dry at room temperature and resuspend the DNA in H_2O at a concentration of 1 µg/µl.
10. Anneal the primer DNA to the denatured template, using 5 µg (5 µl) of alkali-denatured plasmid DNA for each set of four DNA sequencing reactions (i.e., 1.25 µg denatured DNA for the reaction containing the ddA reaction, 1.25 µg for the reaction containing ddG, etc.). Set up annealing reactions as follows:

alkali-denatured DNA	5.0 µg (in 5 µl H_2O)
DMSO (*optional*)	2.0 µl
Sequenase reaction buffer	2.0 µl
sequencing primer	1.0 µl (10 ng)
H_2O	to 11.0 µl

Heat the mixture to 65°C for 2 minutes and then allow it to cool slowly to room temperature. Store the annealed samples on ice until the elongation/chain termination reactions are set up.

11. Proceed to Step 3 in Chapter 12, Protocol 3.

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Protocol 3

Dideoxy-mediated Sequencing Reactions Using Bacteriophage T7 DNA Polymerase (Sequenase)

This protocol describes DNA-sequencing of single-stranded DNA templates in reactions catalyzed by Sequenase (version 2.0). All the materials required for sequencing with Sequenase version 2.0 are contained in kits, sold by USB/Amersham Life Science, which include a detailed protocol describing the reagents and steps used in the sequencing reaction. This kit is invaluable for first-time or occasional sequencers, but it is expensive. After gaining experience, it may turn out to be more economical to purchase Sequenase version 2.0 in bulk and then assemble the remaining reagents locally.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Deionized distilled H_2O (ice cold)

- ddNTP solution (0.5 mM) of each of the four ddNTPs
- dNTP solution (0.5 mM) of each of the four dNTPs
- DTT (dithiothreitol) (100 mM)

5x Labeling mixture and ddNTP extension/termination mixtures (ddCTP, ddTTP, ddATP, and ddGTP)
These reaction mixtures can be assembled by mixing the volumes of the solutions shown in the table below.

5x Labeling Mixture and ddNTP Extension/Termination Mixtures for Sequenase Reactions

REACTION MIXTURE	STOCK SOLUTIONS OF dNTPs AND ddNTPs (ALL VOLUMES IN µl)								OTHER REAGENTS	
	dCTP 0.5 mM	dTTP 0.5 mM	dATP 0.5 mM	dGTP 0.5 mM	ddCTP 0.5 mM	ddTTP 0.5 mM	ddATP 0.5 mM	ddGTP 0.5 mM	5 M NaCl	H_2O
Labeling	15	15	-	15	-	-	-	-	-	955
ddCTP	160	160	160	160	16	-	-	-	10	334
ddTTP	160	160	160	160	-	16	-	-	10	334
ddATP	160	160	160	160	-	-	16	-	10	334
ddGTP	160	160	160	160	-	-	-	16	10	334

The 5x labeling mixture contains unlabeled dGTP, dCTP, and dTTP, each at a concentration of 7.5 mM. Radiolabeled dATP is added at Step 7 to a final concentration of approx. 30 µM. Each of the ddNTP extension/termination mixtures contains all four dNTPs at a concentration of 160 µM and a single ddNTP at a concentration of 0.16 µM. The 5x labeling mixture and extension/termination mixtures should be dispensed in 50-µl aliquots and stored frozen at -20°C.
When sequencing templates that are rich in GC structure, use base analogs such as 7-deaza-2'-dGTP or dITP in place of dGTP. If using 7-deaza-dGTP, simply replace the GTP in all the ddNTP extension/termination mixtures with an equimolar amount of the base analog.
If using dITP, substitute 30 µl of a 0.5 µM stock solution of dITP for dGTP in the 5x labeling mixture and decrease the amount of H_2O to 940 µl. In the ddNTP extension/termination mixtures containing ddCTP, ddTTP, and ddATP, substitute 160 µl of a 0.5 mM stock dITP solution for dGTP. In the extension/termination mixture containing ddGTP, substitute 320 µl of 0.5 mM stock solution of dITP for dGTP, decrease the amount of ddGTP to 3.2 µl, and decrease the volume of H_2O to 187 µl.

⚠
- Formamide loading buffer
- Sequenase dilution buffer
- 5x Sequenase reaction buffer with $MgCl_2$
- 5x Sequenase reaction buffer with $MnCl_2$
- TE (pH 7.6)

Enzymes and Buffers

Sequenase (version 2.0)
Sequenase is supplied by the manufacturer at a concentration of approx. 13 units/µl (approx. 1 mg/ml). Store at -20°C (not in a frost-free freezer).

Yeast inorganic pyrophosphatase
Optional, please see Step 6. This is supplied by the manufacturer (USB/Amersham Life Science) at a concentration of 5 units/ml; it catalyzes the hydrolysis of pyrophosphate to two molecules of orthophosphate. Pyrophosphatase can be mixed with Sequenase at a ratio of 1 unit of pyrophosphatase to 3 units of Sequenase. This is best done by mixing equal volumes of the two enzyme preparations and diluting the mixture with 6 volumes of enzyme dilution buffer. This dilutes Sequenase to its working concentration of approx. 1.6 units/µl and adds enough pyrophosphatase to prevent build-up of pyrophosphate; 2 µl of the mixture of diluted enzymes is required for each sequencing reaction.

Nucleic Acids and Oligonucleotides

Oligonucleotide primer
Use at a concentration of 0.5 pmole/µl (approx. 3.3 ng/µl) in H_2O or TE (pH 7.6).

Template DNAs (1 µg) in TE (pH 7.6)
Single-stranded DNA (1 µg) or 2.0 µg of denatured double-stranded plasmid DNA is required for each set of four sequencing reactions. Linear double-stranded DNA can be denatured and annealed to the primer by mixing the template with an excess of primer and heating the mixture in a boiling water bath for approx. 2 minutes and then plunging the tube into an ice-water bath. The mixture should not be allowed to warm up and should be used without delay.
The concentration of single-stranded DNA in small-scale preparations of bacteriophage M13 recombinants varies from 0.05 µg/ml to 0.5 µg/ml, depending on the growth rate of the particular bacteriophage. Under the conditions normally used for sequencing, the template DNA is present in excess. Small variations in the amounts of template added to different sets of sequencing reactions do not generally affect the quality of the results.

Radioactive Compounds

⚠ [α-^{35}S]dATP (1000 Ci/mmole, 10 mCi/ml)

or

⚠ [α-^{33}P]dATP (1000-3000 Ci/mmole, approx. 20 mCi/ml)

or

⚠ [α-^{32}P]dATP (1000 Ci/mmole, 10 mCi/ml)

Instead of internal labeling with [^{32}P]dATP, carry out sequencing with a 5´ ^{32}P-labeled oligonuclotide primer. In this case, 2 µl (approx. 5 x 10^5 cpm; approx. 0.5 ng) of the radiolabeled primer and 2 µl of H_2O are included in the reaction mixture in place of the unlabeled primer (Step 1) and [^{32}P]dATP (Step 7). All other steps remain the same. The 5´ termini of oligonucleotides are generally labeled by transfer of [γ-^{32}P] from [γ-^{32}P]ATP in a reaction catalyzed by polynucleotide kinase. For details of the labeling reaction, please see Chapter 10, Protocol 2.

METHOD

1. To a 0.5-ml microfuge tube or well of a microtiter plate, add:

single-stranded template DNA (approx. 1 µg/µl)	1 µl
oligonucleotide primer (approx. 1 ng/µl)	3 µl
5x Sequenase reaction buffer containing $MgCl_2$ or $MnCl_2$	2 µl
H_2O	to 10 µl

   IMPORTANT *When sequencing double-stranded plasmid DNAs that have already been denatured and annealed to a sequencing primer (Chapter 12, Protocol 2), ignore the first two steps of this protocol and begin at Step 3.*
2. Incubate the tightly closed tube for 2 minutes at 65°C. Remove the tube from the water bath and allow it to cool to room temperature over the course of 3-5 minutes.
3. While the primer and template are cooling, thaw the 5x labeling and ddNTP extension/termination mixtures and radiolabeled dATP. Store the thawed solutions on ice.
4. Transfer 2.5 µl of each ddNTP extension/termination mixture into separate 0.5-ml microfuge tubes or into individual wells of a microtiter plate color-coded or labeled C, T, A, and G.
5. Make a fivefold dilution of the 5x labeling mixture in ice-cold H_2O. A volume of 2 µl of the diluted labeling mixture is required for each template sequenced.
6. Dilute Sequenase in ice-cold Sequenase dilution buffer, with or without addition of yeast pyrophosphatase, as described above in Materials.
   A volume of 2.0 µl, containing approx. 3.0 units of Sequenase enzyme, is required for each template sequenced. Store the diluted enzyme on ice at all times.
7. To carry out the labeling reaction, add the following to the 10-µl annealing reaction of Step 2:

diluted labeling mixture (from Step 5)	2 µl
0.1 M dithiothreitol	1 µl
[α-^{33}P]dATP, [α-^{32}P]dATP, or [α-^{35}S]dATP	0.5 µl
diluted Sequenase (approx. 1.6 units/µl)	2.0 µl

   Mix the components of the reaction by gently tapping the sides of the tube or microtiter plate and then incubate the reaction for 2-5 minutes at 20°C.
   Store the diluted Sequenase in ice; do not allow it to warm to ambient room temperature. Store the concentrated stock of enzyme supplied by the manufacturer at -20°C. It may lose activity if stored in an ice bucket for hours.
8. Toward the end of the labeling reaction, prepare to set up the termination reactions by prewarming the labeled microfuge tubes or microtiter plates to 37°C. *This step is important!* Then transfer the 3.5 µl of the labeling reaction to the walls of each of the prewarmed labeled microfuge tubes or to the sides of prewarmed microtiter wells containing the appropriate dideoxy terminator mixtures (Step 4 above).
9. Place the microfuge tubes in a microfuge *at room temperature* (use appropriate rotor or adaptors for 0.5-ml tubes or place inside decapitated 1.5-ml tubes), or place the microtiter plates in a centrifuge *at room temperature* equipped with an appropriate adaptor. Centrifuge the C, T, A, and G tubes or plates for a few seconds at 2000 rpm to mix the components of the reactions. Immediately transfer the reactions to a heating block or a water bath for 3-5 minutes at 37°C.
10. Stop the reactions by adding 4 µl of formamide loading buffer.
11. The reactions may be stored for up to 5 days at -20°C or analyzed directly by denaturing gel electrophoresis (Chapter 12, Protocol 8 or Chapter 12, Protocol 9). After heat denaturation (2 minutes at 100°C) and quick cooling on ice, load 3 µl of each of the C, T, A, and G reactions into individual wells of a sequencing gel.

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Protocol 4

Dideoxy-mediated Sequencing Reactions Using the Klenow Fragment of *E. coli* DNA Polymerase I and Single-stranded DNA Templates

The following protocol has its roots in methods devised in the Sanger laboratory in the 1970s, in the early days of dideoxy-DNA sequencing.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Deionized distilled H_2O

- EDTA (10 mM, pH 8.0)

Extension/termination mixtures and chase mixture
These reaction mixtures can be assembled by mixing the volumes (in µl) of the stock solutions of dNTPs and ddNTPs, as shown in the table below.

ddNTP Extension/Termination Mixtures and Chase Mixtures for Klenow Sequencing

REACTION MIXTURE	STOCK SOLUTIONS OF dNTPs (1 mM) (ALL VOLUMES IN µl) dCTP	dTTP	dATP	dGTP	STOCK SOLUTIONS OF ddNTPs (5 mM) (ALL VOLUMES IN µl) ddCTP	ddTTP	ddATP	ddGTP	OTHER REAGENTS TRIS-Cl (1 M, pH 8.0)	EDTA (10 mM, pH 8.0)	H_2O
ddCTP	5	100	-	100	30	-	-	-	10	10	745
ddTTP	100	5	-	100	-	25	-	-	10	10	675
ddATP	100	100	-	100	-	-	5	-	10	10	580
ddGTP	100	100	-	5	-	-	-	25	10	10	750
Chase	245	245	245	245	-	-	-	-	10	10	-
dATP	-	-	100	-	-	-	-	-	10	10	880

The ratios of ddNTP:dNTP in the extension/termination mixtures are 30:1, 25:1, and 25:1 for dCTP, dTTP, and dGTP respectively. In the assembled sequencing reaction, the ratio of ddATP:dATP is approx. 45:1.
Dispense mixtures as 50-µl aliquots and store frozen at -20°C.

⚠ Formamide loading buffer

- Tris-Mg (12-4)
- Tris-Cl (1 M, pH 8.0)

Enzymes and Buffers

Klenow fragment of *E. coli* DNA polymerase I (5 units/µl)
Approximately 2.5 units of enzyme are required for each set of four dideoxy sequencing reactions.

Nucleic Acids and Oligonucleotides

- ddNTP solution (5 mM) of each of the four ddNTPs in H_2O
- dNTP solution (1 mM) of each of the four dNTPs in H_2O

Oligonucleotide primer
Use at a concentration of approx. 0.5 pmole/µl (approx. 3.3 µg/ml) in H_2O.

Template DNA, single-stranded
Use at a concentration of approx. 0.05 pmole/µl, which is equivalent to approx. 0.15 µg/µl of bacteriophage M13 single-stranded DNA.
The concentration of single-stranded DNA in small-scale preparations of bacteriophage M13 recombinants varies from 0.05 to 0.5 µg/ml, depending on the growth rate of the particular bacteriophage. Under the conditions normally used for sequencing, the template DNA is present in excess. Therefore, small variations in the amounts of template added to different sets of sequencing reactions do not generally affect the quality of the results.

Radioactive Compounds

⚠ [α-^{32}P]dATP (3000 Ci/mmole, 10 mCi/ml)

or

⚠ [α-^{33}P]dATP (3000 Ci/mmole, 10 mCi/ml)

or

⚠ [α-^{35}S]dATP (3000 Ci/mmole, 10 mCi/ml)

or

⚠ 5' ^{32}P-labeled oligonucleotide primer
Instead of internal labeling with [^{32}P]dATP, sequencing may be carried out with a 5´ ^{32}P-labeled oligonuclotide primer. In this case, 2 µl (approx. 5 x 10^5 cpm; approx. 0.5 ng) of the radiolabeled primer and 2 µl of H_2O are included in the reaction mixture in place of the unlabeled primer (Step 1) and [^{32}P]dATP (Step 4). All other steps remain the same. The 5´ termini of oligonucleotides are generally labeled by transfer of [γ-^{32}P] from [γ-^{32}P]ATP in a reaction catalyzed by polynucleotide kinase. For details of the kinasing reaction, please see Chapter 10, Protocol 2.

METHOD

1. Transfer the following to a 0.5-ml microfuge tube, or well of a microtiter plate:

single-stranded template DNA (0.1 µg/µl)	5 µl
oligonucleotide primer (1 µg/ml, approx. 160 pmoles/ml)	4 µl
Tris-Mg (12-4)	1 µl

2. Close the top of the tube or seal the microtiter plate and anneal the oligonucleotide primer to the template DNA by incubating the reaction mixture for 5-10 minutes at 55°C.
If necessary, the annealed template and primer may be stored for several months at -20°C.
3. Meanwhile, label four microfuge tubes or four contiguous wells of a 96-well disposable microtiter plate with the letters C, T, A, and G; then add 4 µl of the appropriate ddNTP extension/termination mixture to each tube (i.e., 4 µl of ddCTP mixture to tube/well labeled C, 4 µl of ddTTP mixture to tube/well labeled T, etc.).
4. Place the annealed primer-template solution from Step 2 on ice and add:

[α-^{32}P]dATP *or* [α-^{33}P]dATP *or* [α-^{35}S]dATP	1 µl
Klenow enzyme (approx. 2.5 units)	1 µl

0.1 mM dATP (if [α-^{32}P] or [α-^{33}P] is used)	1 µl
or	
H_2O (if [α-^{35}S]dATP is used)	1 µl

Store the stock of Klenow enzyme at -20°C; do not allow it to warm to ambient room temperature. The enzyme may lose activity if stored in an ice bucket for hours.

5. Transfer 3 µl of the mixture from Step 4 to the walls of each of the C, T, A, and G tubes or to the sides of microtiter wells. Do not allow the radiolabeled mixture to come into contact with the ddNTP extension/termination mixture.
6. Place the microfuge tubes in a microfuge (use appropriate rotor or adaptors for 0.5-ml tubes or place inside decapitated 1.5-ml tubes), or place the microtiter plates in a centrifuge equipped with an appropriate adaptor. Centrifuge the C, T, A, and G tubes or plates for a few seconds at 2000 rpm to mix the components and initiate the extension/termination reactions. Incubate the reactions for 10-12 minutes at 37°C.
Extension/termination and chase reactions catalyzed by the Klenow enzyme work equally well at temperatures ranging from room temperature to 37°C.
7. After 9-11 minutes of incubation, transfer 1 µl of the chase mixture to the side of each C, T, A, and G tube or well. Do not allow the solution to slide into the polymerization reactions. When a total of 10-12 minutes has elapsed, centrifuge the tubes/plates for 2 seconds to introduce the chase mix into the extension/termination reactions. Incubate the reactions for an additional 10-12 minutes at room temperature.
8. After 9-11 minutes of the second incubation, transfer 6 µl of formamide loading buffer to the sides of each C, T, A, G tube or well. Again, take care that the solution does not slide down into the reaction. At the end of the incubation period, centrifuge the tubes or plates to stop the sequencing reactions.
9. The DNA sequencing reactions may be stored for up to 5 days at -20°C or analyzed directly by denaturing gel electrophoresis (Chapter 12, Protocol 8 or Chapter 12, Protocol 9). After heat denaturation (2 minutes at 100°C) and quick cooling on ice, load 3 µl of each of the C, T, A, and G reactions into the individual wells of a sequencing gel.

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Protocol 5

Dideoxy-mediated Sequencing of DNA Using Taq DNA Polymerase

Because sequencing reactions catalyzed by thermostable DNA polymerases such as *Taq* are carried out at elevated temperatures, problems caused by mismatched annealing of primers or templates rich in secondary structure are greatly alleviated.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Deionized distilled H_2O (ice cold)

- ddNTP solution (5 mM) of each of the four ddNTPs
- dNTP solution (1 mM) of each of the four dNTPs
- ⚠ Formamide loading buffer

10x Labeling mixture and ddNTP chain extension/termination mixtures
When sequencing templates that are rich in secondary structure, replace dGTP in the 10x labeling mixture and the ddNTP extension/termination mixtures with an equimolar amount of 7-deaza-2´-dGTP. Assemble the mixtures according to the table below.

10x Labeling Mixture and ddNTP Chain Extension/Termination Mixtures for Use in Conventional DNA-sequencing Reactions Catalyzed by Taq Polymerase

	STOCK SOLUTION OF dNTP AND ddNTPs (ALL VOLUMES IN µl)								OTHER REAGENTS		
REACTION MIXTURE	dCTP 1 mM	dTTP 1 mM	dATP 1 mM	dGTP 1 mM	ddCTP 5 mM	ddTTP 5 mM	ddATP 5 mM	ddGTP 5 mM	TRIS-CL (1 M, pH 8.0)	EDTA (10 mM, pH 8.0)	H_2O
Labeling	1.5	1.5	-	1.5	-	-	-	-	10	10	975
ddCTP	15	15	15	15	90	-	-	-	10	10	830
ddTTP	15	15	15	15	-	240	-	-	10	10	680
ddATP	15	15	7.5	15	-	-	120	-	10	10	800
ddGTP	15	15	15	15	-	-	-	9	10	10	911

The 10x labeling mixture contains dGTP, dCTP, and dTTP, each at a concentration of 1.5 µM.
The ratios of ddNTP:dNTP in the four ddNTP extension/termination mixtures are 30:1, 80:1, 80:1, and 3:1 for C, T, A, and G, respectively.
The 10x labeling mixture and ddNTP extension/termination mixtures should be dispensed in 50-µl aliquots and stored frozen at -20°C.

Enzymes and Buffers

- 5x Dideoxy reaction buffer

Taq (5 units/µl) or a similar thermostable DNA polymerase

Taq dilution buffer
Thermostable enzymes purified from other organisms may require slightly different reaction and dilution buffers. For optimal buffers of individual enzymes, please see the specifications provided by the manufacturer. 1 unit of thermostable DNA polymerase activity is usually defined as that amount which will incorporate 10 nmoles of nucleotide into an acid-precipitable form in 30 minutes at 70-80°C.

Nucleic Acids and Oligonucleotides

Oligonucleotide primer
Use at a concentration of 1.0 pmole/µl (approx. 6.6 ng/µl) in TE (pH 7.6).

Template DNAs (100 ng/µl) in TE (pH 7.6)
Single-stranded DNA (500 ng) or 1.0 µg of denatured double-stranded plasmid DNA is required for each set of four sequencing reactions. Denature and anneal the linear double-stranded DNA to primer by mixing the native template DNA with an excess of primer, heating the mixture in a boiling water bath for approx. 2 minutes, and then plunging the tube into an ice-water bath. Do not allow the mixture to warm up; use immediately.

Radioactive Compounds

⚠ [α-^{32}P]dATP (3000 Ci/mmole, 10 mCi/ml)

or

⚠ [α-^{35}S]dATP (1000 Ci/mmole, 10 mCi/ml)

or

⚠ [α-^{33}P]dATP (2000-4000 Ci/mmole, approx. 10 mCi/ml)

or

5' ^{32}P-labeled oligonucleotide primer
Instead of internal labeling with radiolabeled dATP, carry out sequencing with an oligonucleotide primer labeled at the 5´ end with ^{32}P or ^{33}P. In this case, 1.0-1.5 pmoles of the radiolabeled primer and 2 µl of H_2O are included in the reaction mixture in place of the unlabeled primer (Step 1) and radiolabeled dATP (Step 6). All other steps remain the same. The 5´ termini of oligonucleotides are generally labeled by transfer of [γ-^{32}P] from [γ-^{32}P]ATP in a reaction catalyzed by polynucleotide kinase. For details, please see Chapter 10, Protocol 2.

METHOD

1. To a 0.5-ml microfuge tube or well of a microtiter plate, add:

single-stranded template DNA (250 fmoles) (100 ng/µl)	5.0 µl
oligonucleotide primer (0.5 pmole) (approx. 3.3 ng/µl)	3.0 µl
5x dideoxy reaction buffer	2.0 µl

2. Incubate the tightly closed tube for 2 minutes at 65°C. Remove the tube from the water bath and allow it to cool to room temperature over the course of 3-5 minutes.
3. While the primer and template are cooling, thaw the 10x labeling and ddNTP extension/termination mixtures and radiolabeled dATP. Store the thawed solutions on ice.
4. For each annealing reaction, transfer 4 µl of each ddNTP termination/extension mixture into color-coded 0.5-ml microfuge tubes or into individual wells of a microtiter plate prelabeled C, T, A, and G (i.e., 4 µl of ddCTP mixture to tube/well labeled C, 4 µl of ddTTP mixture into tube/well labeled T, etc.). Store the tubes/microtiter plates on ice.

5. Dilute enough thermostable DNA polymerase enzyme 1:8 for all templates to be sequenced, for example,

Taq DNA polymerase (5-10 units/µl)	1 µl
Taq dilution buffer	7 µl

2 µl (2 units) of diluted enzyme is needed for each set of four sequencing reactions. The final concentration of the enzyme should be approx. 1 unit/µl. Store the diluted enzyme on ice at all times.

6. Add the following to each annealing reaction (Step 2 above):

10x labeling mixture	2 µl
radiolabeled dATP	0.5 µl
diluted DNA polymerase enzyme (approx. 1 unit/µl)	8 µl

Mix the contents of the tube by vortexing and then incubate the reactions for 5 minutes at 45°C.

7. Transfer 4 µl of the labeling reaction to the sides of each of the C, T, A, and G tubes or to the sides of microtiter wells containing the appropriate dideoxy terminator mixtures (Step 4 above).
8. Place the microfuge tubes in a microfuge (use appropriate rotor or adaptors for 0.5-ml tubes or place them inside decapitated 1.5-ml tubes), or place the microtiter plates in a centrifuge equipped with an appropriate adaptor. Centrifuge the C, T, A, and G tubes or plates for a few seconds at 2000 rpm to mix the components of the reactions. Incubate the reactions for 5 minutes at 72°C.
Incubate the tubes/plates in an efficient heating block or place them in contact with H_2O, oil, or other efficient conductor of heat. Tubes/plates placed in an air incubator set at 72°C will not reach optimum temperature for Taq *DNA polymerase during the course of the brief incubation.*
9. Stop the reactions by adding 4 µl of formamide loading buffer.
10. The reactions may be stored at -20°C for up to 5 days or analyzed directly by denaturing gel electrophoresis (Chapter 12, Protocol 8 or Chapter 12, Protocol 9). After heat denaturation (2 minutes at 100°C) and quick cooling on ice, load 3 µl of each of the C, T, A, and G reactions into individual wells of a sequencing gel.

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Protocol 6

Cycle Sequencing: Dideoxy-mediated Sequencing Reactions Using PCR and End-labeled Primers

In this protocol, asymmetric PCR is used to generate single-stranded DNA templates for dideoxy-sequencing.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

ddNTP extension/termination mixtures
Assemble the mixtures according to the table below.

ddNTP Extension/Termination Mixtures for Use in Cycle-sequencing Reactions

ddNTP REACTION MIXTURE	STOCK SOLUTION OF dNTP (1 mM) AND ddNTPS (5 mM) (ALL VOLUMES IN µl)								OTHER REAGENTS		
	dCTP	dTTP	dATP	dGTP	ddCTP	ddTTP	ddATP	ddGTP	TRIS-CL (1 M, PH 8.0)	EDTA (10 mM, PH 8.0)	H_2O
ddCTP	20	20	20	20	80	-	-	-	10	10	820
ddTTP	20	20	20	20	-	160	-	-	10	10	740
ddATP	20	20	20	20	-	-	120	-	10	10	780
ddGTP	20	20	20	20	-	-	-	40	10	10	860

Dispense as 50-µl aliquots; store frozen at -20°C.
The ratios of ddNTP:dNTP in the four extension/temination mixtures are 20:1, 40:1, 30:1, and 10:1 for C, T, A, and G, respectively. These ratios were optimized for reactions catalyzed by AmpliTaq CS DNA polymerase. When using other thermostable DNA polymerases, the ratios may need to be re-optimized.

- ddNTP solutions of the four ddNTPs, each at 5.0 mM
- ⚠ dNTP solutions of the four dNTPs, each at 1.0 mM
- EDTA (0.05 M, pH 8.0)
- ⚠ Formamide loading buffer
- Tris-Cl (1 M, pH 8.0)

Enzymes and Buffers

AmpliTaq CS or other exonuclease-deficient versions of *Taq* DNA polymerase (5 units/µl)

- 5x Cycle-sequencing buffer

Thermostable DNA polymerase
For advice on which enzyme to use, please see Table 12-19 in Protocol 5 in the print version of the manual. This protocol has been written with AmpliTaq CS in mind, but it will work well for thermostable enzymes with similar properties.

Nucleic Acids and Oligonucleotides

- ⚠ Oligonucleotide Primers, radiolabeled at the 5′ terminus with ^{33}P or ^{32}P
  To prepare end-labeled primers, please see Chapter 10, Protocol 2.

Template DNAs
Plasmids, cosmids, bacteriophage λ, and bacteriophage M13 DNAs, purified from large- or small-scale cultures by any of the methods described in Chapters 1-4, can be used as templates. The table below shows the amounts of each type of template required.

Amounts of Various Types of Templates Required in Cycle-sequencing Reactions

TYPE OF TEMPLATE	AMOUNT OF PURIFIED DNA REQUIRED FOR EACH SET OF FOUR DIDEOXYSEQUENCING REACTIONS (FMOLES)
Double-stranded plasmid DNA	20-200
Single-stranded bacteriophage M13 DNA	1-50
Double-stranded bacteriophage λ DNA	20-200
Double-stranded cosmid DNA	50-200

METHOD

1. Transfer 4 µl of appropriate ddNTP extension/termination mixture (please see table in Materials [Buffers and Solutions] section) into 0.5-ml color-coded microfuge tubes or into individual wells of a heat-stable microtiter plate prelabeled C, T, A, and G (i.e., 4 µl of ddCTP mixture into tube/well labeled C, 4 µl of ddTTP mixture into tube/well labeled T, etc.). Store the tubes/microtiter plates on ice.
2. To the side of each tube or well add:

double-stranded template DNA	10-100 fmoles
1.5 pmoles of 5' ^{32}P-end labeled primer*	1.0 µl
5x cycle-sequencing buffer	2.0 µl
H_2O	to 5.0 µl

   **If using a ^{33}P end-labeled primer: 1.5 µl of a standard preparation of ^{33}P-labeled oligonucleotide contains approx. 5.0 pmoles of primer. To maintain the stoichiometry of the components of the sequencing reaction, increase the amount of template DNA approx. 3-fold (30-300 fmoles)* without increasing the total volume of the reaction.
3. Dilute an aliquot of the preparation of AmpliTaq CS DNA polymerase with 1x cycle-sequencing buffer to a final concentration of 0.5-1.0 unit/µl. Add 1 µl of the diluted enzyme to the side of each tube or well.
4. Mix the reagents by flicking the tubes with a finger or shaking the microtiter plate. If necessary, overlay each reaction with a drop of light mineral oil, cap the tubes, and centrifuge them at 2000 rpm for 2 seconds in a microfuge or briefly in a centrifuge with microtiter plate adaptors.
5. Load the tubes or plate into a thermocycler, preheated to 95°C. Then begin thermal cycling according to the program outlined below.
   IMPORTANT *Do not delay in starting the program. Make sure that the samples are not exposed for >3 minutes to 95°C during the loading/preheating step. Otherwise, the DNA polymerase may be inactivated.*

Cycle Number	Denaturation	Annealing	Polymerization
Preheating	60 sec at 95°C		
20-25 cycles	30 sec at 95°C	30 sec at 55°C	60 sec at 72°C
10 cycles	30 sec at 95°C	60 sec at 72°C	60 sec at 72°C

   Times and temperatures may need to be adapted to suit the particular reaction conditions.
6. Remove the tubes or plates from the thermocycler and add 5 µl of formamide loading buffer to each cycle-sequencing reaction.

7. The reactions may be stored for up to 5 days at -20°C or analyzed directly by denaturing gel electrophoresis (Chapter 12, Protocol 8 or Chapter 12, Protocol 9). After heat denaturation (2 minutes at 100°C) and quick cooling on ice, load 3 µl of each of the C, T, A, and G reactions into individual wells of a sequencing gel.

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Protocol 7

Chemical Sequencing

This protocol is based on the classic base-specific cleavage method of DNA sequencing devised in 1977 by Maxam and Gilbert.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Acetic acid (1 M), freshly diluted from glacial acetic acid (17.4 M)
⚠ DMS (dimethylsulfate) (99%) (Gold label 99% from Aldrich)
⚠ DMS (10% v/v) in ethanol
DMS buffer
DMS stop solution
EDTA (0.5 M, pH 8.0)
Ethanol (absolute) chilled to -20°C.
⚠ Formamide
⚠ Formamide loading buffer
⚠ Hydrazine (95%)
Store hydrazine (Eastman Kodak) in small aliquots in tightly capped microfuge tubes at -20°C.
Hydrazine stop solution
NaCl (5 M)
⚠ NaOH (1.2 N) containing 1 mM EDTA
⚠ Piperidine (1 M) in H_2O
This solution should be freshly made by mixing 1 volume of piperidine (10 M; Fisher) with 9 volumes of H_2O in a graduated glass cylinder.
Piperidine formate (1 M)
Prepared by adjusting a 4% solution of formic acid in H_2O to pH 2.0 with 10 M piperidine.
Sodium acetate (3 M, pH 5.2)
Salmon sperm DNA (1 mg/ml) in H_2O
Sheared salmon sperm DNA is the material traditionally used as carrier in the chemical sequencing method. In fact, almost any DNA will serve the purpose equally well. Sheared salmon sperm DNA can be purchased from commercial suppliers (e.g., Sigma), or it can be generated in the laboratory.
⚠ Target DNA, radiolabeled
Prepare at least 5 x 10^5 cpm of DNA, asymmetrically end-labeled with ^{32}P and dissolved at a concentration of approx. 5000 cpm/µl in H_2O. The DNA solution must *be free of salt.*
When the quantity of radiolabeled DNA is limiting, it is possible to determine an unambiguous DNA sequence by carrying out only four (C, C+T, A+G, and G) of the five reactions detailed in the flowchart on page 12.65 of the print version of the manual.
Yeast tRNA (1 mg/ml) in H_2O

Nucleic Acids and Oligonucleotides

Salmon sperm DNA (1 mg/ml) in H_2O
Sheared salmon sperm DNA is the material traditionally used as carrier in the chemical sequencing method. In fact, almost any DNA will serve the purpose equally well. Sheared salmon sperm DNA can be purchased from commercial suppliers (e.g., Sigma), or it can be generated in the laboratory.
⚠ Target DNA, radiolabeled
Prepare at least 5 x 10^5 cpm of DNA, asymmetrically end-labeled with ^{32}P and dissolved at a concentration of approx. 5000 cpm/µl in H_2O. The DNA solution must *be free of salt.*
When the quantity of radiolabeled DNA is limiting, it is possible to determine an unambiguous DNA sequence by carrying out only four (C, C+T, A+G, and G) of the five reactions detailed in the flowchart on page 12.65 of the print version of the manual.
Yeast tRNA (1 mg/ml) in H_2O

METHOD

1. Subject the radiolabeled DNA to the base modification procedures outlined in the flowchart in Table 12-16 on page 12.65 of the print version of the manual.
2. Resuspend each of the four or five lyophilized DNA samples containing the base-modified DNA by vortexing with 100 µl of 1 M piperidine.
3. Close the tops of the tubes securely. Mix the contents of the tubes by vortexing. If necessary, centrifuge the tubes briefly (2000 rpm) to deposit all of the fluid at the bottom.
4. Incubate the tubes for 30 minutes at 90°C. To prevent the tops of the tubes from popping open during heating, either place a heavy weight on the tubes or seal the tops with plastic tape. Alternatively, use a round bath rack with a screw-down pressure plate (e.g., Research Products International).
5. Allow the tubes to cool to room temperature. Open the lids of the tubes, and seal the open tubes with Parafilm. Pierce several holes in the Parafilm with a 21-gauge needle and evaporate the contents of the tubes to dryness in a rotary vacuum evaporator (e.g., Savant SpeedVac).
6. Remove the tubes from the evaporator. Discard the Parafilm and add 20 µl of H_2O to each tube. Close the caps of the tubes, and vortex the tubes for 30 seconds to dissolve the DNA. Centrifuge the tubes briefly to deposit all of the fluid at the bottom. Use a hand-held minimonitor to check that all of the radiolabeled DNA has been washed from the walls of the tubes by the H_2O and is dissolved in the fluid.
7. Once again, evaporate all of the samples to dryness in a rotary vacuum evaporator (see Step 5 above). This step usually takes 15-30 minutes, depending on the efficiency of the evaporator.
8. Repeat Steps 6 and 7.
9. Estimate the amount of radioactivity remaining in each of the tubes by Cerenkov counting, and dissolve the individual modification and cleavage reactions in sequencing gel-loading buffer. An overnight exposure on Kodak XAR-5 film requires approx. 25,000 cpm of reactions that cleave the DNA after only one base (i.e., the C and G reactions), and approx. 50,000 cpm of reactions that cleave after two bases (C+T, A+G, and A>C). Therefore, the modified and cleaved DNAs should be dissolved in sequencing gel-loading buffer so that the C and G reactions contain approx. 25,000 cpm/3 µl and the C+T, A+G, and A>C reactions contain approx. 50,000 cpm/3 µl. Vortex the tubes to dissolve the DNA fully. Centrifuge the tubes briefly to deposit all of the fluid at the bottom. If necessary, the samples may be

stored at -20°C for a few hours while the sequencing gel is prepared.

10. Heat the tubes for 1 minute at 90°C to denature the DNA before quick-cooling on ice. Analyze the reactions by electrophoresis through denaturing polyacrylamide gels as described in Chapter 12, Protocol 8 or Chapter 12, Protocol 9.

REFERENCES

1. Maxam A.M. and Gilbert W. 1977. A new method for sequencing DNA. *Proc. Natl. Acad. Sci.* 74:560-564.
2. Maxam A.M. and Gilbert W. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65:499-560.

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Protocol 8

Preparation of Denaturing Polyacrylamide Gels

In 1978, Fred Sanger and Alan Coulson devised a method to pour and run thin polyacrylamide gels, which are now used ubiquitously to resolve the products of DNA sequencing reactions.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Acrylamide solution (45% w/v)

⚠ Ammonium persulfate (1.6% w/v) in H_2O

Deionized H_2O

Detergent, household dishwashing

Ethanol

⚠ KOH/Methanol solution

⚠ Silanizing fluid
The traditional silanizing fluids (e.g., Sigmacote from Sigma and Repelcote from BDH Inc.) contain dichlorodimethylsilane, which is toxic, volatile, and highly flammable. In recent years, nontoxic alternatives have become available, including Gel Slick (FMC Bioproducts), RainX (Unelko, Scottsdale Arizona), and Acrylease (Stratagene).

10x TBE electrophoresis buffer
TBE is used at a working strength of 1x (89 mM Tris-borate, 2 mM EDTA) for polyacrylamide gel electrophoresis.

⚠ TEMED (N,N,N´,N´-tetramethylethylenediamine)
Electrophoresis-grade TEMED is sold by many manufacturers including Sigma and Bio-Rad. TEMED is hygroscopic and should be stored in a tightly sealed bottle at 4°C.

Urea, solid

METHOD

1. If necessary, remove old silanizing reagent from plates by swabbing them with KOH/methanol solution.
IMPORTANT *To prevent contamination of the glass surfaces by skin oils, wear talc-free gloves at all times and handle the plates by their edges.*
2. Wash the plates and spacers in a warm, dilute solution of dishwashing liquid and then rinse them thoroughly in tap water, followed by deionized H_2O. Rinse the plates with absolute ethanol to prevent water spots, and allow them time to dry.
3. Treat the inner surface of the smaller or notched plate with silanizing solution. Lay the plate, inner surface uppermost, on a pad of paper towels in a chemical fume hood, and pour or spray a small quantity of silanizing fluid onto the surface of the plate. Wipe the fluid over the entire surface with a pad of Kimwipes and then allow the fluid to dry in the air (1-2 minutes). Rinse the plate first with deionized H_2O and then with ethanol, and allow the plate time to dry.
4. Lay the larger (or unnotched) glass plate (clean side up) on an empty test tube rack on the bench and arrange the spacers in place along each side of the glass plate so they are flush with the bottom of the plate.
5. Center the shorter (notched) plate, siliconized side down, on top of larger (unnotched) plate. Make sure that the spacers remain in position at the very edges of the two plates.
6. Clamp the plates together on one side with two or three large (5-cm length) bulldog binder clips. Bind the entire length of the other side and the bottom of the plates with gel-sealing tape to make a watertight seal.
7. Remove the bulldog clips and seal this side of the gel plates with gel-sealing tape.
8. Place the flat side of the sharkstooth comb into the open end of the gel mold so that it fits snugly. Remove the comb and lay the empty gel mold on the test-tube rack.
9. Cover the working area of the bench with plastic-backed protective paper.
10. In a 250-ml side-arm flask, prepare a sequencing gel solution containing the desired concentration of acrylamide as specified in the table below. The volumes given in the table are sufficient for a single 40 x 40-cm sequencing gel and can be proportionally adjusted to accommodate smaller or larger gels.
IMPORTANT *The preparation of the gel must be completed without interruption from this point onward.*

Acrylamide Solutions for Denaturing Gels

	4% GEL	6% GEL	8% GEL	10% GEL
Acrylamide:bis solution (45%)	8.9 ml	13.3 ml	17.8 ml	22.2 ml
10x TBE buffer	10 ml	10 ml	10 ml	10 ml
H_2O	45.8 ml	41.4 ml	36.9 ml	32.5 ml
Urea	42 g	42 g	42 g	42 g

11. Combine all of the reagents and then heat the solution in a 55°C water bath for 3 minutes to help dissolution of the urea.
12. Remove the solution from the water bath and allow it to cool at room temperature for 15 minutes. Swirl the mixture from time to time.
13. Attach the side-arm flask to a vacuum line and de-gas the solution.
14. Transfer the solution to a 250-ml glass beaker. Add 3.3 ml of freshly prepared 1.6% ammonium persulfate and swirl the gel solution gently to mix the reagents.
15. Add 50 µl of TEMED to the gel solution and swirl the solution gently to mix the reagents. Pour the gel solution into the mold directly from the beaker in which it has been prepared. Alternatively, draw approx. 40 ml of the solution into a 60-cc hypodermic syringe. Do not suck air bubbles into the syringe!
Compared with polyacrylamide gels used to resolve proteins, a massive amount of TEMED is used to cast sequencing gels. The large amount of TEMED ensures that polymerization will occur rapidly and uniformly throughout the large surface area of the gel. Because the rate of polymerization is temperature-dependent, cooling the gel solution allows more time for casting the gel. Experienced gel pourers can often cast two or more 40 x 40-cm gels from a single gel solution by judicious precooling.
Work as quickly as possible from here onward because the gel solution will polymerize rapidly. Polymerization can be appreciably slowed by putting the gel solution on ice, a boon for inexperienced gel pourers!
16. Allow a thin stream of gel solution to flow from the beaker or syringe into the top corner of the gel mold while holding the mold at an approx. 45° angle to the horizontal.
17. Lay the mold down on the test-tube rack (please see Figure 12-9 in the print version of the manual).
18. Immediately insert the *flat side* of a sharkstooth comb approx. 0.5 cm into the gel solution. Insert both ends of the comb into the fluid to an equal depth so that the flat surface is level when the gel is standing in a vertical position.
19. Clamp the comb into position using bulldog binder clips. Use the remaining acrylamide/urea solution in the hypodermic syringe/pipette to form a bead of acrylamide across the top of the gel. Allow the gel to polymerize for at least 15 minutes at room temperature.
20. Wash out the 60-cc syringe so that it does not become clogged with polymerized acrylamide.
21. After 15 minutes of polymerization, examine the gel for the presence of a Schlieren line just underneath the flat surface

of the comb. This is a sign that polymerization is occurring satisfactorily. When polymerization is complete (approx. 1 hour after the gel was poured), remove the bulldog clips.

22. The gel can be used immediately (please see Chapter 12, Protocol 11) or stored for up to 24 hours at room temperature or 48 hours at 4°C. To prevent dehydration during storage, leave the tape in place and surround the top of the gel with paper towels dampened with 1x TBE. Cover the paper towels with Saran Wrap. Do not remove the comb at this stage.

REFERENCES

1. Sanger F. and Coulson A.R. 1978. The use of thin acrylamide gels for DNA sequencing. *FEBS Lett.* 87:107-110.

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Protocol 9

Preparation of Denaturing Polyacrylamide Gels Containing Formamide

The inclusion of formamide in sequencing gels eliminates secondary structure in the DNA during electrophoresis. Formamide gels are particularly useful and almost a necessity when sequencing DNA templates with a G/C content >55%. This protocol requires all of the reagents listed in Chapter 12, Protocol 8.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Formamide (100%)

METHOD

1. Clean and assemble glass plates to form a gel mold as described in Steps 1-8 of Chapter 12, Protocol 8.
2. Cover the working area of the bench with plastic-backed protective paper.
3. In a 250-ml side-arm flask, prepare a sequencing gel solution containing the desired concentration of acrylamide as specified in the table below. The volumes given in this table are sufficient for a single 40 x 40-cm sequencing gel and can be proportionally adjusted to accommodate smaller or larger gels.
   IMPORTANT *The preparation of the gel must be completed without interruption from this point onward.*

   Acrylamide Solutions for Denaturing Polyacrylamide Gels Containing Formamid

	4% GEL	6% GEL	8% GEL	10% GEL
Acrylamide:bis solution (45%)	8.9 ml	13.3 ml	17.8 ml	22.2
10x TBE	10 ml	10 ml	10 ml	10 ml
H_2O	20.8 ml	16.4 ml	11.9 ml	7.5 ml
Formamide	25 ml	25 ml	25 ml	25 ml
Urea	42 g	42 g	42 g	42 g

4. Combine all of the reagents and then heat the solution in a 55°C water bath for 3 minutes to help dissolution of the urea.
5. Remove the solution from the water bath and allow it to cool for 15 minutes at room temperature. Swirl the mixture from time to time. Add H_2O to the solution bringing the final volume to 100 ml.
6. Attach the side-arm flask to a vacuum line and de-gas the solution.
7. Transfer the solution to a 250-ml glass beaker. Add 3.3 ml of freshly prepared 1.6% ammonium persulfate and swirl the gel solution gently to mix the reagents.
8. Add 50 µl of TEMED to the gel solution, and swirl the solution gently to mix the reagents. Draw approx. 40 ml of the solution into a 60-ml hypodermic syringe.
9. Pour the gel solution into the mold, as described in Steps 16-22 of Chapter 12, Protocol 8.
   IMPORTANT *Because formamide slows the polymerization reaction substantially, allow the gel to polymerize for 2-3 hours before clamping it in an electrophoresis apparatus.*

REFERENCES

1. Deininger P.L. 1983. Approaches to rapid DNA sequence analysis. *Anal. Biochem* 135:247-263.

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Protocol 10

Preparation of Electrolyte Gradient Gels

Electrolyte gradients are formed when buffers of different concentrations are used in the upper (low electrolyte concentration) and lower (high electrolyte concentration) chambers of the electrophoresis device. Fragments of DNA migrate more slowly as they travel anodically into regions of progressively higher ionic strength. The spacing between bands of DNA is therefore reduced at the bottom of the gel and increased at the top. In consequence, the number of bases that can be read is increased by approx. 30%. This protocol requires all of the reagents listed in Chapter 12, Protocol 8.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Formamide (optional)
Electrolyte gradient gels are poured in 1x TBE buffer and can be run in the presence or absence of formamide. For information on denaturing polyacrylamide gels containing formamide, please see Chapter 12, Protocol 9.

Sodium acetate (3 M, pH 7.0)

METHOD

1. Clean and assemble a set of glass plates to form a gel mold as described in Steps 1-8 of Chapter 12, Protocol 8.
2. Cover the working area of the bench with plastic-backed protective paper.
3. Prepare a denaturing polyacrylamide gel as described in Chapter 12, Protocol 8 or Chapter 12, Protocol 9.
4. Clamp the gel into the electrophoresis device. Fill the upper chamber with 0.5x TBE buffer and the lower chamber with a buffer composed of 2 parts 1x TBE and 1 part 3 M sodium acetate. Load the sequencing reactions and perform electrophoresis as described in Chapter 12, Protocol 11.

REFERENCES

1. Sheen J.Y. and Seed B. 1988. Electrolyte gradient gels for DNA sequencing. *BioTechniques* 6:942-944.

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Protocol 11

Loading and Running DNA-sequencing Gels

The sets of nested DNA fragments generated by DNA sequencing methods are resolved by electrophoresis through thin denaturing polyacrylamide gels. **WARNING** Large voltages are passed through DNA-sequencing gels at substantial amperages. More than enough current is used in these gels to cause severe burns, ventricular fibrillation, central respiratory arrest, and asphyxia due to paralysis of the respiratory muscles. Make sure that the gel boxes used for electrophoresis are well insulated, that all buffer chambers are covered, and that the box is used on a stable bench top that is dry. Always turn off the power to the box before sample loading or dismantling the gel.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 0.5x TBE
- 1x TBE

Nucleic Acids and Oligonucleotides

⚠ DNA sequencing reactions
Carry out the reactions as described in Chapter 12, Protocol 3 to Chapter 12, Protocol 7.

METHOD

1. Use damp paper towels or a wet sponge to wipe away any dried polyacrylamide/urea from the outside of the gel mold. Pipette several ml of 1x TBE buffer along the top of the smaller or notched glass plate and slowly remove the comb from gel. Cut the electrical tape with a scalpel and strip it from the bottom of the gel mold.
   IMPORTANT *Do not remove the tape from the sides of the gel!*
2. Attach the gel mold to the electrophoresis apparatus with bulldog binder clips, plastic-coated laboratory clamps, or, in the case of electrophoretic devices with built-in screw clamps, according to manufacturer's instructions. The smaller or notched plate should be in direct contact with the electrophoresis device. The larger, unnotched plate should face the investigator.
3. Fill the upper and lower reservoirs of the apparatus with the appropriate buffer.

   For standard or formamide-containing gels

   a. Fill the upper and lower buffer reservoirs with 1x TBE. Make sure that the level of the buffer in the lower chamber is well above the bottom of the plates. The level of the buffer in the upper chamber should be well above the level of the upper edge of the shorter or notched plate and be in direct contact with the gel.

   b. Use a 10-ml syringe filled with 1x TBE to rinse the top of the gel. Make sure that excess polyacrylamide and urea are removed from the gel. If necessary, use a syringe needle to scrape off any polyacrylamide sticking to the glass plates. Remove air bubbles under the bottom of the glass plates in the lower reservoir using a Pasteur pipette.

   c. Attach the electrodes to the electrophoresis apparatus and the power supply. The cathode (black lead) should be attached to the upper reservoir and anode (red lead) to the bottom. Attach the built-in thermal sensor (if available) or temperature-monitoring strip. Run the gel at constant wattage (50-70 W) for approx. 45 minutes or until the temperature of the gel reaches 45-50°C. Turn off the power supply and disconnect the electrodes.

   For electrolyte gradient gels

   a. Fill the upper reservoir with 0.5x TBE, and the lower reservoir with a solution consisting of 2 volumes of 1x TBE plus 1 volume of 3 M sodium acetate (please see Chapter 12, Protocol 10).

   b. Wash the well with 0.5x TBE and remove urea/polyacrylamide as described above. Do not pre-run electrolyte gradient gels.
4. Incubate the microfuge tubes containing the sequencing reactions for 2 minutes in a heating apparatus set at 100°C. If the reactions have been carried out in a microtiter plate, remove the cover from the plate and float the open plate in a water bath for 5 minutes at 85°C.
5. While the tubes or plates are incubating, fill a 10-cc syringe fitted with a 22-gauge hypodermic needle with 0.5x or 1x TBE, as appropriate. Squirt the TBE forcibly across the submerged loading surface of the gel to remove any remaining urea and fragments of polyacrylamide from the loading area. Continue squirting until no more urea can be seen in the loading area.
6. Gently insert the sharkstooth comb (teeth downward) into the loading slot. Push the comb down until the points of the teeth just penetrate the surface of the gel.
7. Transfer the microfuge tubes or microtiter plate from the water bath or heating block to ice. Keep the samples at 0°C until they are loaded onto the gel. Quick-cooling to low temperature retards renaturation of the template and radiolabeled strands.
8. Load 1-5 µl (please see Chapter 12, Protocol 3 for recommended volumes) of each sequencing reaction into adjacent slots of the gel.
9. When all of the samples have been loaded, connect the electrodes to the power pack and the electrophoresis apparatus: cathode (black) to the upper reservoir and anode (red) to the bottom reservoir. Run the gel at sufficient constant power to maintain a temperature of 45-50°C.

Gel Size (cm)	Power (W)	Voltage (V)
20 x 40	35-40	approx. 1700
40 x 40	55-75	approx. 2-3000
40 x 40 (electrolyte gradient gel)	75	approx. 2100

The time required to achieve optimal resolution of the sequence of interest must be determined empirically. Monitor the progress of the electrophoretic run by following the migration of the marker dyes in the formamide gel-loading buffer (please see the table below).

Migration Rates of Marker Dyes through Denaturing Polyacrylamide Gels

POLYACRYLAMIDE GEL	BROMOPHENOL BLUE[a]	XYLENE CYANOL FF[a]
5%	35	130
6%	29	106
8%	26	76
10%	12	55
20%	8	28

In electrolyte gradient gels, bromophenol blue migrates progressively more slowly as it travels anodically through the gel. Migration essentially ceases when the dye nears the bottom of the gel. The xylene cyanol tracking dye behaves in a similar fashion. Typically, electrophoresis is continued until the xylene cyanol dye is within 5-10 cm of the bottom of

the gel and the bromophenol blue dye is at the bottom.

[a]*The numbers are the approximate sizes of DNA (in nucleotides) with which the indicated marker dye will comigrate in a standard DNA sequencing gel.*

10. Depending on the distance between the sequence of interest and the oligonucleotide primer, apply a second loading of the sequencing samples to a standard or formamide-containing denaturing polyacrylamide gel approx. 15 minutes after the bromophenol blue in the first set of samples has migrated to the bottom of the gel (1.5-2.0 hours). The sequence obtained from the first loading will be more distal to the primer, whereas that obtained from the second loading will be more proximal. The length of readable sequence can be extended by approx. 35% by reloading the samples into a fresh set of lanes 2 hours after the samples were first loaded.
    a. Turn off the power supply and disconnect the sequencing apparatus.
    b. Replace the buffer in the top and bottom reservoirs.
    c. Denature the samples by heating as described in Step 4 above.
    d. Load the samples.
    e. Reconnect the sequencing apparatus to the power supply.
    f. Run the gel, as before, at sufficient constant power to maintain a temperature of 45-50°C.
11. At the end of the run, follow the procedures described in Chapter 12, Protocol 12 to dismantle the gel and perform autoradiography.

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Protocol 12

Autoradiography and Reading of Sequencing Gels

For information on autoradiography, please see Appendix 9 of the print version of the manual (Volume 3, pages A9.9-9.15). For information on reading of sequencing gels, please see the information panel on Reading an Autoradiograph in the print version on page 12.113.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Sequencing gel-fixing solution

Radioactive Compounds

⚠
- Radioactive ink
  Reusable alternatives to radioactive ink are chemiluminescent markers available from Stratagene (Glogos). The markers can be used multiple times and should be exposed to fluorescent light just prior to a new round of autoradiography.

METHOD

1. At the end of the electrophoretic run (Chapter 12, Protocol 11), turn off the power and disconnect the sequencing apparatus from the power pack. Dispose of the electrophoresis buffer and then remove the gel mold from the apparatus.
2. Lay the gel mold flat on plastic-backed protective bench paper with the smaller (notched) plate uppermost. Allow the gel to cool to <37°C before proceeding.
3. Remove any remaining pieces of gel-sealing tape. Use the end of a metal spatula to pry apart the plates of the mold slowly and gently. The gel should remain attached to the longer (nonsiliconized) glass plate.
4. When the glass plates have been separated, cut off a bottom or top corner on the side of the gel that was loaded first. This landmark serves to orient the gel during subsequent manipulations.
5. Fix the gel in sequencing gel-fixing solution (methanol/acetic acid; please see Materials).
6. Transfer the gel (together with its supporting glass plate), to a shallow tray containing methanol:acetic acid fixing solution. Fix the gel for 30 minutes at room temperature. Do not agitate the fluid while the gel is being fixed.
7. After 30 minutes, lift the glass plate very, very slowly from the fixation solution. Try to keep the plate horizontal until most of the fixation solution has drained away. Lay the plate, gel side uppermost, on a stack of paper towels. Blot excess fixation solution from the glass plate with Kimwipes. Try not to touch the surface of the gel with the Kimwipes. Remove wrinkles and blemishes from the gel by gently caressing its surface with gloved fingers.
8. Prepare a piece of Whatman 3MM CHR paper (or equivalent) that is slightly larger (2-3 cm) than the gel in both length and width. Hold the paper in a bow shape and touch the center of the bow to the center of the gel. Let the paper fall gently onto the surface of the gel and then apply gentle pressure so that the gel becomes firmly attached to the rough surface of the paper.
9. Hold the paper in place with one hand and pick up the supporting glass plate with the other hand. Quickly flip the sandwich over and lay it down on the bench top with the glass plate facing upward. Gently separate the 3MM CHR paper from the glass plate by lifting the plate upward. The gel will stick to the 3MM CHR paper as the glass plate is pulled away.
10. Lay the 3MM CHR paper (gel uppermost) on two pieces of Whatman 3MM paper of the same size. Cut a piece of Saran Wrap slightly longer and wider than the gel and lay it on top of the gel. Try to avoid creases and bubbles. This step is more easily accomplished with the help of another person. Hold the corners of the Saran Wrap and pull outward so that it is tightly stretched. Lower the stretched Saran Wrap onto the surface of the gel. Once the Saran Wrap has touched the gel, do not attempt to remove it, since this can cause the gel to tear. The flat end of an agarose gel comb, a Kimwipe, or a plastic card can be used to remove any bubbles of air trapped between the gel and wrap.
11. Use a paper cutter or sharp pair of scissors to trim all three pieces of Whatman paper and the Saran Wrap to approximately the same size as the gel.
12. Place the sandwich of paper, gel, and Saran Wrap on the gel dryer, with the plastic wrap uppermost. Use a sheet of Mylar to keep the sandwich flat during drying.
13. Following the instructions of the manufacturer of the gel dryer, dry the gel for 30-60 minutes under vacuum at 80°C.
14. Remove the gel from the dryer and peel off the Saran Wrap. The dried gel should feel smooth to the touch and not sticky. A quick remedy to stickiness is to turn a powdered latex glove inside out and use the talcum powder on the inside of the glove to dust the gel. To orient the gel, attach a small adhesive label marked with radioactive or chemiluminescent ink to the 3MM CHR paper in the space created by cutting the bottom corner of the gel (Step 4).
15. In a darkroom, place the dried gel (gel side up) in a spring-loaded metal cassette. Cover the gel with a sheet of unexposed X-ray film. Close the cassette. Establish an autoradiograph by exposing the gel to the film for 16-24 hours at room temperature or -80°C.
16. Develop the autoradiograph according to the recommendations of the manufacturer of the film and read the sequence of the DNA.

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Chapter 13 Mutagenesis

Protocol 1: Preparation of Uracil-containing Single-stranded Bacteriophage M13 DNA

Single-stranded templates of bacteriophage M13 DNA containing 20-30 residues of uracil in place of thymine are generated during growth of the bacteriophage in an F´ strain of *E. coli* carrying mutations in the *ung* and *dut* genes. This DNA is used as a template in the Kunkel method of oligonucleotide-directed mutagenesis (Chapter 13, Protocol 2).

Protocol 2: Oligonucleotide-directed Mutagenesis of Single-stranded DNA

Uracil-containing bacteriophage M13 DNA, generated in Chapter 13, Protocol 1, is used in the classic double-primer method of oligonucleotide-mediated mutagenesis of Zoller and Smith (1984, 1987) to generate a heteroduplex molecule with uracil in the template strand and thymine in the strand synthesized in the in vitro reaction. Transformation of this DNA into an *ung*$^+$ strain results in destruction of the template strand, with consequent suppression of production of wild-type bacteriophages. Up to 80% of the plaques are therefore derived by replication of the uracil-free strand. Because synthesis of this strand has been primed by a mutagenic oligonucleotide, a high proportion of progeny bacteriophages carry the desired mutation.

Protocol 3: In Vitro Mutagenesis Using Double-stranded DNA Templates: Selection of Mutants with *DpnI*

Two oligonucleotides are used to prime DNA synthesis catalyzed by a high-fidelity thermostable polymerase on a denatured plasmid template. The two oligonucleotides both contain the desired mutation and occupy the same starting and ending positions on opposite strands of the plasmid DNA. During several rounds of thermal cycling, both strands of the plasmid DNA are amplified in a linear fashion, generating a mutated plasmid containing staggered nicks on opposite strands.
The products of the linear amplification reaction are treated with the restriction enzyme *Dpn*I, which specifically cleaves fully methylated G^{Me6}ATC sequences. *Dpn*I will therefore digest the bacterially generated DNA used as template for amplification but will not digest DNA synthesized during the course of the reaction in vitro. *Dpn*I-resistant molecules, which are rich in the desired mutants, are recovered by transforming *E. coli* to antibiotic resistance.

Protocol 4: Oligonucleotide-directed Mutagenesis by Elimination of a Unique Restriction Site (USE Mutagenesis)

Two oligonucleotide primers are hybridized to the same strand of a denatured double-stranded recombinant plasmid. One primer (the mutagenic primer) introduces the desired mutation into the target sequences, and the second primer carries a mutation that destroys a unique restriction site in the plasmid. Both primers are elongated in a reaction catalyzed by bacteriophage T4 or T7 DNA polymerase. Nicks in the strand of newly synthesized DNA are sealed with bacteriophage T4 DNA ligase. The product of the first part of the method is a heteroduplex plasmid consisting of a wild-type parental strand and a new full-length strand that carries the desired mutation but no longer contains the unique restriction site.
Residual wild-type plasmid molecules are then linearized by digestion with the appropriate restriction enzyme. The mixture of circular heteroduplex DNA and linear wild-type DNA is then used to transform a strain of *E. coli* that is deficient in repair of mismatched bases. Because linear DNA transforms 10-1000-fold less efficiently than circular DNA, many of the wild-type molecules are unable to reestablish themselves in *E. coli*. The circular heteroduplex molecules, however, begin to replicate. Because the mismatched bases are not repaired, the first round of replication generates a wild-type plasmid that carries the original restriction site and a mutated plasmid that does not. DNA from the first set of transformants is recovered, digested once more with the same restriction enzyme to linearize the wild-type molecules, and then used to transform a standard laboratory strain of *E. coli*. This biochemical selection can be sufficiently powerful to ensure that a high proportion of the resulting transformants carry the desired mutation.

Protocol 5: Rapid and Efficient Site-directed Mutagenesis by the Single-tube Megaprimer PCR Method

The "megaprimer" method of site-directed mutagenesis introduced by Kammann et al. (1989) uses three oligonucleotide primers and two rounds of PCR. One of the oligonucleotides is mutagenic, the other two being forward and reverse primers that lie upstream and downstream from the binding site for the mutagenic oligonucleotide. The mutagenic primer and the nearer of the external primers are used in the first PCR to generate and amplify a mutated fragment of DNA. This amplified fragment - the megaprimer - is used in the second PCR in conjunction with the remaining external primer to amplify a longer region of the template DNA.
This protocol is based on a method developed by Ke and Madison (1997), using forward and reverse external primers with significantly different melting temperatures (T_m). This eliminates the necessity of purifying the template DNA between the two rounds of PCR.

Protocol 6: Site-specific Mutagenesis by Overlap Extension

Four primers and three PCRs are used to create a site-specific mutation by overlap extension. One pair of primers is used to amplify DNA that contains the mutation site together with upstream sequences. The second pair of primers is used in a separate PCR to amplify DNA that contains the mutation site together with downstream sequences. The mutation(s) of interest is located in the region of overlap and therefore in both amplified fragments. The overlapping fragments are mixed, denatured, and annealed to generate heteroduplexes that can be extended and, in a third PCR, amplified into a full-length DNA using two primers that bind to the extremes of the two initial fragments.

Protocol 7: Screening Recombinant Clones for Site-directed Mutagenesis by Hybridization to Radiolabeled Oligonucleotides

Plaques formed by M13 bacteriophages or bacterial colonies transformed by plasmids carrying specific mutations can be detected by hybridization, using a radiolabeled oligonucleotide that forms a perfect duplex with the mutant sequence. Hybridization is carried out under conditions of low stringency that allow the radiolabeled oligonucleotide to anneal to both mutant and wild-type DNAs. A hybrid between the radiolabeled oligonucleotide and the wild-type sequence will contain one or more mismatched base pairs, whereas the hybrid formed between the newly created mutant and the oligonucleotide probe will be perfectly matched. These two types of hybrids usually differ in their thermal stabilities, with mismatched hybrids dissociating at a lower temperature than the corresponding perfect hybrid. Plaques or colonies that continue to hybridize to the probe after several cycles of washing at progressively higher temperatures are likely to contain recombinants carrying the desired mutation.

Protocol 8: Detection of Mutations by Single-strand Conformational Polymorphism

Single-strand conformational polymorphism (SSCP), one of several methods used to scan segments of DNA for mutations, exploits the electrophoretic differences in mobilities between single-stranded mutant and wild-type DNAs.

Protocol 9: Generation of Sets of Nested Deletion Mutants with Exonuclease III

The double-stranded DNA of recombinant plasmid, phagemid, or bacteriophage M13 replicative form DNA is digested with two restriction enzymes whose sites of cleavage both lie between one end of the target DNA and the binding site for universal primer. The enzyme that cleaves nearer the target sequence must generate either a blunt end or a recessed 3´ terminus; the other enzyme must generate a four-nucleotide protruding 3´ terminus. Because only the blunt or recessed 3´ terminus of the resulting linear DNA is susceptible to exonuclease III, digestion proceeds unidirectionally away from the site of cleavage and into the target DNA. The exposed single strands are then removed by digestion with nuclease S1 or mung bean nuclease, and the DNA is then recircularized. If desired, a synthetic linker can be inserted at the site of recircularization.

Protocol 10: Generation of Bidirectional Sets of Deletion Mutants by Digestion with BAL 31 Nuclease

In this method, the nuclease BAL 31 is used to make uni- or bidirectional deletions in a segment of cloned DNA. BAL 31 is a complex enzyme and tends to digest a population of double-stranded DNA targets in an asynchronous fashion, Deletions created by BAL 31 are therefore far more heterogeneous in size than those created by processive enzymes such as exonuclease III. **Warning!** BAL 31 is a tricky enzyme. This protocol is not for the technically challenged! Please see the information panel on BAL31 on page 13.68 in the print version of the manual.

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Protocol 1

Preparation of Uracil-containing Single-stranded Bacteriophage M13 DNA

Single-stranded templates of bacteriophage M13 DNA containing 20-30 residues of uracil in place of thymine are generated during growth of the bacteriophage in an F´ strain of *E. coli* carrying mutations in the *ung* and *dut* genes. This DNA is used as a template in the Kunkel method of oligonucleotide-directed mutagenesis (Chapter 13, Protocol 2).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Ethanol

⚠ PEG 8000 (15% w/v) in 2.5 M NaCl

⚠ Phenol (pH 8.0)

⚠ Phenol:chloroform (1:1, v/v)

Sodium acetate (3 M, pH 5.2)

TE (pH 7.6)

Nucleic Acids and Oligonucleotides

Marker DNA
As a reference, use single-stranded DNA from the original bacteriophage M13 recombinant.

Media

2x YT medium

2x YT medium containing 0.25 µg/ml uridine

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 3, Protocol 3, Chapter 3, Protocol 4, and Chapter 3, Protocol 6.

Step 6 of this protocol requires the reagents listed in Chapter 3, Protocol 1.

Vectors and Bacterial Strains

E. coli strain CJ236 (*dut*$^-$ *ung*$^-$ F´)

E. coli strain TG1, JM109, or equivalent
Please see Step 6.

METHOD

1. Prepare for mutagenesis.
   a. Clone a small fragment of DNA (<500 bp) carrying the target sequence into an appropriate bacteriophage M13 vector such as M13mp18 or mp19.
   b. Isolate single-stranded template DNA and double-stranded replicative form DNA from a freshly grown plaque generated by the recombinant bacteriophage.
   c. Check the fidelity of the recombinant clone by restriction mapping of the replicative form DNA and by DNA sequencing of the single-stranded DNA.
2. Use a sterile Pasteur pipette to transfer a single plaque produced by the bacteriophage M13 recombinant to a microfuge tube containing 1 ml of 2x YT medium.
3. Incubate the tube for 5 minutes at 60°C to kill bacterial cells. Vortex the tube vigorously for 30 seconds to release the bacteriophages trapped in the top agar. Remove dead bacterial cells and fragments of agar by centrifuging the tube at maximum speed for 2 minutes at 4°C in a microfuge.
4. Transfer 50 µl of the supernatant to a 500-ml flask containing 50 ml of 2x YT medium supplemented with 0.25 µg/ml uridine. Add 5 ml of a mid-log-phase culture of *E. coli* strain CJ236 (*dut*$^-$ *ung*$^-$ F´). Incubate the culture with vigorous shaking (300 cycles/minute on a rotary shaker) for 6 hours at 37°C.
5. Pellet the cells by centrifugation at 5000*g* (6470 rpm in a Sorvall SS-34 rotor) for 30 minutes at 4°C. Transfer the supernatant to a fresh 250-ml centrifuge bottle that will fit into a Sorvall GSA rotor or equivalent.
6. Determine the relative titer of the bacteriophage suspension on *E. coli* strain CJ236 (*dut*$^-$ *ung*$^-$ F´) and a strain such as JM109 or TG1 (please see Chapter 3, Protocol 1). The titer on strain CJ236 should be four to five orders of magnitude greater than that on the *dut*$^+$ *ung*$^+$ strain of *E. coli*.
7. Measure the volume of the bacteriophage suspension, and then add 0.25 volume of 2.5 M of NaCl containing 15% (w/v) PEG 8000. Mix the contents of the centrifuge bottle by swirling, and store the bottle on ice for 1 hour.
8. Recover the precipitated bacteriophage particles by centrifugation at 5000*g* (5500 rpm in a Sorvall GSA rotor) for 20 minutes at 4°C. Remove the supernatant by aspiration, and then invert the bottle to allow the last traces of supernatant to drain away. Use a pipette attached to a vacuum line to remove any drops of solution adhering to the walls of the bottle.
9. Resuspend the bacteriophage pellet in 4 ml of TE (pH 7.6). Transfer the suspension to a 15-ml Corex centrifuge tube, and wash the walls of the centrifuge bottle with another 2 ml of TE (pH 7.6). Transfer the washing to the Corex tube. Vortex the suspension vigorously for 30 seconds, and then store the tube on ice for 1 hour.
10. Vortex the suspension vigorously for 30 seconds, and then pellet the bacterial debris by centrifugation at 5000*g* (6470 rpm in a Sorvall SS-34 rotor) for 20 minutes at 4°C.
11. Taking care not to disturb the pellet of bacterial debris, transfer the supernatant to a 15-ml polypropylene tube. Extract the suspension twice with phenol (pH 8.0) and once with phenol:chloroform. Separate the phases by centrifugation at 4000*g* (5800 rpm in a Sorvall SS-34 rotor) for 5 minutes at room temperature. Avoid transferring material from the interface.
12. Transfer the aqueous phase from the final extraction to a glass centrifuge tube (e.g., a 30-ml Corex tube). Measure the volume of the solution, and add 0.1 volume of 3 M of sodium acetate (pH 5.2), followed by 2 volumes of ethanol at 0°C. Mix the contents of the tube thoroughly, and then store the tube on ice for 30 minutes.
13. Recover the DNA by centrifugation at 5000*g* (6470 rpm in a Sorvall SS-34 rotor) for 20 minutes at 4°C. Carefully remove the supernatant. Add 10 ml of 70% ethanol at room temperature, vortex the solution briefly, and recentrifuge.
14. Carefully remove the supernatant by aspiration and store the tube in an inverted position at room temperature until the last traces of ethanol have evaporated. Dissolve the DNA in 200 µl of TE (pH 7.6).
15. Purify the resuspended single-stranded uracil-containing bacteriophage M13 DNA by spun-column chromatography using columns that exclude large DNAs (>100 nucleotides) as described in Appendix 8 of the print version of the manual.
16. Measure the DNA spectrophotometrically at 260 nm (1 OD_{260} = 40 µg/ml). Analyze the size of an aliquot of the DNA (0.5 µg) by gel electrophoresis, using single-stranded DNA of the original bacteriophage M13 recombinant (Step 1) as a size marker.

17. Carry out oligonucleotide-directed mutagenesis as described in Chapter 13, Protocol 2.

REFERENCES

1. Kunkel T.A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci.* 82:488-492.
2. Kunkel T.A., Bebenek K., and McClary J. 1991. Efficient site-directed mutagenesis using uracil-containing DNA. *Methods Enzymol.* 204:125-139.

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Protocol 2

Oligonucleotide-directed Mutagenesis of Single-stranded DNA

Uracil-containing bacteriophage M13 DNA, generated in Chapter 13, Protocol 1, is used in the classic double-primer method of oligonucleotide-mediated mutagenesis of Zoller and Smith (1984, 1987) to generate a heteroduplex molecule with uracil in the template strand and thymine in the strand synthesized in the in vitro reaction. Transformation of this DNA into an *ung*$^+$ strain results in destruction of the template strand, with consequent suppression of production of wild-type bacteriophages. Up to 80% of the plaques are therefore derived by replication of the uracil-free strand. Because synthesis of this strand has been primed by a mutagenic oligonucleotide, a high proportion of progeny bacteriophages carry the desired mutation.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)

10x PE1 buffer

10x PE2 buffer

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Bacteriophage T4 polynucleotide kinase

10x Bacteriophage T4 polynucleotide kinase buffer

Klenow fragment of *E. coli* DNA polymerase I
Any of several different DNA polymerases may be used in the extension reaction (Steps 4 and 5). Other enzymes, such as bacteriophage T4 DNA polymerase, native T7 DNA polymerase, and Sequenase, require shorter incubation times. These enzymes are obligatory when a phosphorylated mutagenic oligonucleotide is used as the single primer in the polymerization/extension reaction.

Nucleic Acids and Oligonucleotides

Bacteriophage M13 universal sequencing primer
Any commercially available universal primer used to prime dideoxy sequencing reactions from (+) strand bacteriophage M13 templates will work well in this protocol.

dNTP solution containing all four dNTPs, each at 2 mM
Use dNTPs of the highest quality to minimize the possibility that contaminating dUTP will be incorporated into the newly synthesized strand of DNA. The concentrated dNTP solutions sold by Pharmacia have worked well in our hands.

Mutagenic bacteriophage M13 single-stranded DNA template

Mutagenic oligonucleotide primer
Before use in site-directed mutagenesis, purify the mutagenic oligonucleotide by Sep-Pak C_{18} column chromatography to remove salts and other impurities (please see Chapter 10, Protocol 6). It is not necessary to purify the oligonucleotide by polyacrylamide gel electrophoresis unless the oligonucleotide is more than 30 nucleotides in length or is to be used for "loop-in" or "loop-out" mutagenesis.

Media

2x YT top agar

YT agar plates

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 13, Protocol 1.

Step 7 of this protocol requires the reagents listed in Chapter 12, Protocol 3 or Chapter 12, Protocol 4.

Step 7 of this protocol may require the reagents listed in Chapter 13, Protocol 7.

Vectors and Bacterial Strains

E. coli strain suitable for transformation (e.g., TG1)

E. coli strain TG1, competent for transformation
Prepare competent cells as described in Chapter 1, Protocol 25 or Chapter 1, Protocol 26.

E. coli strain TG1, overnight culture

METHOD

1. Prepare the single-stranded bacteriophage M13 template as described in Chapter 13, Protocol 1. Purify the uracil-containing template by spun-column chromatography.
2. Phosphorylate the mutagenic oligonucleotide and the universal sequencing primer with bacteriophage T4 polynucleotide kinase. In separate microfuge tubes mix:

synthetic oligonucleotide	100-200 pmoles
10x bacteriophage T4 polynucleotide kinase buffer	2 µl
10 mM ATP	1 µl
bacteriophage T4 polynucleotide kinase	4 units
H_2O	to 20 µl

   Incubate the reactions for 1 hour at 37°C and then heat them for 10 minutes at 68°C to inactivate the polynucleotide kinase.
3. Anneal the phosphorylated mutagenic oligonucleotide and universal sequencing primer to the single-stranded bacteriophage M13 DNA containing the target sequence. Mix:

single-stranded template DNA (approx. 1 µg)	0.5 pmole
phosphorylated mutagenic oligonucleotide	10 pmoles
phosphorylated universal primer	10 pmoles
10x PE1 buffer	1 µl
H_2O	to 10 µl

   Heat the mixture for 5 minutes to 20°C above the theoretical T_m of a perfect hybrid formed by the mutagenic oligonucleotide, calculated from the formula $T_m = 4(G+C) + 2(A+T)$, where (G+C) = the sum of G and C residues in the

oligonucleotide and where (A+T) = the sum of the A and T residues in the oligonucleotide. Transfer the tube containing the reaction mixture to a beaker containing H_2O at 20°C above the T_m. Stand the beaker on the bench, and allow the reaction to cool slowly to room temperature (approx. 20 minutes). Centrifuge the tube briefly (5 seconds) in a microfuge to collect any fluid that has condensed on the walls of the tube.

4. While the annealing reaction cools to room temperature, mix the following reagents in a fresh 0.5-ml microfuge tube:

10x PE2 buffer	1.0 µl
2 mM dNTP solution	1.0 µl
10 mM ATP	1.0 µl
bacteriophage T4 DNA ligase	5 Weiss units
Klenow fragment	2.5 units
H_2O	to 10 µl

Store the mixture on ice until needed.

5. Add 10 µl of the ice-cold reaction mixture from Step 4 to the reaction mixture containing single-stranded DNA and annealed oligonucleotides (Step 3). Incubate the final reaction mixture for 6-15 hours at 16°C.
6. Transfect competent *E. coli* of an appropriate host strain (e.g., TG1) as follows:
   a. Prepare a series of dilutions of the reaction mixture (1:10, 1:100, and 1:500) in 10 mM Tris-Cl (pH 7.6).
   b. To a series of chilled (0°C) Falcon 2059 tubes, transfer 1 µl and 5 µl of (i) the original reaction mixture and (ii) each dilution. Add 200 µl of a preparation of competent TG1 cells to each tube.
   c. Store the mixtures on ice for 30 minutes, and then transfer them for exactly 2 minutes to a water bath equilibrated at 42°C.
   d. Remove the transfected cultures from the water bath, and add 100 µl of a standard overnight culture of TG1 cells. The addition of cells makes it easier to see bacteriophage M13 plaques in the lawn of bacterial cells.
   e. Add 2.5 ml of 2x YT top agar (melted and cooled to 47°C) to each tube, and plate the resulting mixtures on separate YT agar plates. Incubate the plates for 12-16 hours at 37°C to allow plaques to form.
7. Screen plaques by sequencing preparations of single-stranded bacteriophage DNA (please see Chapter 12, Protocol 3 or Chapter 12, Protocol 4). If necessary, the plaques can be screened by hybridization with a radiolabeled oligonucleotide probe to detect mutants that arise at a low frequency (Chapter 13, Protocol 7).

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2. Kunkel T.A., Bebenek K., and McClary J. 1991. Efficient site-directed mutagenesis using uracil-containing DNA. *Methods Enzymol.* 204:125-139.
3. Zoller M.J. and Smith M. 1987. Oligonucleotide-directed mutagenesis: A simple method using two oligonucleotide primers and a single-stranded DNA template. *Methods Enzymol.* 154:329-350.
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Protocol 3

In Vitro Mutagenesis Using Double-stranded DNA Templates: Selection of Mutants with *DpnI*

Two oligonucleotides are used to prime DNA synthesis catalyzed by a high-fidelity thermostable polymerase on a denatured plasmid template. The two oligonucleotides both contain the desired mutation and occupy the same starting and ending positions on opposite strands of the plasmid DNA. During several rounds of thermal cycling, both strands of the plasmid DNA are amplified in a linear fashion, generating a mutated plasmid containing staggered nicks on opposite strands.
The products of the linear amplification reaction are treated with the restriction enzyme *Dpn*I, which specifically cleaves fully methylated $G^{Me6}ATC$ sequences. *Dpn*I will therefore digest the bacterially generated DNA used as template for amplification but will not digest DNA synthesized during the course of the reaction in vitro. *Dpn*I-resistant molecules, which are rich in the desired mutants, are recovered by transforming *E. coli* to antibiotic resistance.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (10 mM)
dNTP solution containing all four dNTPs, each at 5 mM
10x Long PCR buffer (when using mixtures of DNA polymerases)
10x Mutagenesis buffer
Mutagenesis buffer is used in place of Long PCR buffer with DNA polymerases such as Pfu.
⚠ NaOH (1 M)/EDTA (1 mM) (optional)
Sodium acetate (3 M, pH 4.8) (optional)
This solution is used as a neutralizing agent and therefore has a slightly lower pH than most sodium acetate solutions used in molecular cloning. Adjust the pH to 4.8 with glacial acetic acid.
TE (pH 8.0)

Enzymes and Buffers

Bacteriophage T4 DNA ligase (optional)
10x Bacteriophage T4 DNA ligase buffer (optional)
Bacteriophage T4 polynucleotide kinase (optional)
10x Bacteriophage T4 polynucleotide kinase buffer (optional)
DpnI restriction endonuclease
Themostable DNA polymerase (e.g., *Pfu* DNA polymerase)
The conditions described in this protocol are optimized for Pfu*Turbo DNA polymerase. However, they are easly adapted for use with other thermostable polymerases or mixtures of polymerases.*

Nucleic Acids and Oligonucleotides

Oligonucleotide primers
For advice on the design of oligonucleotide primers, please see the introduction to this protocol and the information panel on Mutagenic Oligonucleotides in the print version of the manual. The best results are achieved if the oligonucleotide primers are purified by FPLC or PAGE to reduce the level of contamination with salts (please see Chapter 10, Protocol 1 or Chapter 10, Protocol 5). The purified primers are dissolved in H_2O at a concentration of 20 mM.
Plasmid DNA
The template DNA used for mutagenesis is a circular plasmid containing the gene or cDNA of interest. In general, the shorter the plasmid, the more efficient the amplification of the target DNA. The plasmid DNA should be dissolved at 1 µg/ml in 1 mM Tris-Cl (pH 7.6) containing a low concentration of EDTA (<0.1 mM).

Additional Reagents

Step 14 of this protocol requires the reagents listed in Chapter 1, Protocol 23.
Step 15 of this protocol requires the reagents listed in Chapter 1, Protocol 1.
Step 16 of this protocol requires the reagents listed in Chapter 12, Protocol 3, Chapter 12, Protocol 4, or Chapter 12, Protocol 5.

Vectors and Bacterial Strains

Competent *E. coli* strain with an *hsdR17* genotype (e.g., XL1-Blue, XL2-Blue MRF´, or DH5α)

METHOD

1. Denature the plasmid DNA template in a reaction containing 1-10 µg of plasmid DNA dissolved in 40 µl of H_2O plus 10 µl of 1 M NaOH/1 mM EDTA. Incubate the DNA in the denaturing solution for 15 minutes at 37°C.
2. Add 5 µl of 3 M sodium acetate (pH 4.8) to neutralize the solution. Precipitate the DNA with 150 µl of ice-cold ethanol.
3. Collect the denatured plasmid DNA by centrifugation for 10 minutes at 4°C in a microfuge. Carefully decant the ethanolic supernatant and rinse the pellet with 150 µl of 70% ethanol. Recentrifuge for 2 minutes, decant the supernatant, and allow the last traces of ethanol to evaporate at room temperature. Resuspend the DNA in 20 µl of H_2O.
4. In sterile 0.5-ml microfuge tubes, set up a series of reaction mixtures containing different amounts (e.g., 5, 10, 25, and 50 ng) of plasmid DNA and a constant amount of each of the two oligonucleotide primers.

10x mutagenesis buffer	5 µl
template plasmid DNA	5-50 ng
oligonucleotide primer 1 (20 mM)	2.5 µl
oligonucleotide primer 2 (20 mM)	2.5 µl
dNTP mix	2.5 µl
H_2O	to 50 µl

   Add 2.5 units of *Pfu*Turbo DNA polymerase.
   It is important to add the reagents in the order shown.
5. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil or a bead of paraffin wax to prevent evaporation of the samples during repeated cycles of heating and cooling. Place the tubes in the thermal cycler.
6. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
1 cycle	1 min at 95°C		
2-18 cycles[a]	30 sec at 95°C	1 min at 55°C	2 min/kb of plasmid DNA at 68°C
Last cycle	1 min at 94°C	1 min at 55°C	10 min at 72°C

Times and temperatures may need to be adapted to suit the particular reaction conditions.

[a]*For single-base substitutions, use 12 cycles of linear amplification; for substitution of one amino acid with another (usually two or three contiguous base substitutions), use 16 cycles; for insertions and deletions of any size, use 18 cycles.*

The rate of DNA synthesis is 1.5-2.0 times slower in amplification reactions catalyzed by Pfu *than in reactions catalyzed by* Taq.

7. After amplification of the DNA, place the reactions on ice.
8. Verify that the target DNA was amplified by analyzing 10 µl of each reaction by electrophoresis through a 1% agarose gel containing 0.5 µg/ml ethidium bromide. As standards, load 50 ng of unamplified linearized plasmid DNA and a 1-kb DNA ladder into the outer lanes of the gel.
9. Extract the amplified DNAs twice with phenol:chloroform and precipitate with ethanol.

   Steps 9-12 are optional and are generally used only when the efficiency of mutagenesis is expected to be low (e.g., when constructing insertions and deletions).
10. Resuspend the DNA pellets in the following:

10x bacteriophage T4 polynucleotide kinase buffer	5 µl
10 mM ATP	5 µl
bacteriophage T4 polynucleotide kinase	5 units
H_2O	to 50 µl

   Incubate the reactions for 1 hour at 37°C. Inactivate the kinase enzyme by heating at 68°C for 10 minutes. Extract the phosphorylated DNAs twice with phenol:chloroform and collect the DNAs by ethanol precipitation.
11. Resuspend the pellets of phosphorylated DNA (approx. 0.9 µg each) in 90 µl of TE. Set up a series of ligation reactions containing the phosphorylated DNAs at concentrations ranging from 0.1 to 1 µg/ml.

phosphorylated DNA	(10 ng to 100 ng)
10x bacteriophage T4 DNA ligase buffer	10 µl
10 mM ATP	10 µl
bacteriophage T4 DNA ligase	4 units
H_2O	to 100 µl

    Incubate the reactions for 12-16 hours at 16°C.
12. Extract the ligated DNAs twice with phenol:chloroform and collect the DNA by ethanol precipitation. Resuspend each pellet in 45 µl of H_2O. Add 5 µl of 10x *Dpn*I buffer to each tube.
13. Digest the amplified DNAs by adding 10 units of *Dpn*I directly to the remainder of the amplification reactions (Step 7) or to the phosphorylated and ligated DNAs (Step 12). Mix the reagents by pipetting the solution up and down several times, centrifuge the tubes for 5 seconds in a microfuge, and then incubate them for 1 hour at 37°C.
14. Transform competent *E. coli* with 1, 2, and 5 µl of digested DNA according to the procedure described in Chapter 1, Protocol 23.
15. Prepare plasmid DNA from at least 12 independent transformants. Screen the DNA preparations for mutations by DNA sequencing, by oligonucleotide hybridization (see Chapter 13, Protocol 7), or by restriction digestion of small preparations of plasmid DNA if a site was created or destroyed by the introduced mutation, or if an insertion or deletion was introduced into the template.
16. Sequence the entire segment of target DNA to verify that the desired mutation has been generated and that no spurious mutations occurred during amplification (please see Chapter 12, Protocol 3, Chapter 12, Protocol 4, or Chapter 12, Protocol 5).

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2. Weiner M.P. and Costa G.L. 1994. Rapid PCR site-directed mutagenesis. *PCR Methods Appl.* 4:S131-136.

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Protocol 4

Oligonucleotide-directed Mutagenesis by Elimination of a Unique Restriction Site (USE Mutagenesis)

Two oligonucleotide primers are hybridized to the same strand of a denatured double-stranded recombinant plasmid. One primer (the mutagenic primer) introduces the desired mutation into the target sequences, and the second primer carries a mutation that destroys a unique restriction site in the plasmid. Both primers are elongated in a reaction catalyzed by bacteriophage T4 or T7 DNA polymerase. Nicks in the strand of newly synthesized DNA are sealed with bacteriophage T4 DNA ligase. The product of the first part of the method is a heteroduplex plasmid consisting of a wild-type parental strand and a new full-length strand that carries the desired mutation but no longer contains the unique restriction site.
Residual wild-type plasmid molecules are then linearized by digestion with the appropriate restriction enzyme. The mixture of circular heteroduplex DNA and linear wild-type DNA is then used to transform a strain of *E. coli* that is deficient in repair of mismatched bases. Because linear DNA transforms 10-1000-fold less efficiently than circular DNA, many of the wild-type molecules are unable to reestablish themselves in *E. coli*. The circular heteroduplex molecules, however, begin to replicate. Because the mismatched bases are not repaired, the first round of replication generates a wild-type plasmid that carries the original restriction site and a mutated plasmid that does not. DNA from the first set of transformants is recovered, digested once more with the same restriction enzyme to linearize the wild-type molecules, and then used to transform a standard laboratory strain of *E. coli*. This biochemical selection can be sufficiently powerful to ensure that a high proportion of the resulting transformants carry the desired mutation.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Annealing buffer (13-4)
- 10x Synthesis buffer

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Bacteriophage T4 DNA polymerase or Sequenase

Unique site restriction endonuclease

Nucleic Acids and Oligonucleotides

Mutagenic primer

Selection primer
Both mutagenic and selection primers must anneal to the same strand of the target DNA, and the 5´ end of each primer must be phosphorylated. The oligonucleotide primers should be purified by FPLC or PAGE before use (please see Chapter 10, Protocol 1 or Chapter 10, Protocol 5).
Several companies (CLONTECH, Pharmacia) sell selection primers and some companies (e.g., Pharmacia) also market pairs of "toggle" primers that are used for forward and reverse conversion of restriction sites and allow sequential rounds of mutagenesis without subcloning of the template.

Plasmid DNA
Closed circular plasmid DNA, purified either by chromatography through a commercial resin or by the alkaline lysis method (please see Chapter 1, Protocol 2 or Chapter 1, Protocol 9).

Media

- LB agar plates containing the appropriate antibiotic
- LB medium containing the appropriate antibiotic

Additional Reagents

Steps 7 and 14 of this protocol require the reagents listed in Chapter 1, Protocol 23, Chapter 1, Protocol 24, or Chapter 1, Protocol 26 for the transformation of *E. coli*.

Steps 10 and 16 of this protocol require the reagents listed in Chapter 1, Protocol 1 for the minipreparation of plasmid DNA.

Step 17 of this protocol requires the reagents listed in Chapter 12, Protocol 3, Chapter 12, Protocol 4, or Chapter 12, Protocol 5.

Vectors and Bacterial Strains

E. coli strain with a *mutS* genotype (e.g., BMH 71-18) competent for transformation

E. coli strain with a *mut*$^+$ phenotype competent for transformation
Please see Step 14.

METHOD

1. Mix the following components in a microfuge tube:

10x annealing buffer (13-4)	2 µl
plasmid DNA	0.025 to 0.25 pmole
selection primer	25 pmoles
mutagenic primer	25 pmoles
H_2O	to 20 µl

   Incubate the tube in a boiling water bath for 5 minutes.
2. Immediately chill the tube in ice for 5 minutes. Centrifuge the tube for 5 seconds in a microfuge to deposit the fluid at the base.
3. To the tube of annealed primers and plasmid, add:

10x synthesis buffer	3 µl
bacteriophage T4 DNA polymerase (2-4 units/µl)	1 µl
bacteriophage T4 DNA ligase (4-6 units/µl)	1 µl
H_2O	5 µl

   Mix the reagents well by gentle up and down pipetting. Centrifuge the tube for 5 seconds in a microfuge to deposit the fluid at the base. Incubate the reaction for 1-2 hours at 37°C.
4. Stop the reaction by heating the tube for at least 5 minutes at 70°C to inactivate the enzymes. Store the tube on the bench to allow it cool to room temperature.
5. Adjust the NaCl concentration of the reaction to a level that is optimal for the selected unique site restriction

endonuclease. Use the 10x annealing buffer, a stock of NaCl, or the 10x buffer supplied with the restriction enzyme.

6. Add 20 units of the selective restriction endonuclease to the reaction mixture. Incubate the reaction for at least 1 hour at the appropriate digestion temperature.
   IMPORTANT *The volume of enzymes added to the reactions (including polymerase and ligase) should not exceed 10% of the total reaction volume. Adjust the reaction volume accordingly.*
7. Transform a *mutS E. coli* strain such as BMH 71-18 with the plasmid DNAs contained in the digestion mixture, using one of the transformation procedures described in Chapter 1, Protocol 23 to Chapter 1, Protocol 26.
8. Spread 10, 50, and 250 µl of the transformation mixture onto LB agar plates containing the appropriate antibiotic. Incubate the plates overnight at 37°C. Carry out Step 9 while the plates are incubating.
9. Amplify the plasmids by adding the remaining transformation mixture to 3 ml of LB medium containing the appropriate antibiotic. Incubate the culture overnight at 37°C with shaking.
10. The next day, prepare plasmid DNA from approx. 2.5 ml of the overnight culture (please see Chapter 1, Protocol 1).
11. Digest the plasmid DNA prepared in Step 10 with the selective restriction enzyme:

plasmid DNA	500 ng
10x restriction enzyme buffer	2 µl
unique site restriction endonuclease	20 units
H_2O	to 20 µl

    Incubate the reaction for 2 hours at the appropriate temperature.
12. Add an additional 10 units of the restriction enzyme, and incubate for at least 1 further hour.
13. Assess the extent of digestion by running 5-10 µl of the plasmid DNA on a 1% agarose gel containing 0.5 µg/ml ethidium bromide.
14. Transform competent *mutS*$^+$ *E. coli* cells with either 2-4 µl of the digested plasmid DNA (approx. 50-100 ng) for transformation of chemically treated competent cells or 1 µl of plasmid DNA diluted fivefold with sterile H_2O (approx. 5 ng) for transformation by electroporation (please see Chapter 1, Protocol 23 to Chapter 1, Protocol 26).
15. Spread 10, 50, and 250 µl of the transformation mixture onto LB agar plates containing the appropriate antibiotic. Incubate the plates overnight at 37°C.
16. The next day, prepare minipreparations of plasmid DNA from at least 12 independent transformants. Screen the preparations by restriction endonuclease digestion and agarose gel electrophoresis to identify plasmids that are resistant to cleavage by the selective restriction enzyme.
17. Use DNA sequencing to confirm that the plasmids contain the desired mutation (please see Chapter 12, Protocol 3, Chapter 12, Protocol 4, or Chapter 12, Protocol 5).

REFERENCES

1. Deng W.P. and Nickoloff J.A. 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal. Biochem.* 200:81-88.
2. Zhu L. 1996. In vitro site-directed mutagenesis using the unique restriction site elimination (USE) method. *Methods Mol. Biol.* 57:13-29.

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Protocol 5

Rapid and Efficient Site-directed Mutagenesis by the Single-tube Megaprimer PCR Method

The "megaprimer" method of site-directed mutagenesis introduced by Kammann et al. (1989) uses three oligonucleotide primers and two rounds of PCR. One of the oligonucleotides is mutagenic, the other two being forward and reverse primers that lie upstream and downstream from the binding site for the mutagenic oligonucleotide. The mutagenic primer and the nearer of the external primers are used in the first PCR to generate and amplify a mutated fragment of DNA. This amplified fragment - the megaprimer - is used in the second PCR in conjunction with the remaining external primer to amplify a longer region of the template DNA.
This protocol is based on a method developed by Ke and Madison (1997), using forward and reverse external primers with significantly different melting temperatures (T_m). This eliminates the necessity of purifying the template DNA between the two rounds of PCR.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- dNTP solution containing all four dNTPs, each at a 2.5 mM

Enzymes and Buffers

Thermostable DNA polymerase (Hot-Tub DNA Polymerase [Amersham] or equivalent)
Most thermostable DNA polymerases are supplied in a storage buffer containing 50% glycerol. This solution is very viscous and is difficult to pipette accurately. The best sampling method is to centrifuge the tube containing the enzyme at maximum speed for 10 seconds at 4°C in a microfuge and then to withdraw the required amount of enzyme using a positive-displacement pipette. Use automatic pipetting devices equipped with barrier tips to assemble the components of PCRs.

Nucleic Acids and Oligonucleotides

Primers
Dissolve the forward and reverse external primers in H_2O at a concentration of 100 µM (100 pmoles/µl).
Dissolve the mutagenic primer in H_2O at a concentration of 10 µM (10 pmoles/µl).

Template DNA
Superhelical, double-stranded plasmid DNA, purified by ethidium bromide-CsCl density gradient centrifugation (Chapter 1, Protocol 10 or Chapter 1, Protocol 11) or chromatography on a Qiagen resin (Chapter 1, Protocol 9).
Dissolve the template DNA in TE (pH 8.0) at a concentration of 0.1 µg/ml.

Additional Reagents

Step 6 of this protocol requires the reagents listed in Chapter 1, Protocol 17 or Chapter 1, Protocol 19.

METHOD

1. In a sterile 0.5-ml microfuge tube or amplification tube (on ice), mix the following reagents for the first amplification reaction:

10x amplification buffer	10 µl
plasmid template DNA	200-400 pg
2.5 mM dNTP solution	8 µl
mutagenic primer	10 pmoles
low T_m flanking primer	100 pmoles
thermostable DNA polymerase	0.5 µl (2.5 units)
H_2O	to 100 µl

If the 10x amplification buffer supplied by the manufacturer of the thermostable DNA polymerase does not contain $MgCl_2$, add an appropriate volume of 0.1 M $MgCl_2$ so that the reaction mixture contains the optimum concentration of divalent ion for the particular DNA polymerase being used.
2. If the thermocycler does not have a heated lid, overlay the PCRs with 1 drop (approx. 50 µl) of light mineral oil. Place the tubes in a thermocycler.
3. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

Cycle Number	Denaturation	Annealing	Polymerization
1st cycle	4 min at 94°C	1 min sec at 42-46°C	1 min at 72°C
24 cycles	40 sec at 94°C	1 min sec at 42-46°C	1 min at 72°C
Last cycle	40 sec at 94°C	1 min sec at 42-46°C	5 min at 72°C

Times and temperatures may need to be adapted to suit the particular reaction conditions.
Polymerization should be carried out for 1 minute for every 1000 bp of length of the target DNA.
4. After completion of the first PCR, add to the reaction tube:

high T_m flanking primer	100 pmoles
thermostable DNA polymerase	0.5 µl (2.5 units)
2.5 mM dNTP solution	3 µl

Mix the reagents gently by pipetting the reaction mixture up and down several times. If necessary, centrifuge the tube briefly to deposit the reagents in the bottom.
5. Carry out the second amplification reaction, which consists of 25 cycles with the following two-step temperature profile:
94°C for 40 seconds
72°C for 90 seconds with a final extension step for 5 minutes at 72°C
6. Analyze 5% of the second amplification reaction on an agarose or polyacrylamide gel and estimate the concentration of amplified target DNA.

REFERENCES

1. Kammann M., Laufs J., Schell J., and Gronenborn B. 1989. Rapid insertional mutagenesis of DNA by polymerase chain reaction (PCR). *Nucleic Acids Res.* 17:5404.
2. Ke S.H. and Madison E.L. 1997. Rapid and efficient site-directed mutagenesis by single-tube "megaprimer" PCR

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Protocol 6

Site-specific Mutagenesis by Overlap Extension

Four primers and three PCRs are used to create a site-specific mutation by overlap extension. One pair of primers is used to amplify DNA that contains the mutation site together with upstream sequences. The second pair of primers is used in a separate PCR to amplify DNA that contains the mutation site together with downstream sequences. The mutation(s) of interest is located in the region of overlap and therefore in both amplified fragments. The overlapping fragments are mixed, denatured, and annealed to generate heteroduplexes that can be extended and, in a third PCR, amplified into a full-length DNA using two primers that bind to the extremes of the two initial fragments.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- dNTP solution containing all four dNTPs, each at 20 mM

Enzymes and Buffers

Thermostable DNA polymerase
To avoid the introduction of erroneous bases, use a highly processive thermostable DNA polymerase with 3´-5´ exonuclease "proofreading" capacity in overlap extension mutagenesis. In addition, the DNA polymerase used must not catalyze the nontemplate addition of adenine residues. Thermostable DNA polymerases with the appropriate properties include Pwo *DNA polymerase (Boehringer Mannheim),* rTth *DNA polymerase XL (Perkin-Elmer),* VentR *DNA polymerase (New England Biolabs), and* Pfu *DNA polymerase (Stratagene).*
Mixes of thermostable DNA polymerases designed for use in long PCR are also well suited for overlap extension mutagenesis.

Nucleic Acids and Oligonucleotides

Oligonucleotide Primers
Each primer should be 20-30 nucleotides in length and contain approximately equal numbers of the four bases, with a balanced distribution of G and C residues and a low propensity to form stable secondary structure. The sequences of the mutagenic oligonucleotide primers FM and RM should share at least a 15-bp overlap, and the mismatched base pairs within these primers should be located in the center of the oligonucleotide. The primer sequences at the 5´ and 3´ ends of the DNA fragment to be amplified (i.e., those with wild-type sequences R2, F2) can incorporate unique restriction endonuclease cleavage sites to aid in the subsequent cloning of the mutagenized DNA fragment. For general features of primer design, please see the discussion on Design of Oligonucleotide Primers in the introduction to Chapter 10 in the print version of the manual.
Oligonucleotide primers synthesized on an automated DNA synthesizer can generally be used in overlap extension mutagenesis without further purification.

Template DNA
The template DNA used for mutagenesis is usually a plasmid DNA containing the gene or cDNA of interest. Dissolve the DNA at 1 µg/ml in 10 mM Tris-Cl (pH 7.6) containing a low concentration of EDTA (<0.1 mM).

Additional Reagents

Step 11 of this protocol requires the reagents listed in Chapter 1, Protocol 17 or Chapter 1 Protocol 19.

Step 12 of this protocol requires the reagents listed in Chapter 12, Protocol 3, Chapter 12, Protocol 4, or Chapter 12, Protocol 5.

METHOD

1. Design and synthesize oligonucleotide primers FM, RM, R2, and F2 based on the known sequence of the DNA, as outlined in the protocol introduction and Materials section.
2. In a sterile 0.5-ml microfuge tube or amplification tube, set up PCR 1 by mixing the following reagents:

template DNA	approx. 100 ng
10x amplification buffer	10 µl
20 mM mixture of four dNTPs	1.0 µl
5 µM primer RM (30 pmoles)	6.0 µl
5 µM primer F2 (30 pmoles)	6.0 µl
thermostable DNA polymerase	1-2 units
H_2O	to 100 µl

3. In a second sterile 0.5-ml microfuge tube or amplification tube, set up PCR 2 by mixing the following reagents:

template DNA	approx. 100 ng
10x amplification buffer	10 µl
20 mM mixture of four dNTPs	1.0 µl
5 µM primer FM (30 pmoles)	6.0 µl
5 µM primer R2 (30 pmoles)	6.0 µl
thermostable DNA polymerase	1-2 units
H_2O	to 100 µl

4. If the thermocycler does not have a heated lid, overlay the PCRs with 1 drop (approx. 50 µl) of light mineral oil. Place the tubes in a thermocycler.
5. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table below.

Cycle Number	Denaturation	Annealing	Polymerization
20 cycles	1 min at 94°C	1 min at 50°C	1-3 min at 72°C
Last cycle	1 min at 94°C	10 min at 72°C	

Times and temperatures may need to be adapted to suit the particular reaction conditions.
The length of the polymerization step should be calculated from the polymerization rate of the thermostable DNA polymerase employed and the length of the DNA template to be amplified.
The temperature of the annealing reaction may have to be adjusted depending on the sequence of the mutagenic primers.
6. Analyze 5% of each of the two PCRs on an agarose or polyacrylamide gel and estimate the concentration of amplified target DNAs.
7. (*Optional*) Purify the two PCR products using one of the protocols described in Chapter 5. Including this purification step often increases the yield of the desired amplification product in Step 8 of the protocol and reduces the background

of spurious amplification products.

8. In a sterile 0.5-ml microfuge tube or amplification tube, mix the following reagents in an amplification reaction to join the 5′ and 3′ ends of the target gene:

amplification product PCR 1 (Step 2)	approx. 50 ng
amplification product PCR 2 (Step 3)	approx. 50 ng
10x amplification buffer	10 µl
5 µM primer F2 (30 pmoles)	6.0 µl
5 µM primer R2 (30 pmoles)	6.0 µl
thermostable DNA polymerase	1-2 units
H_2O	to 100 µl

9. If the thermocycler does not have a heated lid, overlay the PCRs with 1 drop (approx. 50 µl) of light mineral oil. Place the tubes in a thermocycler.
10. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed above in Step 5.
11. Analyze 5% of the PCR on an agarose or polyacrylamide acrylamide gel and estimate the concentration of amplified target DNA.
12. Verify the complete sequence of the amplified DNA fragment after cloning to ensure that no mutations other than those in primers FM and RM were introduced during these manipulations.

REFERENCES

1. Higuchi R., Krummel B., and Saiki R.K. 1988. A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. *Nucleic Acids Res.* 16:7351-7367.
2. Ho S.N., Hunt H.D., Horton R.M., Pullen J.K., and Pease L.R. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51-59.

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Protocol 7

Screening Recombinant Clones for Site-directed Mutagenesis by Hybridization to Radiolabeled Oligonucleotides

Plaques formed by M13 bacteriophages or bacterial colonies transformed by plasmids carrying specific mutations can be detected by hybridization, using a radiolabeled oligonucleotide that forms a perfect duplex with the mutant sequence. Hybridization is carried out under conditions of low stringency that allow the radiolabeled oligonucleotide to anneal to both mutant and wild-type DNAs. A hybrid between the radiolabeled oligonucleotide and the wild-type sequence will contain one or more mismatched base pairs, whereas the hybrid formed between the newly created mutant and the oligonucleotide probe will be perfectly matched. These two types of hybrids usually differ in their thermal stabilities, with mismatched hybrids dissociating at a lower temperature than the corresponding perfect hybrid. Plaques or colonies that continue to hybridize to the probe after several cycles of washing at progressively higher temperatures are likely to contain recombinants carrying the desired mutation.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ammonium formate (0.2 M)

Oligonucleotide hybridization solution (13-7)

Oligonucleotide prehybridization solution (13-7)

6x SSC
6x SSC should be warmed to various temperatures. Please see Steps 11, 12, and 13.

TE (pH 7.6)

Enzymes and Buffers

Bacteriophage T4 polynucleotide kinase

10x Bacteriophage T4 polynucleotide kinase buffer

Restriction enzymes
Please see Steps 18 and 19.

Nucleic Acids and Oligonucleotides

Mutagenic oligonucleotide (10 pmoles/µl)

Radioactive Compounds

⚠ [γ-^{32}P]ATP (>5000 Ci/mmole, 10 mCi/ml)

Media

YT agar plates

2x YT top agar

Additional Reagents

Step 4 of this protocol requires the reagents listed in Chapter 10, Protocol 4.

Step 14 of this protocol requires the reagents listed in Chapter 3, Protocol 4.

Steps 15 and 16 of this protocol require the reagents listed in Chapter 12, Protocol 3, Chapter 12, Protocol 4, or Chapter 12, Protocol 5.

Step 17 of this protocol requires the reagents listed in Chapter 3, Protocol 3.

Step 20 of this protocol requires the reagents listed in Chapter 6, Protocol 8 and Chapter 6, Protocol 10.

Vectors and Bacterial Strains

Bacteriophage M13 recombinants, previously mutagenized
Please use plates containing bacteriophage M13 plaques generated in Chapter 13, Protocol 2.

Bacteriophage M13 recombinants, nonmutagenized
These serve as a negative control; please see Step 5.

E. coli strain TG1 or equivalent

METHOD

1. In a sterile microfuge tube, mix:

mutagenic oligonucleotide (10 pmoles/µl)	1 µl
10x bacteriophage T4 polynucleotide kinase buffer	1 µl
10 mCi/ml [γ-^{32}P]ATP (10-50 pmoles)	1 µl
H_2O	6 µl
5-10 units/µl bacteriophage T4 polynucleotide kinase	1 µl

   Incubate the reaction mixture for 30 minutes at 37°C.
2. Dilute the reaction mixture to 100 µl by the addition of 90 µl of TE (pH 7.6) and inactivate the polynucleotide kinase by heating for 10 minutes at 68°C.
3. Measure the efficiency of transfer of ^{32}P to the oligonucleotide and estimate its specific activity by chromatography on DE-81 paper as follows:
   a. Cut a strip of Whatman DE-81 paper approx. 1 cm wide and 7-10 cm long. With a soft-lead pencil, draw a fine line across the strip approx. 1.5 cm from one end. This line marks the origin of the chromatogram.
   b. Spot 1.0 µl of the diluted phosphorylation reaction at the origin. Fill a 250-ml beaker to a depth of approx. 0.5 cm with approx. 25-50 ml of 0.2 M ammonium formate. Place the DE-81 strip vertically in a beaker so that the radioactive sample(s) at the origin is just above the buffer solution. Cover the beaker with a glass plate or aluminum foil and allow the chromatogram to develop until the solvent front has migrated almost to the top of the beaker.
   c. Wrap the strip of DE-81 paper in Saran Wrap and subject it to a very brief period of autoradiography. Use the developed X-ray film as a guide to cut out the radioactive region at the solvent front, the origin, and any other region that contains radioactivity. Measure the amount of radioactivity in each section in a scintillation counter.
4. (*Optional*) Remove unincorporated radiolabel from the oligonucleotide by precipitation with cetylpyridinium bromide as described in Chapter 10, Protocol 4.
   This step is necessary only when background hybridization is a persistent problem. Under normal circumstances, the unfractionated reaction mixture may be used as a probe.

5. Prepare replicas of the bacteriophage M13 plaques that are to be screened with the radiolabeled oligonucleotide as follows:
   a. Transfer plates containing 100-500 plaques to 4°C for at least 30 minutes.
   Include at least one plate that contains plaques of the original wild-type recombinant bacteriophage M13. This plate serves as a negative control in the hybridization and washing steps.
   b. When the plates are thoroughly chilled, remove them from the cold room and immediately lay a numbered, dry nitrocellulose or nylon filter on the agar surface of each plate. Use an 18-gauge hypodermic needle to make a series of holes in each filter and the underlying agar. These holes will later serve to key the filters to the plates.
   c. After 30 seconds to 4 minutes, use blunt-ended forceps to peel each filter carefully from its plate. Spread out all of the filters (plaque side up) on a pad of paper towels. Wrap the plates in Saran Wrap, and store them at 4°C until they are needed.
   d. When the filters have dried (approx. 30 minutes at room temperature), bake them for 1 hour at 80°C in a vacuum oven.
6. Transfer all of the filters to a heat-sealable plastic bag (e.g., Sears Seal-A-Meal), or to an evaporating dish of the appropriate diameter, or to a hybridization roller bottle. Add oligonucleotide prehybridization solution (13-7) (approx. 10 ml/82-mm filter in bags or dishes; 5 ml/82-mm filter in bottles). Seal the bag, cover the evaporating dish with Saran Wrap, or cap the roller bottle, and incubate the filters for 1-2 hours at 65°C.
7. Discard the prehybridization solution, and replace it with oligonucleotide hybridization solution (13-7) (approx. 5 ml/82-mm filter). Reseal the bag, recover the evaporating dish, or recap the hybridization roller bottle, and incubate the hybridization reaction for 4-6 hours at the appropriate temperature.
   Perform hybridization with the radiolabeled oligonucleotide at a temperature 5-10°C below the T_m *estimated from the following formula:*
   $T_m = 4(G+C) + 2(A+T)$
   where (G+C) is the sum of G and C residues in the oligonucleotide, and where (A+T) is the sum of the A and T residues in the oligonucleotide.
8. At the end of the hybridization period, quickly transfer the filters to a tray containing 200-300 ml of 6x SSC at room temperature. Cover the tray with Saran Wrap, and place it on a rotating shaker for 15 minutes. Replace the washing fluid every 5 minutes. Meanwhile, transfer the remainder of the radioactive hybridization solution from the bag, dish, or roller bottle to a disposable plastic tube. Close the tube tightly, and store the radioactive solution at -20°C until it is needed for rescreening positive plaques (Step 13).
9. At the end of the washing period, quickly transfer the filters to a piece of Saran Wrap stretched on the bench. Cover the filters with another piece of Saran Wrap. Fold the edges of the two pieces of Saran Wrap together to form a tight seal. Apply adhesive dot labels marked with radioactive or chemiluminescent ink to the outside of the package, and generate an autoradiograph by exposing the package of filters to X-ray film for 1-2 hours at -70°C, using an intensifying screen.
   IMPORTANT *Do not allow the filters to dry on the Saran Wrap.*
10. Compare the pattern of hybridization with the distribution of plaques. At this stage, it is normal to find that virtually every plaque hybridizes to the probe. Typically, however, some plaques hybridize more strongly than others, and these often turn out to be the plaques carrying the desired mutation.
11. Transfer the filters to a plastic box containing 100-200 ml of 6x SSC that has been warmed to 10°C below the T_m. Agitate the filters in the solution for 2 minutes (please see note below), and then transfer them to a piece of Saran Wrap as described in Step 9. Establish another autoradiograph. At this stage, it is often possible to identify two types of plaques: those whose radioactive signal has decreased in intensity and those that show no change in intensity.
12. Repeat the cycles of washing and autoradiography, increasing the temperature of the washing solution by 2-10°C in each cycle. The aim is to find a temperature that does not markedly affect perfect hybrids but causes dissociation of mismatched hybrids (such as those formed between the mutagenic oligonucleotide and the original wild-type sequence).
13. Positively hybridizing plaques usually contain a mixture of both mutant and wild-type sequences. It is therefore *essential* to plaque-purify the bacteriophages from positively hybridizing plaques as follows:
   a. Touch the blunt end of a sterile, disposable wooden toothpick to the surface of a positively hybridizing plaque.
   b. Drop the toothpick into a sterile tube containing 1 ml of sterile TE (pH 7.6). Store the tube for 10-15 minutes at room temperature, shaking it from time to time to dislodge bacteriophage particles.
   c. Make a series of tenfold dilutions of the bacteriophage suspension with TE (pH 7.6). Mix 10 µl of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions with 100-µl aliquots of an overnight culture of an appropriate strain of *E. coli* (e.g., TG1).
   d. Add 2.5 ml of 2x YT top agar (melted and cooled to 45°C) to each culture, and plate the entire mixture on a single YT agar plate. Incubate the plate for 16 hours at 37°C to allow plaques to form.
   e. Rescreen the plaques with the radiolabeled oligonucleotide as described in Steps 5-12. In this second round of screening, there is no need to increase the temperature of the washing solution in a stepwise fashion. Instead, the filters can be transferred directly from the washing solution at room temperature (Step 8) to 6x SSC previously warmed to the discriminatory temperature found empirically in Step 12.
14. Pick two plaques from each of three independent putative mutants. Prepare single-stranded DNA from small-scale cultures infected with bacteriophages derived from each of these plaques as described in Chapter 3, Protocol 4.
15. Carry out DNA sequencing by the dideoxy-mediated chain-termination method (please see Chapter 12, Protocol 3 or Chapter 12, Protocol 4) through the region containing the target sequence. Use either a universal sequencing primer or a custom-synthesized primer that binds 50-100 nucleotides upstream of the mutation site.
16. When the presence of the mutation has been confirmed, verify the sequence of the entire region of the target DNA cloned in the bacteriophage M13 vector to ensure that no adventitious mutations have been generated during propagation of the recombinant in bacteriophage M13. Often, this requires the synthesis of custom-designed sequencing primers that are complementary to segments of the target DNA spaced approx. 200-400 bp apart.
17. Isolate bacteriophage M13 replicative form DNA from a culture infected with plaque-purified recombinant bacteriophages (Step 14) that carry the desired mutation and show no other changes in sequence in the target region. For methods to isolate and purify bacteriophage M13 replicative form DNA, please see Chapter 3, Protocol 3.
18. Recover the mutated target sequence by digestion of bacteriophage M13 replicative form DNA with the appropriate restriction enzyme(s) and preparative gel electrophoresis. Clone the target DNA into the desired vector.
19. Use several different restriction enzymes to digest aliquots of either a recombinant that carries the original (nonmutagenized) target sequence or the recombinant that carries the mutagenized target sequence.
20. Separate the resulting fragments by gel electrophoresis, and transfer them to a solid support (e.g., nitrocellulose or nylon membrane) as described in Chapter 6, Protocol 8. Carry out Southern hybridization at 10°C below the T_m, using the ^{32}P-labeled mutagenic oligonucleotide as a probe. Wash the filter under the discriminatory conditions and establish an autoradiograph.
   The final autoradiograph should show hybridization only to the relevant fragments of the mutagenized target DNA.

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2. Zoller M.J. and Smith M. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Methods Enzymol.* 100:468-500.

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Protocol 8

Detection of Mutations by Single-strand Conformational Polymorphism

Single-strand conformational polymorphism (SSCP), one of several methods used to scan segments of DNA for mutations, exploits the electrophoretic differences in mobilities between single-stranded mutant and wild-type DNAs.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 10x Amplification buffer
- dNTP solution (PCR grade) containing all four dNTPs, each at a concentration of 1 mM (pH 7.0)
- ⚠ Formamide loading buffer
- 6x Gel-loading buffer I
- 10x TBE electrophoresis buffer
  Use TBE at a working strength of 1x (89 mM Tris-borate, 2 mM EDTA) for polyacrylamide gel electrophoresis.

Enzymes and Buffers

Restriction enzymes (optional)

Thermostable DNA polymerase
Taq *DNA polymerase is recommended.*

Nucleic Acids and Oligonucleotides

Human genomic DNA to be screened for point mutations
Dissolve the DNA at 10 µg/ml in TE (pH 7.6).

Oligonucleotide primers, forward and reverse (35 µM each) in TE (pH 7.6)

Radioactive Compounds

⚠ [α-^{32}P]dCTP (3000 Ci/mmole, 10 mCi/ml)

METHOD

1. In a sterile 0.5-ml microfuge tube, mix in the following order:

1 mM dNTP solution	1 µl
10x amplification buffer	2 µl
35 µM 5'-oligonucleotide solution	1 µl (35 pmoles)
35 µM 3'-oligonucleotide solution	1 µl
10 µCi/µl [α-^{32}P]dCTP	1 µl
human genomic DNA	10 µl (100 ng)
thermostable DNA polymerase	1-3 units
H_2O	to 20 µl

   [α-^{32}P]dCTP is incorporated in the PCRs to label the amplified DNA uniformly. ^{32}P-labeled oligonucleotide primers can be used in place of [α-^{32}P]dCTP to produce an end-labeled DNA.
   If possible, set up control reactions using two DNA samples known to contain alleles that differ in sequence by one or more base pairs and that are known to resolve on SSCP gels. In addition, set up a contamination control in which no template DNA is added to the reaction.
2. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil to prevent evaporation of the samples during repeated cycles of heating and cooling. Alternatively, place a bead of paraffin wax into the tube if using a hot start protocol. Place the tubes in the thermal cycler.
3. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table. For advice on thermal cycler programs, please see Chapter 8, Protocol 1.

Cycle Number	Denaturation	Annealing/Polymerization
30 cycles	5-30 sec at 94°C	0.5-1 min at 68°C
Last cycle	1 min at 94°C	5-10 min at 68°C

   Times and temperatures may need to be adapted to suit the particular reaction conditions.
4. While the thermal cycler program is running, prepare a 5.5% polyacrylamide gel containing 10% (v/v) glycerol in 1x TBE gel buffer.

10x TBE gel buffer	10 ml
29:1% acrylamide:bisacrylamide solution	18 ml
10% ammonium persulfate	0.5 ml
glycerol	10 ml
H_2O	61.5 ml

   Mix the reagents by gentle swirling or stirring.
   This volume of gel solution is sufficient for one polyacrylamide gel of standard size (40 x 40-cm plates with 0.4-mm spacers). The volume of the gel solution can be increased or decreased as needed for other gel sizes.
   Use the same stock of 10x TBE gel buffer to prepare enough 1x TBE gel buffer to fill the tanks of the electrophoresis apparatus.
5. Assemble and tape together two 40 x 40-cm glass electrophoresis plates with 0.4-mm spacers.
   To obtain maximum resolution of single-stranded DNA conformers, it is important to use "thin-gel" spacers that are less than or equal to 0.4 mm in thickness.
6. Add 100 µl of TEMED to the gel solution. Mix the solution by gently swirling the flask, and pour the gel.
   Work quickly as the acrylamide solution will polymerize rapidly. For instructions on pouring thin gels, please see Chapter 12, Protocol 8.
7. Assemble the polymerized gel into an electrophoresis apparatus at room temperature. Fill the buffer tanks with 1x TBE gel buffer made from the same stock as the gel solution.
8. (*Optional*) If the amplified DNA fragment is to be digested with a restriction enzyme, remove the PCR tubes from the thermal cycler at the end of the run and place them on ice. Set up the following restriction enzyme digestion:

PCR solution	5 µl
10x restriction enzyme buffer	4 µl
restriction enzyme (2-50 units)	2 µl
H_2O	29 µl

   Incubate for 1-2 hours at the temperature appropriate for the restriction enzyme.

9. Dilute either 1.5 µl of the original PCR (from Step 3) or 5 µl of the restriction-enzyme-digested PCR (from Step 8) into 20 µl of sucrose gel-loading buffer. Dilute similar aliquots into 20 µl of formamide dye mix.
10. Boil the formamide-containing samples for 6 minutes, and then plunge the tubes directly into ice.
11. Use a Pasteur pipette or a Hamilton syringe to wash out the wells of the polyacrylamide gel with 1x TBE gel buffer. With a drawn-out glass capillary tube or a micropipettor equipped with a gel-loading tip, load 2 µl of each sample on the polyacrylamide gel.
12. Apply 6-7 V/cm (approx. 250 V [and 15 mA] for a 40 x 40-cm gel) to the gel for approx. 14 hours.
13. At the completion of electrophoresis, separate the glass plates, and transfer the gel to a sheet of Whatman 3MM filter paper. Dry the gel on a vacuum dryer for 30-60 minutes.
14. Subject the dried gel to autoradiography for 4-16 hours at room temperature without an intensifying screen.

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2. Orita M., Suzuki Y., Sekiya T., and Hayashi K. 1989. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 5:874-879.

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Protocol 9

Generation of Sets of Nested Deletion Mutants with Exonuclease III

The double-stranded DNA of recombinant plasmid, phagemid, or bacteriophage M13 replicative form DNA is digested with two restriction enzymes whose sites of cleavage both lie between one end of the target DNA and the binding site for universal primer. The enzyme that cleaves nearer the target sequence must generate either a blunt end or a recessed 3´ terminus; the other enzyme must generate a four-nucleotide protruding 3´ terminus. Because only the blunt or recessed 3´ terminus of the resulting linear DNA is susceptible to exonuclease III, digestion proceeds unidirectionally away from the site of cleavage and into the target DNA. The exposed single strands are then removed by digestion with nuclease S1 or mung bean nuclease, and the DNA is then recircularized. If desired, a synthetic linker can be inserted at the site of recircularization.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- dNTP solution containing dATP, dGTP, and dTTP, each at 0.5 mM in 25 mM Tris-Cl (pH 8.0)
  Ethanol (100%, ice cold, and 70%, room temperature)
- 10x Exonuclease III buffer
- Nuclease S1 stop mixture (13-9)
- ⚠ Phenol:chloroform
- Sodium acetate (3 M, pH 5.2)

Enzymes and Buffers

Exonuclease III
The quality of exonuclease III varies from manufacturer to manufacturer and should be checked in analytical digests before large-scale preparation of nested deletion templates.

Klenow mixture (sufficient for 30 samples)

H_2O	20 µl
1 M $MgCl_2$	6 µl
0.1 M Tris-Cl (pH 7.6)	3 µl
Klenow fragment	3 units

Prepare this mixture on ice just before use.

Ligase mixture (sufficient for 24 samples)

H_2O	550 µl
10x bacteriophage T4 ligation buffer	100 µl
5 mM rATP	100 µl
PEG 8000 (30% w/v)	250 µl
bacteriophage T4 DNA ligase	5 Weiss units

Prepare this mixture on ice just before use.

Nuclease S1 reaction mixture

H_2O	172 µl
10x S1 buffer	27 µl
Nuclease S1	60 units

Prepare this mixture just before use.
Mung bean nuclease can be substituted for nuclease S1 in this reaction mixture.

Restriction enzymes (two)

Nucleic Acids and Oligonucleotides

Target DNA
Analyze the sequence of the target DNA for the presence of suitable restriction sites. Clone the DNA to be digested into a plasmid or bacteriophage vector that contains as few nonessential sequences as possible. Analyze an aliquot of the preparation of recombinant plasmid DNA that is to be used as a substrate for mutagenesis by agarose gel electrophoresis in TAE buffer (please see Chapter 5, Protocol 1). The plasmid must be >90% superhelical molecules. Repurify the preparation if any linear or >10% nicked or relaxed plasmids are detected. Because exonuclease III will initiate digestion from single-strand nicks, it is important that the template DNA consist predominantly of closed circular molecules. Purification of the template has the added advantage of removing small pieces of DNA and RNA from the closed circular DNA preparation. These can interfere with digestion by exonuclease III.

Additional Reagents

Step 14 of this protocol requires the reagents listed in Chapter 1, Protocol 24, Chapter 1,Protocol 25, or Chapter 1, Protocol 26 (for transformation).

Step 15 of this protocol requires the reagents listed in Chapter 1, Protocol 1 or Chapter 1, Protocol 4 (for minipreparation of plasmid DNA) or in Chapter 3, Protocol 3 (for preparation of replicative form of M13 DNA).

Step 16 of this protocol requires the reagents listed in Chapter 12, Protocol 3, Chapter 12, Protocol 4, or Chapter 12, Protocol 5.

METHOD

1. Digest 10 µg of target DNA (recombinant bacteriophage M13 replicative form DNA, phagemid DNA, or plasmid DNA) with two restriction enzymes that cleave the polycloning site between the primer-binding site of the vector and the target DNA.
2. Purify the DNA by standard extraction with phenol:chloroform and precipitation with ethanol. Carefully remove the supernatant, and add 0.5 ml of 70% ethanol to the pellet.
   Rinsing the pellet with ethanol is important because sodium ions inhibit exonuclease III (Hoheisel 1993).
3. Recover the washed pellet of DNA by centrifuging at maximum speed for 2 minutes at 4°C in a microfuge, and then carefully remove the supernatant. Incubate the open tube on the bench to allow the last traces of ethanol to evaporate, and then dissolve the DNA in 60 µl of 1x exonuclease III buffer. Store the dissolved DNA on ice.
4. Place 7.5 µl of nuclease S1 reaction mixture in each of 25 0.5-ml microfuge tubes or in 25 wells of a 96-well microtiter

plate with U-shaped wells. Store the microtiter plate or microfuge tubes on a bed of ice.

5. Incubate the DNA solution prepared in Step 3 for 5 minutes at 37°C. Transfer 2.5 µl of the solution to the first microfuge tube or well of the microtiter plate containing the nuclease S1 reaction mixture.
6. To the remainder of the DNA solution, add 150 units of exonuclease III per pmole of recessed 3´ termini (1 unit of exonuclease III will generate 1 nmole of acid-soluble total nucleotide in 30 minutes at 37°C). Tap the tube to mix the contents and immediately return the tube to the 37°C water bath.
7. At 30-second intervals, remove 2.5-µl samples of the DNA solution and place them in successive microfuge tubes or wells of the microtiter plate containing the nuclease S1 reaction mixture.
8. When all of the samples have been harvested, incubate the microfuge tubes or microtiter plate containing the nuclease S1 and digested plasmid DNA for 30 minutes at 30°C.
9. Add 1 µl of nuclease S1 stop mixture (13-9) to each of the microfuge tubes or wells and incubate the reaction mixtures for 10 minutes at 70°C.
10. Transfer the microfuge tubes or microtiter plate to a bed of ice and analyze aliquots of each of the samples by agarose gel electrophoresis.
11. Pool the samples containing DNA fragments of the desired size. Add 1 µl of Klenow mixture for each 10 µl of pooled sample and incubate the reaction mixture for 5 minutes at 37°C.
12. For each 10 µl of pooled sample, add 1 µl of 0.5 mM dNTPs. Continue incubation for 15 minutes at room temperature.
13. Add 40 µl of T4 bacteriophage ligase mixture for each 10 µl of pooled sample. Mix and continue incubation for 2 hours at room temperature.
14. Transform the appropriate *E. coli* host with aliquots of the ligated DNA.
15. Prepare minipreparations of bacteriophage M13 replicative form DNA, plasmid, or phagemid DNA from at least 24 randomly selected plaques or colonies.
16. Linearize the DNAs by digestion with an appropriate restriction enzyme and analyze their sizes by electrophoresis through a 1% agarose gel. Include the original plasmid or bacteriophage DNA that has been linearized by restriction enzyme digestion as a marker. Choose clones of an appropriate size for sequencing (Chapter 12) or additional restriction enzyme mapping.

REFERENCES

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Protocol 10

Generation of Bidirectional Sets of Deletion Mutants by Digestion with BAL 31 Nuclease

In this method, the nuclease BAL 31 is used to make uni- or bidirectional deletions in a segment of cloned DNA. BAL 31 is a complex enzyme and tends to digest a population of double-stranded DNA targets in an asynchronous fashion, Deletions created by BAL 31 are therefore far more heterogeneous in size than those created by processive enzymes such as exonuclease III. **Warning!** BAL 31 is a tricky enzyme. This protocol is not for the technically challenged! Please see the information panel on BAL31 on page 13.68 in the print version of the manual.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 5x BAL 31 buffer (13-10)
- dNTP solution of all four dNTPs, each at 0.5 mM
- EGTA (0.5 M, pH 8.0)

Ethanol

- 6x Gel-loading buffer
- ⚠ Phenol:chloroform (1:1, v/v)
- Sodium acetate (3 M, pH 5.2)
- TE (pH 7.6)

Enzymes and Buffers

Bacteriophage T4 DNA polymerase

BAL 31 nuclease

Klenow fragment of *E. coli* DNA polymerase I

Restriction endonucleases

Please see Steps 3, 23, and 31.

Nucleic Acids and Oligonucleotides

Marker DNA for gel electrophoresis

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 1, Protocol 17 to Chapter 1, Protocol 19, or the reagents listed in Chapter 3, Protocol 6.

Step 2 of this protocol requires the reagents listed in Chapter 1, Protocol 9.

Step 27 of this protocol requires the reagents listed in Chapter 5, Protocol 4 to Chapter 5, Protocol 7.

Step 30 of this protocol requires the reagents listed in Chapter 1, Protocol 23 to Chapter 1, Protocol 26, or the reagents listed in Chapter 3, Protocol 6 or Chapter 3, Protocol 8.

Step 31 of this protocol requires the reagents listed in Chapter 1, Protocol 1, or the reagents listed in Chapter 3, Protocol 3.

Step 34 of this protocol requires the reagents listed in Chapter 12, Protocol 3, Chapter 12, Protocol 4, or Chapter 12, Protocol 5.

METHOD

1. Clone the target fragment into an appropriate plasmid or bacteriophage M13 vector.
   If deletion mutants are to be constructed from both termini of the target DNA, it will be necessary to clone the parental target DNA in both orientations with respect to the polycloning site in an appropriate vector.
2. Purify the closed circular recombinant DNA(s) by column chromatography on Qiagen columns (or their equivalent) and precipitation with ethanol. Redissolve the DNA in the smallest practical volume of Tris/EDTA.
   It is essential to use highly purified closed circular DNA (i) to minimize the contribution of contaminating RNA and small fragments of E. coli *chromosomal DNA to the total concentration of termini in the reaction and (ii) to eliminate nicked circular molecules, which are degraded by BAL 31 from the site of the nick.*
3. Digest 30 µg of the closed circular DNA to completion with a restriction endonuclease that cleaves at one end of the target DNA. This site defines the common point from which the nested deletions will begin. Use agarose gel electrophoresis to verify that digestion with the restriction endonuclease is complete.
4. Purify the DNA by extraction with an equal volume of phenol:chloroform. Separate the aqueous and organic phases by centrifugation at maximum speed for 3 minutes at 0°C in a microfuge, and then transfer the aqueous phase to a fresh microfuge tube.
5. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ice-cold ethanol. Store the tube for 10 minutes at 0°C, and then recover the DNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge.
6. Remove the supernatant, and wash the pellet of DNA carefully with 70% ethanol at room temperature. Dry the pellet at room temperature, and dissolve it in TE (pH 7.6) at a concentration of 1 µg/µl. Store the DNA at -20°C.
7. In a microfuge tube, mix:

linearized DNA (1 µg/µl)	4 µl
H_2O	48 µl
5x BAL 31 buffer (13-10)	13 µl

   Dispense 9 µl of this mixture into each of seven separate microfuge tubes.
   Because the ratio of fast and slow forms of BAL 31 varies from preparation to preparation, it is essential to assay the activity of the particular batch of enzyme that will be used to generate deletions.
8. Make a series of seven twofold dilutions of BAL 31 in 1x BAL 31 buffer (13-10). Enzyme dilution is best carried out by placing seven aliquots (2 µl) of 1x BAL 31 buffer (13-10) on the surface of a piece of Parafilm lying on a bed of ice or on a cold block. Use a disposable micropipette tip to mix 2 µl of the BAL 31 preparation under test with the first drop. Use a fresh tip to transfer 2 µl of the mixture to the next drop, and again mix. Continue in this fashion until the enzyme has been added to all of the drops. Working quickly, add 1 µl of each of the last six dilutions to six of the microfuge tubes containing the linear DNA being tested. Do not add enzyme to the seventh tube.
9. Incubate all of the microfuge tubes (including the tube that received no enzyme) for 30 minutes at 30°C.
10. Add 1 µl of 200 mM EGTA (pH 8.0) to each tube, and then heat the tubes for 5 minutes at 65°C.
11. Mix each of the samples with 3 µl of agarose gel-loading buffer and analyze the size of the DNAs by electrophoresis through a 0.8% agarose gel containing 0.5 µg/ml ethidium bromide.
12. Examine the gel by UV illumination, and determine the dilution of enzyme just sufficient to digest the DNA to the point where only a smear of small (200-bp) fragments is detectable. This dilution of BAL 31 will be used in the large-scale digestion (Step 15).

13. Mix:

linearized DNA (1 µg/µl)	20 µl
H_2O	240 µl
5x BAL 31 buffer (13-10)	65 µl

Incubate the mixture in a water bath at 30°C.

14. While the mixture is warming to 30°C, prepare a set of eight microfuge tubes, each containing 5 µl of 200 mM EGTA (pH 8.0). Label the tubes 1.5 minutes, 3.0 minutes, 4.5 minutes, etc.
15. Add 36 µl of the appropriate dilution of BAL 31 (please see Step 12) to the reaction mixture prepared in Step 13. Quickly mix the enzyme by tapping the side of the tube, and then return the tube to the water bath set at 30°C and start a stopwatch.
16. At 1.5-minute intervals, transfer 45 µl of the reaction mixture to the appropriately labeled microfuge tube. Store the tubes on ice until all of the samples have been collected.
17. Heat the tubes for 5 minutes at 65°C to inactivate the BAL 31.
18. Add 5 µl of 3 M sodium acetate (pH 5.2) to each tube, followed by 100 µl of ice-cold ethanol. Mix the solution by vortexing, and store the tubes on ice for 20-30 minutes.
19. Recover the DNAs by centrifugation at maximum speed for 10 minutes at 4°C. Remove the supernatants, and wash the pellets with 200 µl of ice-cold 70% ethanol. Centrifuge for a further 2 minutes.
20. Carefully remove the supernatants, and stand the open tubes at room temperature until all of the ethanol has evaporated. Dissolve each of the pellets in 23 µl of TE (pH 7.6).
21. Add to each of the DNA preparations:

0.5 mM dNTP solution	3 µl
10x polymerase buffer	3 µl
bacteriophage T4 DNA polymerase (approx. 5 units)	1 µl

Incubate the reactions for 15 minutes at room temperature, and then add approx. 1 µl (approx. 5 units) of the Klenow fragment. Continue the incubation for a further 15 minutes at room temperature.

22. Purify the DNAs by extraction with phenol:chloroform, and then precipitate the DNAs with ethanol as described in Steps 18-20. Dissolve each of the DNAs in 16 µl of TE (pH 7.6).
23. To each DNA, add 2 µl of the appropriate 10x restriction enzyme buffer and 8 units of a restriction enzyme that will separate the target DNA from the vector. Incubate the reactions for 1 hour at the appropriate temperature.
24. At the end of the incubation, transfer an aliquot (3 µl) from each digest to a fresh microfuge tube. Store the remainder of the digests on ice until needed in Step 27.
25. Add 1 µl of sucrose gel-loading buffer to each 3-µl aliquot, and load the contents of each tube into the wells of an agarose gel cast in 0.5x TBE and containing 0.5 µg/ml ethidium bromide. The wells at the sides of the gel should contain markers of the appropriate size.
26. Separate the target fragments from the vector DNA by electrophoresis. Examine the gel by UV illumination, and determine which of the samples has been digested to an appropriate size by BAL 31.
27. Pool the samples (from Step 24) containing target DNA of the appropriate size, and isolate the target fragments by preparative gel electrophoresis. Recover the target DNA fragments from the gel using one of the methods described in Chapter 5, Protocol 4 to Chapter 5, Protocol 7.
28. Estimate the amount of purified target DNA from the intensity of ethidium-bromide-mediated fluorescence.
29. Ligate the deleted target fragments with a plasmid, phagemid, or bacteriophage M13 vector (please see Chapter 1 or Chapter 3) that carries one blunt end and one terminus that is compatible with the restriction enzyme used in Step 23.
30. Transform (plasmids or phagemids) or transfect (bacteriophage M13 replicative form DNA) competent *E. coli* of an appropriate strain with small aliquots or dilutions of the ligation mixture. The next day, grow small-scale cultures of 12 transformed colonies or bacteriophage M13 plaques, chosen at random.
31. Purify plasmid, phagemid, or bacteriophage M13 replicative form DNA from each of the 12 cultures by using one of the methods described in Chapter 1 or Chapter 3. Digest the DNAs with a restriction enzyme(s) that will liberate the target fragment from the vector.
32. Analyze the size of the target fragment liberated from each of the DNAs by agarose gel electrophoresis, using size markers of an appropriate size.
33. If the results are satisfactory (i.e., if the target fragments fall within the desired size range), pick a large number of individual transformed colonies or plaques and determine the size of the inserts as described above. Preserve those cultures that carry recombinants of the desired size.
34. Determine the exact endpoints of the deletion in each mutant by DNA sequencing (please see Chapter 12, Protocol 3, Chapter 12, Protocol 4, or Chapter 12, Protocol 5).

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Chapter 14 Screening Expression Libraries

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Protocol 1

Screening Expression Libraries Constructed in Bacteriophage λ Vectors

An expression library constructed in a bacteriophage λ vector is plated on an appropriate *E. coli* strain in the absence of isopropylthio- β-D-galactoside (IPTG). After 2-4 hours, the plates are moved to 37°C (to stabilize any fusion proteins that are temperature sensitive), and filters impregnated with IPTG are laid on top of the developing plaques. After incubation for a further 2-4 hours, the filters, which contain imprints of the phage-encoded proteins, are removed and probed with antibody. The plates are stored at 4°C until the results of immunological screening are available.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Blocking buffer
- ⚠ Chloroform
- IPTG (10 mM)
  Prepare just before use in Step 4.
- SM
  Store SM at room temperature in 50-ml aliquots. Discard each aliquot after use to reduce the possibility of contamination.
- TNT buffer
  Approximately 1 liter of TNT buffer is required per 10 filters screened. Store TNT buffer at room temperature.

  Washing buffers
  Use TNT buffer containing 0.1% (w/v) bovine serum albumin or TNT buffer containing 0.1% (w/v) bovine serum albumin and 0.1% (v/v) Nonidet P-40
  These buffers should not contain sodium azide.

Radioactive Compounds

- ⚠ ^{125}I-labeled protein A or ^{125}I-labeled immunoglobulin (optional)
- ⚠ Radioiodinated secondary antibody
  Use this antibody if detecting the antigen-antibody complexes with a radiolabeled secondary antibody in Step 15.

Antibodies

- Chemiluminescent screening reagents
- Chromogenic screening AP reagents
- Chromogenic screening HRP reagents

  Primary antibody

Media

- LB agar plates
  Each 90-mm Petri dish should contain 30-35 ml of agar medium, whereas each 150-mm plate should contain approx. 50 ml. The plates must be dry; otherwise, the top agarose will peel off when the nitrocellulose filter is removed. Usually, 2-day-old plates that have been dried for an additional 1-2 hours at 37°C with their lids slightly open work well. LB agar plates without ampicillin are used in the screening procedure because E. coli *Y1090*hsdR *grows slowly in the presence of the antibiotic.*
- LB top agarose
  Melt the top agarose by microwaving for a short period of time and then cooling to 47°C. Dispense the molten top agar as 3-ml aliquots (for 90-mm plates) or 7.5-ml aliquots (for 150-mm plates) in sterile tubes. Place the aliquots in a 47°C heating block or water bath to prevent the top agar from gelling.

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 2, Protocol 1.

Step 17 of this protocol requires the reagents listed in Chapter 2, Protocol 2.

Vectors and Bacterial Strains

Bacteriophage λ expression library
Construct a cDNA library in an appropriate expression vector as described in Chapter 11 or purchase the library from a commercial supplier. Before beginning the screening experiment, determine the titer of the bacteriophage solution as described in Chapter 2, Protocol 1.

E. coli
Different bacterial host strains used in expression screening require different growth media. Before plating the host strain to be used, become familiar with its genotype and any unique growth requirements.

METHOD

1. Using a single colony of the appropriate strain of *E. coli* as inoculum, prepare a plating culture as described in Chapter 2, Protocol 1.
2. Calculate the number of plates that will be required to screen the library, assuming 0.5 x 10^4 to 2 x 10^4 plaques per 90-mm plate or 0.5 x 10^4 to 5 x 10^4 plaques per 150-mm plate. Arrange a set of sterile tubes (13 x 100 mm) in a rack; use a fresh tube for each plate. In each tube, mix 0.1 ml of the plating bacteria with 0.1 ml of SM containing the desired number of plaque-forming units of the bacteriophage λ expression library. Incubate the infected bacteria for 20 minutes at 37°C.
3. Add to each tube 2.5 ml (90-mm plate) or 7.5 ml (150-mm plate) of molten top agarose, and immediately pour the mixture onto an LB agar plate. Incubate the infected plates for 3.5 hours at 42°C.
4. Number the nitrocellulose filters with a soft-lead pencil or a ballpoint pen. Use gloves to handle the filters because skin oils will prevent the transfer of proteins. Soak the filters in 10 mM IPTG for a few minutes. Use blunt-ended forceps (e.g., Millipore forceps) to remove the filters from the solution, and allow them to dry at room temperature on a pad of Kimwipes.
5. Remove one plate at a time from the incubator, and quickly overlay it with an IPTG-impregnated nitrocellulose filter. Do not move the filter once contact is made with the plate. Put the plate in the 37°C incubator and repeat the above procedure until all plates contain a nitrocellulose filter.
   It is important to place filters on the plates one at a time so that the temperature of the plates does not drop below 37°C. The growth of bacteriophages is severely retarded at temperatures <30°C.
6. Incubate the plates for at least 4 hours at 37°C.
7. Remove the lids from the plates and continue the incubation for a further 20 minutes at 37°C. This step strengthens the bond between the soft agarose and the agar plate.

8. Move the plates in small batches to room temperature. Use an 18-gauge needle attached to a syringe containing waterproof black ink to mark each filter in at least three asymmetric locations by stabbing through it and into the agar underneath.
9. Use blunt-ended forceps to peel the filters off the plates and immediately immerse them in a large volume of TNT buffer. Rinse away any small remnants of agarose by gently agitating the filters in the buffer. The speed of the shaking incubator should be high enough to prevent the filters from sticking to one another.
   If large areas of the top agarose stick to the nitrocellulose filters, chill the plates for 30 minutes at 4°C or 5 minutes at -20°C before peeling the filters off the surface of the agarose.
10. After all of the filters have been transferred to the TNT buffer, wrap the plates in plastic film and store them at 4°C until the results of the immunological screening are available.
    IMPORTANT *Do not allow the filters to dry out during any of the subsequent steps. Antibodies bound nonspecifically and reversibly to wet filters become permanently attached if the filters dry out. It is also essential that the filters do not stick to one another when they are immersed in the various buffers and antibody solutions. This problem can be minimized by dividing the filters into small batches (e.g., five filters per batch) and using a separate large Petri dish or crystallizing dish for each batch. The Petri dishes can be stacked on top of one another on a slowly rotating platform shaker.*
11. When all of the filters have been rinsed, transfer them one at a time to a fresh batch of TNT buffer. After transfer, agitate the buffer and filters gently for a further 30 minutes at room temperature.
12. Use blunt-ended forceps to transfer the filters individually to a glass tray(s) containing blocking buffer (7.5 ml for each 82-mm filter, 15 ml for each 138-mm filter). When all of the filters have been submerged, agitate the buffer and filters slowly on a rotary platform for 30 minutes at room temperature.
13. Use blunt-ended forceps to transfer the filters to a fresh glass tray(s) containing the primary antibody diluted in blocking buffer (7.5 ml for each 82-mm filter, 15 ml for each 138-mm filter). Use the highest dilution of antibody that gives acceptable background yet still allows detection of 50-100 pg of denatured antigen. When all of the filters have been submerged, agitate the solution gently on a rotary platform for 2-4 hours at room temperature.
14. Wash the filters for 10 minutes in each of the buffers below, in the order given. Transfer the filters individually from one buffer to the next. Use 7.5 ml of each buffer for each 82-mm filter and 15 ml for each 138-mm filter.
    TNT buffer containing 0.1% bovine serum albumin
    TNT buffer containing 0.1% bovine serum albumin and 0.1% Nonidet P-40
    TNT buffer containing 0.1% bovine serum albumin
15. Detect the antigen-antibody complexes with the chosen radiochemical, chromogenic, or chemiluminescent reagent. For radiochemical screening, use approx. 1 µCi of ^{125}I-labeled protein A or immunoglobulin per filter. Radiolabeled protein A is available from commercial sources (specific activity 2-100 µCi/µg). Radioiodinated secondary antibody is available commercially or can be prepared. For chromogenic screening, antibodies coupled to horseradish peroxidase (HRP) or alkaline phosphatase (AP) that react with species-specific determinants on primary antibodies are available from commercial sources and should be used at the recommended dilution in accordance with the manufacturer's instructions. Typically, 5 µl of conjugated antiserum is used for each 82-mm filter in 7.5 ml of blocking buffer (without sodium azide). Chemiluminescence is the most sensitive method for detecting immunopositive plaques. Chemiluminescent detection is quick, produces a permanent record of the screened filter (an X-ray film or phosphoimage), and is sensitive (1-10 pg of antigen in a plaque can be detected).

Radiochemical screening

a. Dilute radiolabeled ligands in blocking buffer (7.5 ml for each 82-mm filter, 15 ml for each 138-mm filter).
b. Incubate the filters for 1 hour at room temperature, and then wash them several times in TNT buffer before establishing autoradiographs.

Continue at Step 16.

Chromogenic screening with AP-conjugated antibodies

a. Gently agitate the filters for 1.5-2 hours at room temperature in the solution of AP-conjugated antibody.
b. Wash the filters as described in Step 14.
c. Prepare stock solutions of BCIP (50 mg/ml in 100% dimethylformamide) and NBT (50 mg/ml in 70% dimethylformamide). These solutions are stable when stored in the dark.
d. Prepare the BCIP/NBT developing solution just before use as follows:
   i. Add 33 µl of the NBT solution to 5 ml of AP buffer and mix well.
   ii. Add 16.5 µl of the stock solution of BCIP. Mix again. Protect the solution from strong light and use it within 1 hour.
e. Blot the washed filters on paper towels.
f. Incubate the filters (Step d) in the BCIP/NBT developing solution (7.5 ml for each 82-mm filter, 15 ml for each 138-mm filter) for several hours at room temperature.
g. Rinse the filters briefly in two changes of H_2O. An intense purple color will develop at the site of antigen-antibody complexes.

Continue at Step 16.

Chromogenic screening with HRP-conjugated antibodies

a. Gently agitate the filters in the HRP-conjugated antibody solution for 1.5-2 hours at room temperature.
b. Wash the filters as described in Step 14.
c. To prepare developing solution, dissolve 60 mg of 4-chloro-1-naphthol in 20 ml of ice-cold methanol. Just before use, mix the solution with 100 ml of 10 mM Tris-Cl (pH 7.5), 150 mM NaCl containing 60 µl of 30% H_2O_2.
d. Blot the washed filters on paper towels and wash them briefly in 10 mM Tris-Cl (pH 7.5) containing 150 mM NaCl.
e. Incubate the filters for 15-20 minutes at room temperature in the 4-chloro-1-naphthol developing solution (10 ml for each 82-mm filter, 25 ml for each 138-mm filter).
f. Wash the filters in two changes of H_2O. An intense purple color will develop at the site of antigen-antibody complexes.

Continue at Step 16

Chemiluminescent screening

a. Gently agitate the filters for 1.5-2 hours in the AP- or HRP-conjugated antibody solution at room temperature.
b. Wash the filters as described in Step 14.
c. Prepare the chemiluminescent substrates according to the manufacturer's instructions.
d. Incubate the washed filters in the chemiluminescent substrates for 1-5 minutes (again, consult the manufacturer's recommendations for optimal exposure times).
e. Drain the excess solution from the filters, and immediately wrap the filters in Saran Wrap. Do not allow the filters to dry out.
f. Establish an autoradiogram. Typically, the initial exposure is 1 minute. This interval provides enough information to establish the proper exposure time.

Continue at Step 16.

16. Identify the locations of positive plaques or, if made, compare the duplicate filters, searching for coincident signals. For screens involving radiolabeled or chemiluminescent probes, compare the resulting autoradiograms with the agar plates on a light box. For screens involving chromogenic reagents that leave a visible positive residue on the filter, carry out the following steps:
    a. Lay a sheet of Saran Wrap or Mylar film over the filters.
    b. On the surface of the Saran Wrap, mark the locations of the holes in the filters and the locations of antigen-positive clones with different colored waterproof markers. Label the Saran Wrap to identify the plates from which the filters were derived.
    c. Place the sheet of Saran Wrap on a light box, and align the plates containing the original bacteriophage λ plaques on top of it.
    d. Identify the area containing the positive plaque, and remove a plug of agar from this area using the large end of a Pasteur pipette. Transfer the plug to 1 ml of SM containing 2 drops of chloroform.

e. Keep the sheet of Saran Wrap, which provides a permanent record of the locations of the positive plaques. The colored spots on the original filters fade quite rapidly.

17. Allow the bacteriophage particles to elute from the agar plug for several hours at 4°C. Measure the titer of the bacteriophages in the eluate, and then replate them so as to obtain approx. 3000 plaques per 90-mm plate. Rescreen the plaques as described above (from Step 4 onward), and repeat the process of screening and plating until a homogeneous population of immunopositive recombinant bacteriophages is obtained.

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3. Young R.A. and Davis R.W. 1991. Gene isolation with λgt11 system. *Methods Enzymol.* 194:230-238.

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Protocol 2

Screening Expression Libraries Constructed in Plasmid Vectors

A cDNA library constructed in a plasmid expression vector of the pUC, pUR, or pEX series is plated on agar medium and then replicated onto filters, which are transferred to plates containing IPTG. After 2-4 hours of induction, the colonies are lysed with chloroform and then screened with appropriate antibodies. The antigen-antibody complexes are detected by standard radiochemical, chromogenic, or chemiluminescent methods.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Blocking buffer
- ⚠ Chloroform
- Lysis buffer (14-2)
  Add DNase I and lysozyme to this buffer just before use in Step 11.
- SM
  Store SM at room temperature. Discard each aliquot after use to reduce the possibility of contamination.
- TNT buffer
  Approximately 1 liter of TNT buffer is required per 10 filters screened. Store TNT buffer at room temperature.

  Washing buffers
  Use TNT buffer containing 0.1% (w/v) bovine serum albumin or TNT buffer containing 0.1% (w/v) bovine serum albumin and 0.1% (v/v) Nonidet P-40.
  These buffers should not contain sodium azide.

Radioactive Compounds

- ⚠ ^{125}I-labeled protein A or ^{125}I-labeled immunoglobulin (optional)
- ⚠ Radioiodinated secondary antibody
  Use this antibody if detecting the antigen-antibody complexes with a radiolabeled secondary antibody in Step 19.

Antibodies

- Chemiluminescent screening reagents
- Chromogenic screening AP reagents
- Chromogenic screening HRP reagents

  Primary antibody

Media

- Rich media agar plates
  Include the appropriate antibiotic for the expression system or vector used to construct the cDNA library. Each 90-mm Petri dish should contain 30-35 ml of agar medium, whereas each 150-mm plate should contain approx. 50 ml. The plates must be dry; otherwise, the top agarose will peel off when the nitrocellulose filter is removed. Usually, 2-day-old plates that have been dried for an additional 1-2 hours at 37°C with their lids slightly open work well.
- Rich media agar plates containing 1 mM IPTG
  Plates containing IPTG are required if the expression vector carries the lac *promoter.*

Vectors and Bacterial Strains

Plasmid expression library
Construct a cDNA library in an appropriate expression vector as described in Chapter 11 or purchase the library from a commercial supplier.

Additional Reagents

Step 19 of this protocol requires the reagents listed in Chapter 14, Protocol 1.

METHOD

1. Use sterile blunt-ended forceps (e.g., Millipore forceps) to place a sterile nitrocellulose filter, numbered side down, on an LB (or SOB) plate. Remove the filter from the plate, invert it, and replace it, numbered side up.
2. Apply the bacteria in a small volume of liquid (<0.5 ml containing up to 20,000 bacteria for a 138-mm filter, <0.2 ml containing up to 10,000 bacteria for an 82-mm filter). Spread the liquid over the surface of the filter with a sterile bent glass rod. Leave a border 2-3 mm wide at the edge of the filter free of bacteria. Store the plates at room temperature until all of the liquid has been absorbed.
3. Invert the plates and incubate them until very small (0.1-mm diameter) colonies appear (8-10 hours).
   Grow colonies containing expression vectors carrying the lac *promoter at 37°C. Grow colonies containing expression vectors carrying the bacteriophage λp_R promoter at 30°C to prevent the expression of fusion proteins.*
4. Wet a numbered, sterile nitrocellulose filter by touching it, numbered side up, to the surface of a fresh agar plate containing the appropriate antibiotic. Leave the filter in contact with the surface of the agar. The numbers on the set of replica filters should correspond to those on the master filters.
5. Use sterile blunt-ended forceps to remove the master filter gently from one of the agar plates (Step 3) and place it, colony side up, on the stack of sterile Whatman 3MM papers.
6. Carefully place the second, correspondingly numbered, wetted filter numbered side down on top of the master filter, being careful not to move the filters once contact has been made. Place a circle of 3MM paper on top of the filter sandwich. Place the bottom of an empty Petri dish on top of the 3MM paper and exert hand pressure on the sandwich.
7. Use an 18-gauge needle to make a characteristic set of registration holes in the filters while they are sandwiched together. Gently peel the filters apart. Transfer the replica filter to the plate used for wetting (Step 4). Transfer the master filter, colony side up, to a fresh agar plate containing the appropriate antibiotic.
   If required, several replicas can be made from a single master filter. However, if the master filter is to be used to make more than two replicas, reincubate it for a few hours to allow the colonies to regenerate. Generally, it is best to make only two replicas from a single master to avoid problems caused by smearing of the colonies.
8. Repeat Steps 4-7 until all the master filters have been replicated.
9. Induce the expression of a gene cloned into an expression vector carrying the *lac* promoter as described below.
   a. Incubate the plates (masters and replicas) at 37°C until colonies 1-2 mm in diameter have appeared. Colonies on the master plates generally reach the desired size more rapidly.
   b. Allow the master plates to cool to room temperature on the laboratory bench, wrap them in plastic film, and store them at 4°C until the results of the immunological screens are available.
   c. Transfer the replica filters numbered side up to fresh agar plates, prewarmed to 37°C, containing IPTG at a concentration of 1 mM. Incubate the IPTG-containing plates for a further 2-4 hours.

d. To induce synthesis in expression vectors that carry the bacteriophage λ p_R promoter (e.g., the pEX vectors), transfer the filters to a series of prewarmed agar plates and incubate them for 2-4 hours at 42°C.

10. Use blunt-ended forceps to remove the nitrocellulose filters from the plates and place them on damp paper towels *in a chemical fume hood*. Cover the filters with an inverted plastic box. Place an open glass Petri dish containing chloroform under the box with the filters. Expose the bacterial colonies on the filters to chloroform vapor for 15 minutes.
11. Transfer small groups of filters to Petri dishes containing Lysis buffer (14-2) (6 ml per 82-mm filter, 12 ml per 138-mm filter). When all of the filters have been submerged, stack the Petri dishes on a rotary platform and agitate the Lysis buffer (14-2) by gentle rotation of the platform. Lysis of the bacterial colonies takes 12-16 hours at room temperature.
12. Transfer the filters to Petri dishes or glass trays containing TNT buffer. Incubate the filters for 30 minutes at room temperature.
13. Repeat Step 12 using fresh TNT buffer.
14. Transfer the filters, one by one, to a glass tray containing TNT buffer. Use Kimwipes to remove the residue of the colonies from the surfaces of the filters.
    IMPORTANT *Do not allow the filters to dry out during any of the subsequent steps. Antibodies bound nonspecifically and reversibly to wet filters become permanently attached if the filters dry out. It is also essential that the filters do not stick to one another when they are immersed in the various buffers and antibody solutions. This problem can be minimized by dividing the filters into small batches (e.g., five filters per batch) and using a separate large Petri dish for each batch. The Petri dishes can be stacked on top of one another on a slowly rotating platform shaker.*
15. When all of the filters have been rinsed, transfer them one at a time to a fresh batch of TNT buffer. After transfer, agitate the buffer and filters gently for a further 30 minutes at room temperature.
16. Use blunt-ended forceps to transfer the filters individually to glass trays or Petri dishes containing blocking buffer (7.5 ml for each 82-mm filter, 15 ml for each 138-mm filter). When all of the filters have been submerged, agitate the buffer slowly on a rotary platform for 30 minutes at room temperature.
17. Use blunt-ended forceps to transfer the filters to fresh glass trays or Petri dishes containing the primary antibody diluted in blocking buffer (7.5 ml for each 82-mm filter, 15 ml for each 138-mm filter). Use the greatest dilution of antibody that gives acceptable background yet still allows detection of 50-100 pg of denatured antigen. When all of the filters have been submerged, agitate the solution gently on a rotary platform for 2-4 hours at room temperature.
18. Wash the filters for 10 minutes in each of the buffers below in the order given. Transfer the filters individually from one buffer to the next. Use 7.5 ml of each buffer for each 82-mm filter and 15 ml for each 138-mm filter.
    TNT buffer containing 0.1% bovine serum albumin
    TNT buffer containing 0.1% bovine serum albumin and 0.1% Nonidet P-40
    TNT buffer containing 0.1% bovine serum albumin
19. Detect the antigen-antibody complexes with the chosen radiochemical, chromogenic, or chemiluminescent reagent. For radiochemical screening, use approx. 1 µCi of ^{125}I-labeled protein A or immunoglobulin per filter. Radiolabeled protein A is available from commercial sources (specific activity 2-100 µCi/µg). Radioiodinated secondary antibody is available commercially or can be prepared. For chromogenic screening, antibodies coupled to horseradish peroxidase (HRP) or alkaline phosphatase (AP) that react with species-specific determinants on primary antibodies are available from commercial sources and should be used at the recommended dilution in accordance with the manufacturer's instructions. Typically, 5 µl of conjugated antiserum is used for each 82-mm filter in 7.5 ml of blocking buffer (without sodium azide). Chemiluminescence is the most sensitive method for detecting immunopositive colonies. Chemiluminescent detection is quick, produces a permanent record of the screened filter (an X-ray film or phosphoimage), and is sensitive (1-10 pg of antigen in a colony can be detected).

Radiochemical screening

a. Dilute radiolabeled ligands in blocking buffer (7.5 ml for each 82-mm filter, 15 ml for each 138-mm filter).
b. Incubate the filters for 1 hour at room temperature, and then wash them several times in TNT buffer before establishing autoradiographs.

Continue at Step 20.

Chromogenic screening with AP-conjugated antibodies

a. Gently agitate the filters for 1.5-2 hours at room temperature in the solution of AP-conjugated antibody.
b. Wash the filters as described in Step 18.
c. Prepare stock solutions of BCIP (50 mg/ml in 100% dimethylformamide) and NBT (50 mg/ml in 70% dimethylformamide). These solutions are stable when stored in the dark.
d. Prepare the BCIP/NBT developing solution just before use as follows:
   i. Add 33 µl of the NBT solution to 5 ml of AP buffer and mix well.
   ii. Add 16.5 µl of the stock solution of BCIP. Mix again. Protect the solution from strong light and use it within 1 hour.
e. Blot the washed filters on paper towels.
f. Incubate the filters (Step d) in the BCIP/NBT developing solution (7.5 ml for each 82-mm filter, 15 ml for each 138-mm filter) for several hours at room temperature.
g. Rinse the filters briefly in two changes of H_2O. An intense purple color will develop at the site of antigen-antibody complexes.

Continue at Step 20.

Chromogenic screening with HRP-conjugated antibodies

a. Gently agitate the filters for 1.5-2 hours at room temperature in the solution of HRP-conjugated antibody.
b. Wash the filters as described in Step 18.
c. To prepare developing solution, dissolve 60 mg of 4-chloro-1-naphthol in 20 ml of ice-cold methanol. Just before use, mix the solution with 100 ml of 10 mM Tris-Cl (pH 7.5), 150 mM NaCl containing 60 µl of 30% H_2O_2.
d. Blot the washed filters on paper towels and wash them briefly in 10 mM Tris-Cl (pH 7.5) containing 150 mM NaCl.
e. Incubate the filters for 15-20 minutes at room temperature in the 4-chloro-1-naphthol developing solution (10 ml for each 82-mm filter, 25 ml for each 138-mm filter).
f. Wash the filters in two changes of H_2O. An intense purple color will develop at the site of antigen-antibody complexes.

Continue at Step 20.

Chemiluminescent screening

a. Gently agitate the filters for 1.5-2 hours at room temperature in the solution of AP- or HRP-conjugated antibody.
b. Wash the filters as described in Step 18.
c. Prepare the chemiluminescent substrates according to the manufacturer's instructions.
d. Incubate the washed filters in the chemiluminescent substrates for 1-5 minutes (again, consult the manufacturer's recommendations for optimal exposure times).
e. Drain the excess solution from the filters, and immediately wrap the filters in Saran Wrap. Do not allow the filters to dry out.
f. Establish an autoradiogram. Typically, the initial exposure is for 1 minute. This interval provides enough information to establish the proper exposure time.

Continue at Step 20.

20. Identify the locations of positive colonies or, if made, compare the duplicate filters, searching for coincident signals. For screens involving radiolabeled or chemiluminescent probes, compare the resulting autoradiograms with the agar plates on a light box. For screens involving chromogenic reagents that leave a visible positive residue on the filter, carry out the following steps.
    a. Lay a sheet of Saran Wrap or Mylar film over the filters.
    b. On the surface of the Saran Wrap, mark the locations of the holes in the filters and the locations of antigen-positive clones with different colored waterproof markers. Label the Saran Wrap to identify the plates from which the filters were derived.
    c. Place the sheet of Saran Wrap on a light box, and align the plates containing the original bacterial colonies on top of it.

d. Identify the areas containing the positive colonies, and transfer a segment of each putative colony to 1 ml of LB medium containing the appropriate antibiotic. Incubate the cultures for 12-16 hours at the appropriate temperature.

e. Keep the sheet of Saran Wrap, which provides a permanent record of the locations of the positive colonies. The colored spots on the original filters fade quite rapidly.

21. Repeat the process of plating and screening until a homogeneous population of immunopositive colonies is obtained.
22. Validate the clones isolated by immunological screening.

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1. Helfman D.M. and Hughes S.H. 1987. Use of antibodies to screen cDNA expression libraries prepared in plasmid vectors. *Methods Enzymol.* 152:451-457.

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Protocol 3

Removal of Cross-reactive Antibodies from Antiserum: Pseudoscreening

Antibodies directed against bacterial and phage-encoded proteins can be removed from sera by incubation with nitrocellulose filters impregnated with lysates of uninfected or phage-infected *E. coli.*

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Blocking buffer

Antibodies

Antibody preparation that is to be used for screening

Media

- LB agar plates
  Each 90-mm Petri dish should contain 30-35 ml of agar medium. Each 150-mm plate should contain approx. 50 ml of agar medium. The plates must be dry; otherwise, the top agarose will peel off when the nitrocellulose filter is removed. Usually, 2-day-old plates that have been dried for an additional 1-2 hours at 37°C with the lids slightly open work well.
- LB top agarose
  Melt the top agarose just before use by microwaving for a short period of time and then cooling to 47°C. Dispense the molten top agar as 3-ml aliquots (for 90-mm plates) or 7.5-ml aliquots (for 150-mm plates) in sterile tubes. Place the aliquots in a 47°C heating block or water bath to prevent the top agar from gelling.

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 2, Protocol 1.

Step 2 of this protocol requires the reagents listed in Chapter 14, Protocol 1.

Vectors and Bacterial Strains

Bacteriophage λ vector
Use the expression vector and bacterial strain that was used to produce the cDNA library of interest.

METHOD

1. On ten LB agar plates, plate out nonrecombinant bacteriophage λ so as to obtain semiconfluent lysis of the bacterial lawn (please see Chapter 2, Protocol 1).
2. Prepare imprints of the lysed lawns on nitrocellulose filters as described in Steps 5-12 of Protocol 1, but omitting the treatment with IPTG.
3. Dilute the preparation of antibody that is to be used for screening 1:10 with blocking buffer.
4. Incubate the filters for 6 hours with the diluted antibody. The treated antibody may be stored at 4°C in the presence of 0.05% (w/v) sodium azide until used for immunological screening.

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Protocol 4

Removal of Cross-reactive Antibodies from Antiserum: Incubation with *E. coli* Lysate

This protocol describes how antibodies that react with bacterial-encoded proteins may be removed from polyclonal antisera by incubation with a bacterial lysate.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Blocking buffer
- Cell resuspension buffer (14-4)

Antibodies

Antibody preparation that is to be used for screening

Media

- LB medium

Vectors and Bacterial Strains

E. coli strain Y1090*hsdR*
This strain can be obtained from Stratagene, Life Technologies, or the ATCC (www.atcc.org).

METHOD

1. Grow a 100-ml culture of *E. coli* strain Y1090*hsdR* to saturation in LB medium.
2. Harvest the cells by centrifugation at 5000*g* (5500 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C.
3. Resuspend the cells in 3 ml of cell resuspension buffer. Freeze and thaw the suspension several times, and then sonicate it at full power for six periods of 20 seconds, each at 0°C.
4. Centrifuge the extract at maximum speed for 10 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh tube. Store the lysate at -20°C.
5. Just before using the lysate, dilute the preparation of antibody that is to be used for screening 1:10 with blocking buffer.
6. Add 0.5 ml of lysate for every milliliter of antibody preparation to be processed. Incubate the mixture for 4 hours at room temperature on a slowly rotating wheel. The treated antibody may be stored at 4°C in the presence of 0.05% (w/v) sodium azide until used for immunological screening.

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Protocol 5

Removal of Cross-reactive Antibodies from Antiserum: Affinity Chromatography

This protocol describes a method for removing antibodies that react with bacterially encoded proteins by passing a crude preparation of immunoglobulins through a column containing immobilized bacterial proteins.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Cell lysis buffer (14-5)
- ⚠ NaOH (1 N)
- TBS
- TBS containing 0.2% (w/v) sodium azide

  Triton X-100

Enzymes and Buffers

- Lysozyme
  Use a molecular biology grade of lysozyme. Add solid lysozyme to assist lysis of bacterial cells.
- Pancreatic DNase I
  Add solid DNase I to the bacterial cell lysate to digest chromosomal DNA.

Antibodies

Antibody preparation that is to be used for screening
This protocol works best when using an IgG fraction, prepared by chromatography of the antiserum on protein A-Sepharose.

Media

Growth medium
One liter of growth medium appropriate for the E. coli *strain of choice is required.*

Vectors and Bacterial Strains

E. coli strain used as host for preparation of expression library

METHOD

1. Grow a 1-liter culture of the appropriate strain of *E. coli* (e.g., Y1090*hsdR*, XL1-Blue, or DH1) to stationary phase.
2. Recover the bacteria by centrifugation at 4000*g* (5000 rpm in a Sorvall GSA rotor) for 20 minutes at 4°C.
3. Pour off the medium, and stand the centrifuge tubes in an inverted position to allow the last traces of medium to drain away.
4. Resuspend the pellet in 100 ml of Cell lysis buffer (14-5).
5. Add 200 mg of lysozyme, and incubate the bacterial suspension for 20 minutes at room temperature.
6. Add 1 mg of pancreatic DNase I and 200 µl of Triton X-100.
7. Incubate the bacterial suspension for 1 hour at 4°C, or until the turbidity clears and the viscosity decreases.
8. Centrifuge the bacterial lysate at 8000*g* (8200 rpm in a Sorvall SS-34 rotor) for 20 minutes at 4°C. Carefully decant the supernatant into a fresh flask.
9. Adjust the pH of the supernatant to 9.0 with 1 M NaOH.
10. Determine the concentration of protein in the lysate using the Lowry, Bradford, or other method of measurement.
11. Chill the extract to 0°C, and then bind the bacterial proteins to cyanogen-bromide-activated Sepharose 4B or to Affi-Gel 10 according to the manufacturer's instructions.
12. Before use, equilibrate the Sepharose 4B or Affi-Gel 10 resin containing conjugated *E. coli* proteins in TBS containing 0.2% (w/v) sodium azide.
13. Use 1 ml of settled volume of resin coupled to *E. coli* antigen for each milligram of IgG protein to be purified by affinity chromatography. Mix the IgG and the coupled resin and incubate for 12-18 hours at room temperature on a rotating wheel device.
14. Load the slurry into a chromatography column. Recover the antibody by washing the column with TBS. Collect fractions (0.2 column volume each) until the OD_{280} drops to zero. Pool the fractions containing antibody, and store the pool at -20°C until it is used for immunological screening.

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Protocol 6

Identifying DNA-binding Proteins in Bacteriophage λ Expression Libraries

This protocol describes how to identify cloned cDNAs encoding proteins that bind to specific DNA sequences. The methods used are very similar to those used for immunological screening of expression libraries except that the nitrocellulose filters carrying immobilized proteins are screened with ^{32}P-labeled double-stranded DNA rather than with antibodies.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ ATP (100 mM)
Make two ATP solutions, 10 mM and 50 mM, respectively, by dilution of the 100 mM stock into 25 mM Tris-Cl (pH 8.0).

10x Binding buffer

1x Binding buffer containing 1 mM DTT (dithiothreitol)
This solution is used to dilute full-strength Denaturation Solution (14-6) to generate the series of washes in the protocol. Please see Steps 11-13 to calculate the volume of solution required.

1x Binding buffer containing 1 mM DTT (dithiothreitol) and 0.25% nonfat dry milk
Approximately 75 ml of 1x binding buffer is needed per 82-mm filter screened or 180 ml per 138-mm filter. Do not scrimp on the amounts of this buffer used in the washing procedure.

1x Binding buffer containing 5% (w/v) nonfat dry milk
About 10 ml of this blocking solution is needed per 82-mm filter screened or 25 ml per 138-mm filter.

1x Binding buffer containing 0.25% (w/v) nonfat dry milk
About 10 ml of this blocking solution is needed per 82-mm filter screened or 25 ml per 138-mm filter.

Denaturation solution (14-6) (freshly made)

DTT (dithiothreitol) (1 M)

EDTA (0.5 M)

Ethanol

10x Kinase/ligase buffer

⚠ Phenol:chloroform (1:1, v/v)

Screening buffer
About 10 ml of screening buffer is needed for each 82-mm filter or 25 ml for each 138-mm filter.

⚠ SDS (20% w/v)

Sodium acetate (3 M, pH 5.2)

TE (pH 8.0)

Enzymes and Buffers

Bacteriophage T4 DNA ligase

Bacteriophage T4 polynucleotide kinase

Nucleic Acids and Oligonucleotides

Synthetic oligonucleotides
Single-stranded oligonucleotides of complementary sequence, 20-25 nucleotides in length, should be purified by gel electrophoresis and Sep-Pak chromatography as described in Chapter 10, Protocol 1 and dissolved in TE (pH 7.6) at a concentration of 0.2 mg/ml. When reannealed, the central region of the oligonucleotides should form a double-stranded monomeric version of the site that has been established by gel retention or Southwestern blotting to be optimum for binding of the target protein. At least one complementary pair of "mutant" oligonucleotides is also required in the second round of screening. When annealed, the central region of the "mutant" oligonucleotide duplex should form a defective version of the optimum binding site that is unable to bind the target protein. Sequential screening with a positive probe (the optimum double-stranded binding sequence) and a negative probe (the closely related but mutated double-stranded sequence) eliminates most false positives.

Both pairs of oligonucleotides should be designed with protruding, cohensive termini that can be ligated to one another.

Radioactive Compounds

⚠ [γ-^{32}P]ATP (10 mCi/ml, 5000 Ci/mmole)

Additional Reagents

Steps 8 and 10 of this protocol require the reagents listed in Chapter 14, Protocol 1.

METHOD

1. Set up two separate phosphorylation reactions, each containing one of the synthetic oligonucleotides to be annealed:

oligonucleotide	200 ng
10x kinase/ligase buffer	2.5 µl
100 mM dithiothreitol	2.5 µl
[γ-^{32}P]ATP	100 µCi
H_2O	to 23 µl
bacteriophage T4 polynucleotide kinase (8-10 units/µl)	2 µl

   Incubate the reactions for 1 hour at 37°C.
2. Mix the two phosphorylation reactions together. Anneal the oligonucleotides by incubating the mixture in the following sequence, which is most conveniently carried out in a thermal cycler:
   2 minutes at 85°C
   15 minutes at 65°C
   15 minutes at 37°C
   15 minutes at 22°C
   15 minutes at 4°C
3. Add 4 µl of bacteriophage T4 DNA ligase (1 Weiss unit/µl) and 1 µl of 50 mM ATP. Incubate the mixture for 12 hours at 16°C.
4. Add 0.5 M EDTA (pH 8.0) to a final concentration of 5 mM.

5. Separate the labeled oligonucleotides from unused [γ-^{32}P]ATP, single-stranded oligonucleotides, and unligated double-stranded oligonucleotides by spun-column chromatography through a Sephadex G-75 column.
6. Estimate the specific activity of the final probe.
   The specific activity should be ≥ 2 x 10^6 cpm/pmole.
7. Analyze the size of the radiolabeled DNA by nondenaturing polyacrylamide gel electrophoresis and autoradiography. If all has gone well in the above annealing and radiolabeling experiment, the concatenated DNA should form a ladder of polymers of the original duplex oligonucleotide.
8. Prepare agar plates containing plaques of the bacteriophage λ expression library and numbered nitrocellulose filters exactly as described in Chapter 14, Protocol 1, Steps 1-8.
9. Use blunt-ended forceps (e.g., Millipore forceps) to remove the numbered nitrocellulose filters from the lawn of plaques and place them on Whatman 3MM paper with the side exposed to the plaques facing upward. Allow the filters to dry for 15 minutes at room temperature.
10. Lay a second (numbered) filter impregnated with IPTG on each agar plate (please see Step 4 of Chapter 14, Protocol 1). Use an 18-gauge needle to make holes in each filter in the same locations as the holes used to key the first filter to the lawn. Incubate the plates for 2 additional hours at 37°C and then remove the filters. Allow them to dry at room temperature as described in the preceding step.
    IMPORTANT *Carry out all subsequent steps at 4°C. The first set of filters is probed directly without denaturation with guanidine HCl (i.e., omitting Steps 11-13), whereas the second set is processed as described in Steps 11-14.*
11. Place the second set of numbered filters in a 12 x 8-inch baking dish containing Denaturation Solution (14-6) at 4°C. Agitate the filters gently on a platform shaker for 5 minutes at 4°C. Decant the Denaturation Solution (14-6), and replace it with fresh solution. Agitate the filters for an additional 5 minutes at 4°C.
12. Decant the second batch of Denaturation Solution (14-6) into a graduated cylinder. Dilute the solution with an equal volume of 1x binding buffer containing dithiothreitol. Pour this solution into a clean glass dish, and transfer the filters to the solution one at a time, making sure that each filter becomes thoroughly exposed to the diluted Denaturation Solution (14-6) containing dithiothreitol.
13. Repeat the process described in Step 12 four more times, diluting the denaturation solution by a factor of 2 each time. The concentrations of guanidine HCl in the solutions are therefore 3 M (Step 11), 1.5 M, 0.75 M, 0.375 M, 0.187 M, and 0.094 M. Finally, wash the filters twice in 1x binding buffer containing dithiothreitol.
14. Place both sets of numbered filters (i.e., denatured and nondenatured) in 1x binding buffer containing 5% nonfat dried milk. Agitate the filters gently for 30 minutes at 4°C.
15. Rinse the filters in 1x binding buffer containing 0.25% nonfat dried milk.
16. In a crystallizing dish, add the ^{32}P-labeled concatenated DNA probe from Step 5 to screening buffer to make a hybridization solution (approx. 10 ml for each 82-mm filter or approx. 25 ml for each 138-mm filter).
17. Transfer the filters to the radiolabeled probe solution in the crystallizing dish. Incubate the filters with gentle agitation on a rotating platform for 2-12 hours at 4°C.
18. Wash the filters for 5 minutes at 4°C in a large volume (25 ml for each 82-mm filter, 60 ml for each 138-mm filter) of binding buffer containing 1 mM dithiothreitol and 0.25% nonfat dried milk.
19. Repeat Step 18 twice more.
20. Decant the final wash buffer. Arrange the damp filters on a sheet of Saran Wrap. Cover the filters with another sheet of Saran Wrap. Apply adhesive labels marked with radioactive ink or chemiluminescent markers to several asymmetric locations on the Saran Wrap.
    Cover the radioactive labels with Scotch Tape to prevent contamination with the radioactive ink of the film holder or intensifying screen.
21. Establish an autoradiograph.
22. Pick positive plaques, and rescreen them with specific and nonspecific probes as discussed in the introduction to this protocol.

REFERENCES

1. Singh H. 1993. Specific recognition site probes for isolating genes encoding DNA-binding proteins. *Methods Enzymol.* 218:551-567.
2. Singh H., Clerc R.G., and LeBowitz J.H. 1989. Molecular cloning of sequence-specific DNA binding proteins using recognition site probes. *BioTechniques* 7:252-261.

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Protocol 7

Preparation of Lysates Containing Fusion Proteins Encoded by Bacteriophage λ Lysogens: Lysis of Bacterial Colonies

In this protocol, a bacterial lysogen is constructed from a recombinant bacteriophage λ encoding a fusion protein of interest. The resulting lysogenic colonies are induced to synthesize the fusion protein, which is then isolated in preparation for functional and biochemical analyses.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- IPTG (1 M)
  Approximately 40 µl of 1 M IPTG is required per lysogen induced.
- Lysogen extraction buffer
  Add PMSF to the lysogen extraction buffer just before it is used in Steps 13 and 18. Approximately 100 ml of lysogen extraction buffer is needed per 20 lysogens induced.
- NaCl (5 M)
  Store the solution of NaCl at 4°C.

Enzymes and Buffers

- Lysozyme (10 mg/ml)
  This solution should be freshly prepared for use in Step 15.

Media

- LB agar plates containing 50 µg/ml ampicillin
- LB medium
- LB medium containing 50 µg/ml ampicillin
- LB medium containing 10 mM $MgCl_2$
- LB medium containing 10 mM $MgCl_2$, 0.2% (w/v) maltose, and 50 µg/ml ampicillin

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 2, Protocol 3.

Vectors and Bacterial Strains

Bacteriophage λgt11, λ gt18-23, λ ZAP, and λ ZipLox recombinant
This protocol has been optimized for a bacteriophage λgt11 recombinant that expresses a recombinant fusion protein (identified by methods outlined in Chapter 14, Protocol 1 or Chapter 14, Protocol 6 or by hybridization and DNA sequence analysis). Recombinants constructed in many other bacteriophage λ expression vectors can be used to establish lysogens essentially as described here.

E. coli strains Y1090*hsdR* and Y1089
These strains, which are available from the ATCC (www.atcc.org), are maintained on LB agar plates containing 50 µg/ml ampicillin. The Y1090 and Y1089 strains carry mutations in the lon *gene, which encodes an ATP-dependent protease. Fusion proteins expressed in these strains are often more stable than those expressed in strains lacking this mutation. Y1089 carries the* hflA *mutation, which dramatically increases the frequency of lysogenization by bacteriophage λ. In addition, Y1089 lacks a suppressor tRNA gene; thus, the amber mutation in the* S *gene (lysis gene) of λgt11 is not suppressed in this strain, allowing large amounts of bacteriophage-λ-encoded gene products (including LacZ fusion proteins) to accumulate to high levels within an induced cell. F*

METHOD

1. Make a plate stock of each of the recombinant bacteriophage(s) of interest using the methods described in Chapter 2, Protocol 2 or Chapter 2, Protocol 3. The titer of the stocks, measured on *E. coli* strain Y1090*hsdR*, should be $>10^{10}$ pfu/ml.
2. Grow a 2-ml culture of *E. coli* strain Y1089 to saturation in LB medium containing 10 mM $MgCl_2$, 0.2% maltose, and ampicillin (50 µg/ml).
3. Dilute 50 µl of the saturated culture with 2 ml of LB medium containing 10 mM $MgCl_2$. Transfer four 100-µl aliquots of the diluted culture to fresh culture tubes.
4. To each of three of the tubes, add 1 x 10^7, 5 x 10^7, and 2 x 10^8 plaque-forming units of the bacteriophage stock. The fourth tube should receive no bacteriophage. Incubate all four tubes for 20 minutes at 37°C to allow virus attachment.
5. Dilute 10 µl of each of the four cultures with 10 ml of LB medium. Immediately plate aliquots (100 µl) of each of the four diluted cultures onto LB agar plates containing 50 µg/ml ampicillin. Incubate the plates for 18-24 hours at 32°C.
6. Use sterile toothpicks or inoculating loops to transfer a series of individual colonies onto two LB plates that contain 50 µg/ml ampicillin. Incubate one plate at 32°C and the other plate at 42°C for 12-16 hours. Clones that give rise to colonies at 32°C but not at 42°C are lysogenic for recombinant bacteriophage λgt11. Usually, 10-70% of the colonies tested are lysogens.
7. Inoculate 2 ml of LB medium containing 50 µg/ml ampicillin with individual bacteriophage λgt11 lysogens. Grow the cultures for 12-16 hours at 32°C with vigorous agitation (300 cycles/minute in a rotary shaker).
8. Add 50 µl of each culture to 4 ml of prewarmed (32°C) LB medium containing 50 µg/ml ampicillin. Continue incubation at 32°C with vigorous agitation.
9. Grow the cultures until the OD_{600} = 0.45 (approx. 3 hours of incubation).
   IMPORTANT *The OD_{600} of the cultures should not exceed 0.5 (approx. 2 x 10^8 bacteria/ml) before induction of the lysogens.*
10. Transfer the cultures to a shaking water bath equilibrated to 44°C. Incubate the cultures for 15 minutes at 44°C.
11. Add IPTG to each culture to a final concentration of 10 mM, and then incubate the cultures for 1 hour at 37°C with vigorous agitation.
12. Transfer 1.5-ml aliquots of each of the induced lysogenic cultures to two microfuge tubes. Immediately centrifuge the tubes at maximum speed for 30 seconds at 4°C in a microfuge.
13. Remove the medium by aspiration, and then rapidly resuspend the bacterial pellets by vortexing in 100 µl of lysogen extraction buffer.
14. Close the caps of the tubes, and place the tubes in liquid nitrogen.
15. After 2 minutes, recover the tubes from the liquid nitrogen. Hold the tubes in one hand to warm them until the lysates just thaw, and then immediately add to each tube 20 µl of 10 mg/ml lysozyme. Store the tubes for 15 minutes in an ice bath.
16. Add 250 µl of 5 M NaCl to each tube. Mix the contents by flicking the side of each tube with a finger. Incubate the tubes for 30 minutes at 4°C on a rotating wheel.

17. Centrifuge the tubes at maximum speed for 30 minutes at 4°C in a microfuge.
18. Float a Millipore filter (Type VS, 0.025-µm pore size) on the surface of a Petri dish (150 mm) filled with lysogen extraction buffer at 4°C.
19. Transfer the supernatants from the centrifuge tubes to the upper surface of the filter. Up to 20 different samples can be applied to the same filter.
20. After 1-2 hours at 4°C, transfer the dialyzed samples to fresh microfuge tubes, which can be stored at -70°C until needed.
21. Analyze the cell lysates directly for the presence of DNA-binding proteins, for example, in methylation protection experiments, by DNase I footprinting, or in gel electrophoresis DNA-binding assays (electrophoretic mobility-shift assays) (please see Chapter 17).

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1. Singh H. 1993. Specific recognition site probes for isolating genes encoding DNA-binding proteins. *Methods Enzymol.* 218:551-567.
2. Snyder M., Elledge S., Sweetser D., Young R.A., and Davis R.W. 1987. λgt 11: Gene isolation with antibody probes and other applications. *Methods Enzymol.* 154:107-128.

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Protocol 8

Preparation of Lysates Containing Fusion Proteins Encoded by Bacteriophage λ: Lytic Infections on Agar Plates

A lytic infection by a recombinant bacteriophage is established in soft agarose. Following induced expression, the recombinant fusion protein is recovered from the infected cells.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Control overlay solution
  6 ml is required for each recombinant plaque analyzed.
- IPTG overlay solution
  12 ml is required for each recombinant plaque analyzed.
- $MgSO_4 \cdot 7H_2O$ (1 M)
- SM

Media

- LB agar plates (150 mm)
  Freshly poured plates that have been equilibrated to room temperature give the best results.
- LB medium containing 50 µg/ml ampicillin
- LB top agarose containing 10 mM $MgSO_4$ and 50 µg/ml ampicillin

Vectors and Bacterial Strains

Bacteriophage λgt11 recombinant
Prepare a stock of recombinant bacteriophage λ of known titer by soaking an individual plaque in approx. 100 µl of SM for at least 2 hours at room temperature or by preparing a plate lysate (please see Chapter 2, Protocol 3).

E. coli strain Y1090*hsdR*
This strain is available from the ATCC (www.atcc.org) and is maintained on LB-agar plates containing 50 µg/ml ampicillin.

METHOD

1. Inoculate 50 ml of LB medium containing 50 µg/ml ampicillin with a single colony of *E. coli* Y1090*hsdR*. Grow the culture overnight at 37°C with moderate agitation (250 cycles/minute in a rotary shaker).
2. Transfer the culture to a centrifuge tube, and centrifuge the cells at 4000*g* (5800 rpm in a Sorvall SS-34 rotor) for 10 minutes at room temperature.
3. Discard the supernatant, and resuspend the cell pellet in 20 ml of 10 mM $MgSO_4$. Measure the OD_{600} of a 1/100 dilution of the resuspended cells and prepare a plating stock by diluting the resuspended cells to a final concentration of 2.0 OD_{600}/ml with 10 mM $MgSO_4$.
4. Transfer three 0.2-ml aliquots of the plating stock of *E. coli* Y1090*hsdR* to fresh tubes. To two of the tubes, add 2 x 10^5 to 5 x 10^6 pfu of the recombinant bacteriophage λgt11 stock. The third tube serves as an uninfected control. Incubate the tubes for 20 minutes at 37°C to allow the virus to attach to the cells.
5. Add 7.5 ml of molten LB top agarose containing 10 mM $MgSO_4$ and 50 µg/ml ampicillin to one of the tubes. Mix the contents, and immediately pour the top agarose onto a 150-mm LB agar plate.
6. Repeat Step 5 with each of the remaining tubes.
7. Incubate the agar plates for 4 hours at 42°C.
8. Remove the plates from the incubator. Add 6 ml of control overlay solution to one of the infected plates. Add 12 ml of IPTG overlay solution to the two remaining plates.
9. Return the plates to the 42°C incubator for 3-5 hours.
10. Remove the plates from the incubator and transfer the overlay solutions into individual sterile tubes.
11. Detect the fusion protein in the overlay solution by immunoblotting or by DNA-binding assays if the original phage was isolated as described in Chapter 14, Protocol 6.
12. Purify the β-galactosidase fusion protein from the overlay solution by affinity chromatography using commercially available kits (e.g., Promega ProtoSorb), or as described in Chapter 15, Protocol 1. Dialyze the overlay solution before purification of the protein to remove IPTG.

REFERENCES

1. Huang S.H. and Jong A. 1994. Efficient induction and preparation of fusion proteins from recombinant phage λgt11 clones. *Trends Genet.* 10:183.

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Protocol 9

Preparation of Lysates Containing Fusion Proteins Encoded by Bacteriophage λ: Lytic Infections in Liquid Medium

This rapid method is used to screen bacteriophage λgt11 clones for the production of immunodetectable fusion proteins. After optimizng the conditions of infection and induction, the method can be used to produce preparative amounts of a fusion protein.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Cell lysis buffer (14-9)
  100 µl is required for each plaque analyzed.
- IPTG (1 M)
  70 µl is required for each plaque analyzed.
- $MgSO_4 \cdot 7H_2O$ (1 M)
- ⚠ PMSF (phenylmethysulfonyl fluoride) (100 mM)
- SM
  Discard each aliquot after use to minimize contamination.

Media

- LB medium containing 50 µg/ml ampicillin

Vectors and Bacterial Strains

Bacteriophage λgt11 recombinant
Prepare stocks of bacteriophage λ recombinants by soaking individual plaques in approx. 1 ml of SM for at least 2 hours at room temperature or by preparing a plate lysate (please see Chapter 2, Protocol 3).

E. coli strain Y1090*hsdR*
This strain is available from the ATCC (www.atcc.org).

METHOD

1. Inoculate 50 ml of LB medium containing 50 µg/ml ampicillin with a single colony of *E. coli* Y1090*hsdR*. Grow the culture overnight at 37°C with moderate agitation (250 cycles/minute in a rotary shaker).
2. Transfer the culture to a centrifuge tube, and centrifuge the cells at 4000*g* (5800 rpm in a Sorvall SS-34 rotor) for 10 minutes at room temperature.
3. Discard the supernatant, and resuspend the cell pellet in 25 ml of 10 mM $MgSO_4$. Store the bacterial suspension on ice until required.
4. In sterile 15-ml tubes, mix 8 ml of LB containing 50 µg/ml ampicillin, 400 µl of bacterial suspension, and 100 µl of phage lysate.
5. Place the tubes in a 37°C shaking water bath for 2 hours.
6. Transfer a 1-ml aliquot of each culture of infected cells to a sterile microfuge tube. Store the tightly capped tubes in liquid nitrogen. Add 70 µl of 1 M IPTG to the remainder of the infected cultures and continue the incubation at 37°C.
7. At hourly intervals thereafter, withdraw 1-ml aliquots of each culture of infected cells to microfuge tubes. Store the tightly capped tubes in liquid nitrogen. Collect aliquots in this fashion for a period of 4 hours.
8. Incubate the remainder of the infected cultures for a further 12 hours at 37°C. Remove a final 1-ml sample from each culture. Place the tightly capped tubes in liquid nitrogen for 30 minutes.
9. Thaw all the samples and collect the infected bacteria by centrifugation at maximum speed for 1 minute at room temperature in a microfuge. Decant and discard the bacterial medium.
10. Add 100 µl of Cell Lysis Buffer (14-9) to each tube and rapidly resuspend the cell pellets by vigorous vortexing.
11. Place the samples in a boiling-water bath for 3 minutes. Transfer the samples to room temperature, add 1 µl of 100 mM PMSF, and mix the contents of the tubes by vortexing.
12. Analyze the samples directly by SDS-polyacrylamide gel electrophoresis and immunoblotting. Just before electrophoresis, spin the samples at maximum speed for 1 minute in a microfuge. Load a 25-µl aliquot of the supernatant from this spin onto the SDS-polyacrylamide gel.

REFERENCES

1. Runge S.W. 1992. Rapid analysis of λgt11 fusion proteins without subcloning or lysogen induction. *BioTechniques* 12:630-631.

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Chapter 15 Expression of Cloned Genes in *Escherichia coli*

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Protocol 1

Expression of Cloned Genes in *E. coli* Using IPTG-inducible Promoters

This protocol describes how (1) to clone cloned sequences encoding open reading frames in plasmids carrying IPTG-inducible promoters, (2) to optimize expression of target proteins in transformants carrying these recombinants, and (3) to scale-up production of foreign proteins.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Coomassie Brilliant Blue stain or Silver stain
- IPTG (1 M)
- 1x SDS gel-loading buffer

Nucleic Acids and Oligonucleotides

Gene or cDNA fragment of interest

Media

- LB agar plates containing 50 µg/ml ampicillin
- LB medium containing 50 µg/ml ampicillin

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 8, Protocol 7.

Step 2 of this protocol requires the reagents listed in Chapter 1, Protocol 17 or Chapter 1, Protocol 19.

Step 3 of this protocol requires the reagents listed in Chapter 1, Protocol 23 to Chapter 1, Protocol 26.

Step 4 of this protocol requires the reagents listed in Chapter 12, Protocol 3.

Vectors and Bacterial Strains

E. coli strain suitable for transformation and carrying either the *lacI*^{q} or *lacI*^{q1} allele
Some IPTG-inducible expression vectors carry the lacI^{q} *allele on the expression plasmid (e.g., pMAL and pGEX). These can be used in any laboratory strain of* E. coli *(e.g., JM101, DH5F', and TG1).*

IPTG-inducible expression vector
*Other examples include pGEM-3Z (Promega), pGEX-1 (Pharmacia), pKK223-3 (Pharmacia), pMEX (U.S. Biochemicals), p*Trc *99A (Pharmacia), and pMAL (New England Biolabs).*

Positive control plasmid (e.g., an IPTG-inducible vector known to express a LacZ fusion protein of defined size)

METHOD

1. Modify by PCR (Chapter 8, Protocol 7), or isolate by restriction enzyme digestion, a fragment of DNA carrying 5'- and 3'-restriction enzyme sites compatible with sites in an IPTG-inducible expression vector.
   Constructs generated by PCR should be sequenced to ensure that no spurious mutations were introduced during the amplification reactions.
2. Ligate the DNA fragment containing the cDNA/gene of interest into the expression vector (Chapter 1, Protocol 17 or Chapter 1, Protocol 19).
3. Transform an *E. coli* strain containing the *lacI*^{q} allele with the recombinant plasmid. If the plasmid vector itself carries the *lacI* gene, then any appropriate strain of *E. coli* can be used. Plate aliquots of the transformation reaction on LB agar containing 50 µg/ml ampicillin. Incubate the cultures overnight at 37°C.
4. Screen transformants by colony hybridization and/or restriction enzyme analysis, oligonucleotide hybridization, or direct DNA sequence analysis (please see Chapter 12, Protocol 3) of plasmid minipreparations.
5. Inoculate 1-ml cultures (LB medium containing 50 µg/ml ampicillin) with 1 or 2 colonies containing the empty expression vector, the positive control plasmid (pGEX-1), and the recombinant expression plasmid. Incubate the cultures overnight at the appropriate temperature (20-37°C).
6. Inoculate 5 ml of LB medium containing 50 µg/ml ampicillin with 50 µl of each overnight culture. Incubate the cultures for >2 hours at 20-37°C in a shaking incubator until cells reach mid-log growth (A_{550} of 0.5-1.0).
   It is important to monitor the number of bacteria inoculated into the growth medium, the length of time cells are grown before induction, and the density to which cells are grown after induction.
7. Transfer 1 ml of each uninduced culture (zero-time aliquot) to microfuge tubes. Immediately process the zero-time aliquots as described in Steps 9 and 10.
8. Induce the remainder of each culture by adding IPTG to a final concentration of 1 mM and continue incubation at 20-37°C with aeration.
   But please see the notes below Step 8 in the print version of the manual concerning optimization of the IPTG concentration and the induction temperature).
9. *At various time points during the induction period (e.g., 1, 2, 4, and 6 hours), transfer 1 ml of each culture to a microfuge tube, measure the A_{550} in a spectrophotometer, and centrifuge the tubes at maximum speed for 1 minute at room temperature in a microfuge. Remove the supernatants by aspiration.*
10. *Resuspend each pellet in 100 µl of 1x SDS gel-loading buffer, and heat the samples to 100°C for 3 minutes. Centrifuge the tubes at maximum speed for 1 minute at room temperature in a microfuge, and store them on ice until all of the samples are collected and ready to load on a gel.*
11. *Warm the samples to room temperature and load 0.15 OD_{550} units (of original culture) or 40 µg of each suspension on a 10% SDS-polyacrylamide gel.*
12. *Run the gel at 8-15 V/cm until the bromophenol blue reaches the bottom of the resolving gel.*
13. *Stain the gel with Coomassie Brilliant Blue or silver, or carry out an immunoblot to visualize the induced protein.*
14. *For large-scale expression and purification of the target protein, inoculate 50 ml of LB containing 50 µg/ml ampicillin in a 250-ml flask with a colony of E. coli containing the recombinant construct. Incubate the culture overnight at 20-37°C.*
15. *Inoculate 450-500 ml of LB containing 50 µg/ml ampicillin in a 2-liter flask with 5-50 ml of overnight culture of E. coli. Incubate with shaking at 20-37°C until the culture has reached the mid-log phase of growth (A_{550} = 0.5-1.0).*
16. *Induce expression of the target protein based on the optimal values of IPTG concentration, incubation time, and incubation temperature determined in the previous section.*
17. *After the induced cells have grown for the proper length of time, harvest the cells by centrifugation at 5000g (5500 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C and proceed with a purification protocol:*
    - Chapter 15, Protocol 5 if the expressed protein is a fusion with glutathione *S*-transferase
    - Chapter 15, Protocol 6 if the expressed protein is a fusion with maltose-binding protein
    - Chapter 15, Protocol 7 if the expressed protein contains a polyhistidine tag

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Protocol 2

Expression of Cloned Genes in *E. coli* Using the Bacteriophage T7 Promoter

This protocol describes how (1) to clone cloned sequences encoding open reading frames in plasmids carrying bacteriophage T7 promoters, (2) to optimize expression of target proteins in transformants carrying these recombinants, and (3) to scale-up production of foreign proteins.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Coomassie Brilliant Blue stain or Silver stain
- IPTG (1 M)
- 1x SDS gel-loading buffer

Nucleic Acids and Oligonucleotides

Gene or cDNA fragment of interest

Media

- NZCYM agar plates containing 50 µg/ml ampicillin
- NZCYM medium containing 50 µg/ml ampicillin

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 8, Protocol 7.

Step 2 of this protocol requires the reagents listed in Chapter 1, Protocol 17 or Chapter 1, Protocol 19.

Step 3 of this protocol requires the reagents listed in Chapter 1, Protocol 23 to Chapter 1, Protocol 26.

Step 4 of this protocol requires the reagents listed in Chapter 12, Protocol 3.

Vectors and Bacterial Strains

E. coli strains HMS174(DE3) or BL21(DE3)

pET vector or equivalent
For the many variations of this plasmid series, please refer to the Novagen catalog (or Web Site at www.novagen.com).

Positive control plasmid (e.g., carrying a bacteriophage T7 promoter that controls expression of a fusion protein of defined size)

METHOD

1. Modify by PCR (Chapter 8, Protocol 7), or isolate by restriction enzyme digestion, a fragment of DNA carrying 5'- and 3'-restriction enzyme sites compatible with sites in a bacteriophage T7 promoter expression plasmid (e.g., pET vectors).
2. Ligate the DNA fragment containing the cDNA/gene of interest into the expression vector (Chapter 1, Protocol 17 or Chapter 1, Protocol 19).
3. Transform *E. coli* strain BL21(DE3) or HMS174(DE3) with aliquots of the ligation reaction. Select for ampicillin-resistant transformants by plating aliquots of the transformation reaction on NZCYM agar plates containing 50 µg/ml ampicillin. Incubate the plates overnight at 37°C.
4. Screen the transformants by colony hybridization and/or restriction enzyme analysis, oligonucleotide hybridization, or direct DNA sequence analysis (please see Chapter 12, Protocol 3) of plasmid minipreparations.
5. Inoculate 1-ml cultures (NZCYM medium containing 50 µg/ml ampicillin) with a transformed colony containing positive control vectors, negative control vectors, and one containing the recombinant vector. Incubate the cultures overnight at 37°C to obtain a saturated culture.
6. Inoculate 5 ml of NZCYM medium containing 50 µg/ml ampicillin in a 50-ml flask with 50 µl of a saturated culture. Incubate the cultures for 2 hours at 37°C.
   It is important to monitor the number of bacteria inoculated into the growth medium, the length of time cells are grown before induction, and the density to which cells are grown after induction.
7. Transfer 1 ml of each culture (zero-time aliquot) to a microfuge tube. Immediately process the zero-time aliquots as described in Steps 9 and 10.
8. Induce the remainder of each culture by adding IPTG to a final concentration of 1.0 mM and continue incubation at 20-37°C with aeration.
9. At 0.5, 1, 2, and 3 hours after induction, transfer 1 ml of each culture to a microfuge tube, measure the A_{550} in a spectrophotometer, and centrifuge the tubes at maximum speed for 1 minute at room temperature in a microfuge. Remove the supernatants by aspiration.
10. Resuspend each pellet in 100 µl of 1x SDS gel-loading buffer, and heat the samples to 100°C for 3 minutes. Centrifuge the tubes at maximum speed for 1 minute at room temperature in a microfuge, and store them on ice until all of the samples are collected and ready to load on a gel.
11. Warm the samples to room temperature and load 0.15 OD_{550} units (of original culture) or 40 µg of each suspension on a 10% SDS-polyacrylamide gel.
12. Run the gel at 8-15 V/cm until the bromophenol blue reaches the bottom of the resolving gel.
13. Stain the gel with Coomassie Brilliant Blue or silver, or carry out an immunoblot to visualize the induced protein.
14. For large-scale expression and purification of the target protein, inoculate 50 ml of NZCYM containing 50 µg/ml ampicillin in a 250-ml flask with individual colonies of *E. coli* containing the recombinant and control plasmids. Incubate the cultures overnight at 37°C.
15. Inoculate 450-500 ml of NZCYM containing 50 µg/ml ampicillin in a 2-liter flask with 5-50 ml of overnight culture of *E. coli* containing the recombinant plasmid. Incubate the culture with shaking at 37°C until the culture has reached the mid-log phase of growth (A_{550} = 0.5-1.0).
16. Induce expression of the target protein based on the optimal values of IPTG concentration, incubation time, and incubation temperature determined in the previous section.
17. After the induced cells have grown for the proper length of time, harvest the cells by centrifugation at 5000*g* (5500 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C and proceed with a purification protocol:
    - Chapter 15, Protocol 5 if the expressed protein is a fusion with glutathione *S*-transferase
    - Chapter 15, Protocol 6 if the expressed protein is a fusion with maltose-binding protein
    - Chapter 15, Protocol 7 if the expressed protein contains a polyhistidine tag

REFERENCES

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Protocol 3

Expression of Cloned Genes in *E. coli* Using the Bacteriophage λ p_L Promoter

This protocol describes how (1) to clone cloned sequences encoding open reading frames in plasmids carrying bacteriophage λ p_L promoters, (2) to optimize expression of target proteins in transformants carrying these recombinants, and (3) to scale-up production of foreign proteins.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Coomassie Brilliant Blue stain or Silver stain
- 1x SDS gel-loading buffer

L-Tryptophan (10 mg/ml)

Nucleic Acids and Oligonucleotides

Gene or cDNA fragment of interest

Media

- LB agar plates
- LB medium
  Depending on the vector used, the LB medium and agar plates will require supplementation with various antibiotics.
- LB medium heated to 65°C
  Optional, please see Step 13.
- M9 minimal medium
  After sterilization, supplement this medium with 0.5% (w/v) glucose, 0.2% (w/v) casamino acids, and antibiotics as needed. This medium is used with tryptophan-inducible c*I-gene-based* p_L *expression vectors.*

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 8, Protocol 7.

Step 2 of this protocol requires the reagents listed in Chapter 1, Protocol 17 or Chapter 1, Protocol 19.

Step 3 of this protocol requires the reagents listed in Chapter 1, Protocol 23 to Chapter 1, Protocol 26.

Step 4 of this protocol requires the reagents listed in Chapter 12, Protocol 3.

Vectors and Bacterial Strains

E. coli harboring either the ts857 allele or wild-type allele of the bacteriophage λ *c*I gene
In strain M5219, the c*Its857 allele is inducible by heat shock. Strain GI724 (ATCC 55151* c*I gene) harbors a tryptophan-inducible wild-type* c*I gene.*

p_L Expression vector (e.g., pHUB, pPLc, pKC30, pAS1, pCQV2, pAL-781, and pTrxFus)

Positive control (e.g., a p_L expression vector encoding a fusion protein of defined size)

METHOD

1. Modify by PCR (Chapter 8, Protocol 7), or isolate by restriction enzyme digestion, a fragment of DNA carrying 5'- and 3'-restriction enzyme sites compatible with sites in a p_L expression vector.
   Constructs generated by PCR should be sequenced to ensure that no spurious mutations were introduced during the amplification reactions.
2. Ligate the DNA fragment containing the cDNA/gene of interest into the expression vector (Chapter 1, Protocol 17 or Chapter 1, Protocol 19).
3. Transform an *E. coli* strain containing the c*Its*857 allele or wild-type *c*I gene with aliquots of the ligation reaction. Plate aliquots of the transformation reaction on LB medium containing the appropriate selective antibiotic (usually ampicillin at 50 µg/ml), and incubate the cultures overnight at 30°C (strains harboring the c*Its*857 allele) or at 37°C (strains harboring a tryptophan-inducible wild-type *c*I gene).
4. Screen the transformants by colony hybridization and/or restriction enzyme analysis, oligonucleotide hybridization, or direct DNA sequence analysis of plasmid minipreparations (please see Chapter 12, Protocol 3).
5. Determine the optimum conditions for the induction of target protein expression, which is driven by the down-regulation of the *c*I repressor protein, either by an increase in temperature or by the presence of tryptophan.
   It is important to monitor the number of bacteria inoculated into the growth medium, the length of time cells are grown before induction, and the density to which cells are grown after induction.

   When using a temperature-inducible system

   a. Inoculate 1-ml cultures of LB medium containing the appropriate antibiotics with 1 or 2 colonies of *E. coli* (carrying the c*Its*857 allele) containing the empty expression vector, and 1 or 2 colonies containing the recombinant expression vector. Incubate the cultures overnight at 30°C.
   b. Inoculate 10 ml of LB medium containing antibiotic in a 50-ml flask with 50 µl of an overnight culture. Grow the culture to the mid-log phase of growth (A_{550} = 0.5-1.0) at 30°C.
   c. Transfer 1 ml of each uninduced culture (zero-time aliquot) to microfuge tubes. Immediately process the zero-time aliquots as described in Steps 6 and 7.
   d. Induce the remainder of each culture by shifting the incubation temperature to 40°C. Proceed to Step 6.

   When using a tryptophan-inducible system

   a. Inoculate 1-ml cultures of supplemented M9 medium with 1 or 2 colonies of *E. coli* (carrying the *c*I wild-type allele) containing the empty expression vector, and 1 or 2 colonies containing the recombinant expression vector. Incubate the cultures overnight at 37°C.
   b. Inoculate 10 ml of supplemented M9 medium in a 50-ml flask with 50 µl of the overnight cultures. Grow the cultures to the mid-log phase of growth (A_{550} = 0.5-1.0) at 37°C.
   c. Transfer 1 ml of each uninduced culture (zero-time aliquot) to microfuge tubes. Immediately process the zero-time aliquots as described in Steps 6 and 7.
   d. Induce the remainder of each culture by adding tryptophan to a final concentration of 100 µg/ml and continue incubation at 37°C.
6. At various time points during the induction period (e.g., 1, 2, 4, and 6 hours), transfer 1 ml of each culture to a

microfuge tube, measure the A_{550} in a spectrophotometer, and centrifuge the tubes at maximum speed for 1 minute at room temperature in a microfuge. Remove the supernatants by aspiration.

7. Resuspend each pellet in 100 µl of 1x SDS gel-loading buffer, and heat the samples to 100°C for 3 minutes. Centrifuge the tubes at maximum speed for 1 minute at room temperature in a microfuge and store them on ice until all of the samples are collected and ready to load on a gel.
8. Warm the samples to room temperature and load 0.15 OD_{550} units (of original culture) or 40 µg of each suspension on a 10% SDS-polyacrylamide gel.
9. Run the gel at 8-15 V/cm until the bromophenol blue reaches the bottom of the resolving gel.
10. Stain the gel with Coomassie Brilliant Blue or silver, or carry out an immunoblot to visualize the induced protein.
11. For large-scale expression and purification of the target protein, inoculate 50 ml of LB containing antibiotic or supplemented M9 medium in a 250-ml flask with a colony of *E. coli* containing the recombinant construct. Incubate the cultures overnight at 30°C or at 37°C, respectively.
12. Inoculate 450 ml of LB plus antibiotic or supplemented M9 medium in a 2-liter flask with 50-ml overnight cultures of *E. coli* containing the recombinant plasmids. Incubate the cultures with agitation at 30°C or 37°C, respectively, until the cultures have reached the mid-log phase of growth (A_{550} = 0.5-1.0). Induce the culture according to either Step 13 or 14, as appropriate.
13. Induce the *E. coli* culture carrying the c*Its*857 allele by the addition of 500 ml of LB medium heated to 65°C. Incubate the culture at 40°C for the optimum time period determined in the previous section.
14. Induce the *E. coli* culture carrying the c*I* allele by the addition of tryptophan to 100 µg/ml. Incubate the culture at 37°C for the optimum time period determined in the previous section.
15. After the induced cells have grown for the proper length of time, harvest the cells by centrifugation at 5000*g* (5500 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C and proceed with a purification protocol:
    - Chapter 15, Protocol 5 if the expressed protein is a fusion with glutathione *S*-transferase
    - Chapter 15, Protocol 6 if the expressed protein is a fusion with maltose-binding protein
    - Chapter 15, Protocol 7 if the expressed protein contains a polyhistidine tag

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Protocol 4

Expression of Secreted Foreign Proteins Using the Alkaline Phosphatase Promoter (*phoA*) and Signal Sequence

Secretion of a foreign protein into the periplasmic space of *E. coli* is accomplished by fusing the coding sequence downstream from a segment of DNA encoding the *phoA* signal peptide. The signal peptide is removed as the foreign protein is exported from the cytoplasm, in a cleavage reaction catalyzed by signal peptidase.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Coomassie Brilliant Blue stain or Silver stain
- Micronutrients (used in 10x MOPS salts)
- 10x MOPS salts (used in induction medium)
- Neutral phosphate buffer (1 M) (used in induction medium)
  An equimolar mix of 1 M Na_2HPO_4 and 1 M NaH_2PO_4.
- 1x SDS gel-loading buffer

Nucleic Acids and Oligonucleotides

Gene or cDNA fragment of interest

Media

- Induction medium
- LB agar plates containing 50 µg/ml ampicillin
- LB medium containing 50 µg/ml ampicillin

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 8, Protocol 7.

Step 2 of this protocol requires the reagents listed in Chapter 1, Protocol 17 or Chapter 1, Protocol 19.

Step 3 of this protocol requires the reagents listed in Chapter 1, Protocol 23 to Chapter 1, Protocol 26.

Step 4 of this protocol requires the reagents listed in Chapter 12, Protocol 3.

Vectors and Bacterial Strains

E. coli strain
Essentially any strain of E. coli *can be used to express genes cloned in* phoA *vectors.*
The various strains should be grown at different temperatures to maximize expression levels and protein stability (please see Chapter 15, Protocol 1).

pTA1529 or pBAce
Plasmid pTA1529 is described by Oka et al. (1985). pBAce is available from C.C. Wang (Department of Pharmaceutical Chemistry, University of California, San Francisco).

METHOD

1. Modify by PCR (Chapter 8, Protocol 7), or isolate by restriction enzyme digestion, a fragment of DNA carrying 5'- and 3'-restriction enzyme sites compatible with the polycloning sites in pTA1529 or pBAce.
2. Ligate the DNA fragment containing the cDNA/gene of interest into the expression vector (Chapter 1, Protocol 17 or Chapter 1, Protocol 19).
3. Transform an appropriate *E. coli* strain with aliquots of the ligation reaction. Plate aliquots of the transformation reaction on LB agar containing 50 µg/ml ampicillin, and incubate the cultures overnight at 37°C.
4. Screen the transformants by colony hybridization and/or restriction enzyme analysis, oligonucleotide hybridization, or direct DNA sequence analysis (please see Chapter 12, Protocol 3) of plasmid minipreparations.
5. Inoculate 1-ml cultures of LB medium containing 50 µg/ml ampicillin with one to two colonies containing the empty expression vector, and one to two colonies containing the recombinant and control plasmids. Incubate the cultures overnight at 37°C.
6. Inoculate 5 ml of induction medium containing 50 µg/ml ampicillin in a 50-ml flask with 50 µl of an overnight culture. Incubate the cultures with shaking at 20-37°C.
   It is important to monitor the number of bacteria inoculated into the growth medium, the length of time cells are grown before induction, and the density to which cells are grown after induction.
7. At various time points after inoculation (e.g., 0, 6, 12, 18, and 24 hours), transfer 1 ml of each culture to a microfuge tube, measure the A_{550} in a spectrophotometer, and centrifuge the tubes at maximum speed for 1 minute at room temperature in a microfuge. Remove the supernatants by aspiration.
8. Resuspend each pellet in 100 µl of 1x SDS gel-loading buffer, and heat the samples to 100°C for 3 minutes. Centrifuge the tubes at maximum speed for 1 minute at room temperature in a microfuge, and store them on ice until all of the samples are collected and ready to load on a gel.
9. Warm the samples to room temperature and load 0.15 OD_{550} units (of original culture) or 40 µg of each suspension on a 10% SDS-polyacrylamide gel.
10. Run the gel at 8-15 V/cm until the bromophenol blue reaches the bottom of the resolving gel.
11. Stain the gel with Coomassie Brilliant Blue or silver, or carry out an immunoblot to visualize the induced protein.
12. For large-scale expression and purification of the target protein, inoculate 25 ml of LB medium containing 50 µg/ml ampicillin in a 125-ml flask with a colony of *E. coli* containing the recombinant *phoA* construct. Incubate the culture overnight with agitation at 37°C.
13. Collect the cells by centrifugation at 5000*g* (6500 rpm in a Sorvall SS-34 rotor) for 15 minutes. Resuspend the cell pellet in 25 ml of induction medium, and collect the cells again by centrifugation.
14. Resuspend the washed cells in 2.5 ml of fresh induction medium, and inoculate the cells into 500 ml of induction medium in a 2-liter flask. Incubate the large-scale culture at the optimum temperature and for the optimum time determined in the previous section.
15. After the cells have grown for the proper length of time, harvest the cells by centrifugation at 4000*g* (5000 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C.

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Protocol 5

Purification of Fusion Proteins by Affinity Chromatography on Glutathione Agarose

Recombinant proteins, constructed in pGEX vectors, are fused to glutathione *S*-transferase (GST) and can be purified to near homogeneity by affinity chromatography on glutathione-agarose. Bound GST-fusion proteins are readily displaced from the column by elution with buffers containing free glutathione.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- DTT (dithiothreitol) (1 M)
- Glutathione elution buffer
- PBS (4°C)

Triton X-100 (0.2% v/v)

Enzymes and Buffers

DNase (5 mg/ml)

- Lysozyme

Protease for cleavage of fusion protein
Use thrombin, enterokinase, or Factor Xa solution and follow the manufacturer's instructions for the preparation and storage of these proteases.

RNase (5 mg/ml)

Vectors and Bacterial Strains

E. coli cells expressing a recombinant GST fusion protein (cell pellet generated in Step 17 of Chapter 15, Protocol 1 or Chapter 15, Protocol 2)

METHOD

1. Gently invert the container of the glutathione-agarose resin to mix the slurry.
2. Transfer an aliquot of the slurry to a 15-ml polypropylene tube (2 ml of the slurry will be needed for each 100 ml of the original bacterial culture).
3. Centrifuge the tube at 500*g* (2100 rpm in a Sorvall SS-34 rotor) for 5 minutes at 4°C. Carefully remove and discard the supernatant.
4. Add 10 bed volumes of cold PBS to the resin, and mix the slurry by inverting the tube several times. Centrifuge the tube at 500*g* (2100 rpm in a Sorvall SS-34 rotor) for 5 minutes at 4°C. Carefully remove and discard the supernatant.
5. Add 1 ml of cold PBS per milliliter of resin to make a 50% slurry. Mix the slurry by inverting the tube several times. Keep the suspension on ice until the cell extract has been prepared.
6. Resuspend the cell pellet (e.g., from Chapter 15, Protocol 1, Step 17) in 4 ml of PBS per 100 ml of cell culture.
7. Add lysozyme to a final concentration of 1 mg/ml and incubate the cell suspension on ice for 30 minutes.
8. Add 10 ml of 0.2% Triton X-100. Use a syringe to inject the solution forcibly into the viscous cell lysate. Shake the tube vigorously several times to mix the solution of detergent and cell lysate. Add DNase and RNase to the tube each to a final concentration of 5 µg/ml, and continue the incubation with rocking for 10 minutes at 4°C. Remove the insoluble debris by centrifugation at 3000*g* (5000 rpm in a Sorvall SS-34 rotor) for 30 minutes at 4°C. Collect the supernatant (cell lysate) in a fresh tube. Add dithiothreitol to a final concentration of 1 mM.
   It may be necessary to pass the supernatant through a 0.45-µm filter to prevent clogging of the resin during purification of the GST-fusion protein.
9. Combine the cell lysate with an appropriate amount of the 50% slurry of glutathione-agarose resin in PBS. Use 2 ml of slurry for each 100 ml of bacterial culture used to make the protein extract. Shake the mixture gently for 30 minutes at room temperature.
10. Centrifuge the mixture at 500*g* (2100 rpm in a Sorvall SS-34 rotor) for 5 minutes at 4°C. Carefully remove the supernatant. Save a small amount of the supernatant to analyze by SDS-polyacrylamide gel electrophoresis.
11. Wash unbound proteins from the resin by adding 10 bed volumes of PBS to the pellet, and mix by inverting the tube several times.
12. Centrifuge at 500*g* (2100 rpm in a Sorvall SS-34 rotor) for 5 minutes at 4°C. Carefully remove the supernatant. Save a small amount of the supernatant to analyze by SDS-polyacrylamide gel electrophoresis.
13. Repeat Steps 11 and 12 two more times.
14. Bound GST fusion protein may be eluted from the resin using glutathione elution buffer. Alternatively, GST fusion proteins may be cleaved while still bound to the gel with an appropriate protease such as thrombin, enterokinase, or Factor Xa, liberating the protein of interest from the GST moiety.

    Elution of the fusion protein using glutathione

    a. Elute the bound protein from the resin by adding 1 bed volume of glutathione elution buffer to the pellet. Incubate the tube with gentle agitation for 10 minutes at room temperature.

    b. Centrifuge the tube as in Step 12. Transfer the supernatant (which contains the eluted fusion protein) to a fresh tube.

    c. Repeat Steps a and b twice more, pooling all three supernatants.
    Depending on the particular fusion protein being purified, a significant amount of protein may remain bound to the gel following the elution steps. The volume of elution buffer and the elution times may vary among fusion proteins. Additional elutions may be required. Monitor the eluates for GST protein by SDS-polyacrylamide gel electrophoresis.

    Proteolytic cleavage of the target protein from the bound GST moiety

    a. Add a protease such as thrombin, enterokinase, or Factor Xa (as appropriate for the cleavage site within the fusion protein) to the beads. Use 50 units of the appropriate protease in 1 ml of PBS for each milliliter of resin volume. Mix the solution by inverting the tube several times, and incubate the mixture with shaking for 2-16 hours at room temperature. The exact time should be determined empirically using small-scale reactions.

    b. Centrifuge the tube at 500g (2100 rpm in a Sorvall SS-34 rotor) for 5 minutes at 4°C. Carefully transfer the supernatant to a fresh tube.
15. Analyze the protein profile of each step (cell extract, washes, and elution) on a 10% SDS-polyacrylamide gel.

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Protocol 6

Purification of Maltose-binding Fusion Proteins by Affinity Chromatography on Amylose Resin

Foreign proteins fused to maltose-binding protein can be readily purified to near homogeneity by affinity chromatography on resins containing cross-linked polysaccharides such as amylose.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Cell lysis buffer (15-6) (approx. 150 ml)
- Cell wash buffer (15-6) (approx. 250 ml)
- Column elution buffer (approx. 100 ml)
- Column wash buffer (approx. 250 ml)

$MgCl_2$ (0.1 mM, approx. 250 ml)

⚠ PMSF (100 mM)
Add 17.4 mg of PMSF per milliliter of isopropanol. Store at -20°C.An alternative to PMSF (4-[2-aminoethyl]-benzenesulfonylfluoride, hydrochloride; Pefabloc SC) is available from Boehringer Mannheim. Pefabloc is an irreversible serine protease inhibitor, is used at the same concentration as PMSF, but is nontoxic and stable in aqueous buffer solutions.

- 1x SDS gel-loading buffer
- Tris-Cl (10 mM, pH 7.1)

Enzymes and Buffers

DNase (5 mg/ml)

- Lysozyme

Protease for cleavage of fusion protein
Use thrombin, enterokinase, or Factor Xa solution and follow the manufacturer's instructions for the preparation and storage of these proteases.

RNase (5 mg/ml)

Vectors and Bacterial Strains

E. coli cells expressing a recombinant MBP fusion protein (cell pellet generated in Step 17 of Chapter 15, Protocol 1 or Chapter 15, Protocol 2)

METHOD

1. Pour a 3 x 6-cm column of amylose agarose. Equilibrate in 10 mM Tris-Cl (pH 7.1) at 4°C.
2. Resuspend the cell pellet in 1/10 original culture volume (typically 50 ml) of ice-cold cell wash buffer. Collect the cells by centrifugation at 5000*g* (5500 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C and again resuspend the pellet in 1/10 original culture volume (typically 50 ml) of ice-cold cell wash buffer.
3. Prepare the cell lysate.

If an MBP vector without a signal sequence was used (pMal-c2)

a. Lyse cells by sonication with a microtip sonicator, using three bursts of 10 seconds each. Use a power setting of approx. 30 W and keep the cells cold (0°C) during sonication.

b. Add PMSF to a final concentration of 1 mM and clarify the solution by centrifugation at 87,000*g* (35,000 rpm in a Beckman Ti60 rotor) for 30 minutes at 4°C.
Proceed to purify the fusion protein from the supernatant by amylose-agarose chromatography as described in Step 4.

If an Mbp vector with a signal sequence was used (pMal-p2)

a. Collect the cells by centrifugation at 5000*g* (5500 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C and resuspend the cell pellet in 1/20 original culture volume (typically 25 ml) of ice-cold cell wash buffer.

b. Add PMSF to 1 mM. Stir the cell suspension for 15 minutes at room temperature. Collect the cells by centrifugation at 17,200*g* (12,000 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C.

c. Spread the cell pellet around the sides of the centrifuge tube. Add ice-cold 0.1 mM $MgCl_2$ solution (100 ml/liter original culture volume) and stir the suspension for 10 minutes at 4°C.

d. Centrifuge the shocked cells at 17,200*g* (12,000 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C. Add 10 mM Tris-Cl (pH 7.1) until the pH of the supernatant is 7.1.

e. Filter the supernatant through a 100-ml disposable Nalgene filter (0.45-µm nitrocellulose membrane), and dialyze the filtered solution against 100 volumes 10 mM Tris-Cl (pH 7.1) at 4°C.
Proceed to purify the fusion protein from the supernatant by amylose-agarose chromatography as described in Step 4.

4. Pour the supernatant from Step 3 over the column. Rinse the column with 100 ml of 10 mM Tris-Cl (pH 7.1). Pass 100 ml of column wash buffer through the column.
5. Elute the bound fusion protein with 50 ml of column elution buffer and collect 1-ml fractions.
6. Analyze aliquots of the collected fractions by SDS-polyacrylamide gel electrophoresis to determine the location of the fusion protein in the series of elution fractions. Pool the fractions containing the fusion protein, and store them at -70°C.
7. Use the appropriate protease to cleave the fusion protein from the MBP moiety.
   a. Add thrombin, enterokinase or Factor Xa (as appropriate for the cleavage site within the fusion protein) to the beads. Use 50 units of the appropriate protease in 1 ml of PBS for each milliliter of resin volume. Mix the solution by inverting the tube several times, and incubate the mixture with shaking for 2-16 hours at room temperature.
   b. Centrifuge the tube at 500*g* (2100 rpm in a Sorvall SS-34 rotor) for 5 minutes at 4°C. Carefully transfer the supernatant to a fresh tube.
   The protein of interest can be separated from the protease by conventional chromatography or by SDS-polyacrylamide gel electrophoresis.

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Protocol 7

Purification of Histidine-tagged Proteins by Immobilized Ni^{2+} Absorption Chromatography

Recombinant proteins engineered to have a polyhistidine tail at either the carboxyl or amino terminus can easily be purified in one step by affinity chromatography on a resin carrying chelated nickel ions. Chromatography can be carried out in column or batch formats. After unbound proteins are washed away, the target protein is eluted using imidazole, which typically preserves the antigenic and functional features of the protein.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Binding buffer (15-7) (pH 7.8)
- Imidazole elution buffer (pH 6.0)

Triton X-100 (10% v/v)

- Wash buffer (15-7) (pH 6.0)

Enzymes and Buffers

DNase (5 mg/ml)

- Lysozyme

RNase (5 mg/ml)

Gels

⚠ Polyacrylamide gel (10%) containing SDS
For preparation of SDS-polyacrylamide gels used in the separation of proteins, please see Appendix 8 in the print version of the manual.

Vectors and Bacterial Strains

E. coli cells expressing a recombinant MBP fusion protein (cell pellet generated in Step 17 of Chapter 15, Protocol 1 or Chapter 15, Protocol 2)

METHOD

1. Gently invert the bottle of Ni^{2+}-charged chromatography resin to mix the slurry, and transfer 2 ml to a small polypropylene or glass column. Allow the resin to pack under gravity flow.
2. Wash the resin with 3 column volumes of sterile H_2O.
   One column volume is equivalent to the volume of the settled bed of resin.
3. Equilibrate the resin with 3 column volumes of Binding buffer (15-7) (pH 7.8). The column is now ready for use in Step 9.
4. Resuspend the cell pellet (e.g., from Chapter 15, Protocol 1, Step 17) in 4 ml of Binding buffer (15-7) (pH 7.8) per 100 ml of cell culture.
5. Add lysozyme to a final concentration of 1 mg/ml and incubate the cell suspension on ice for 30 minutes.
   IMPORTANT *Protease inhibitors may be added, but do not add EDTA or other chelators, which will remove the Ni^{2+} from the affinity resin, destroying its ability to bind histidine.*
6. Incubate the mixture on a rocking platform for 10 minutes at 4°C.
7. Add Triton X-100, DNase, and RNase to the tube to final concentrations of 1%, 5 µg/ml, and 5 µg/ml, respectively, and continue the incubation with rocking for another 10 minutes at 4°C.
8. Remove the insoluble debris by centrifugation at 3000*g* (5000 rpm in a Sorvall SS-34 rotor) for 30 minutes at 4°C. Collect the supernatant (cell lysate) in a fresh tube.
   It may be necessary to pass the supernatant through a 0.45-µm filter to prevent clogging of the resin during purification of the GST-fusion protein.
9. Allow the binding buffer above the resin to drain to the top of the column.
10. Immediately load the cell lysate (Step 8) onto the column. Adjust the flow rate to 10 column volumes per hour.
    Ni^{2+} affinity resins will typically bind approx. 8-12 mg of protein per milliliter of resin. The amount of polyhistidine-tagged protein produced in E. coli *will vary depending on the target protein, but it is typically in the range of 1-10 mg of protein per 100 ml of cell culture.*
11. Wash the column with 6 column volumes of Binding buffer (15-7) (pH 7.8).
12. Wash the column with 4 volumes of Wash buffer (15-7) (pH 6.0). Continue washing the column until the A_{280} of the flowthrough is <0.01.
13. Elute the bound protein with 6 volumes of 10 mM Imidazole elution buffer. Collect 1-ml fractions from the column, and monitor the A_{280} of each fraction.
14. Repeat Step 13 using imidazole elution buffers containing increasing concentrations of imidazole (i.e., 50 mM, 100 mM, and 150 mM imidazole).
    Alternatively, elute the protein using a continuous gradient of increasing imidazole concentration from 10 mM to 100 mM. Most His-tagged proteins will elute between 50 mM and 100 mM imidazole.
15. Assay the fractions of interest for the presence of the polyhistidine-tagged protein by analyzing 20-µl aliquots by electrophoresis through a 10% SDS-polyacrylamide gel.

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Protocol 8

Purification of Expressed Proteins from Inclusion Bodies

The expression of foreign proteins at high levels in *E. coli* often results in the formation of inclusion bodies composed of insoluble aggregates of the expressed protein. The inclusion bodies are recovered from bacterial lysates by centrifugation and are washed with Triton X-100 and EDTA to remove as much bacterial protein as possible from the aggregated foreign protein.To obtain soluble protein, the washed inclusion bodies are dissolved in denaturing agents and the released protein is then refolded by gradual removal of the denaturing reagents by dilution or dialysis. The procedure given here has been used to solubilize prorennin inclusion bodies. However, each protein may require a slightly different procedure, which must be determined empirically.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Cell lysis buffer I (15-8)
- Cell lysis buffer II (15-8), ice cold

  Deoxycholic acid
  Use a protein grade of this bile acid/detergent.
- ⚠ HCl (12 M) (concentrated)
- Inclusion-body solubilization buffer I
- Inclusion-body solubilization buffer II
- ⚠ KOH (10 N)
- ⚠ PMSF (100 mM)
- 1x and 2x SDS gel-loading buffer
- Tris-Cl (0.1 M, pH 8.5) with urea
  For use in Method 2 only; please see Step 7. Prepare 0.1 M Tris-Cl (pH 8.5) with increasing concentrations of urea (e.g., 0.5, 1, 2, and 5 M). Make the solution fresh from solid urea and use immediately. Do not use solutions containing urea that have been stored for any period of time, because the urea decomposes.

Enzymes and Buffers

DNase I (1 mg/ml)

- Lysozyme (10 mg/ml)
  Prepare the solution fresh in Tris-Cl (pH 8.0).

Vectors and Bacterial Strains

E. coli cells expressing the protein of interest
Grow 1 liter of E. coli *cells that have been transformed by any of the methods in Chapter 15, Protocol 1 to Chapter 15, Protocol 4 and now expresses the protein of interest as inclusion bodies.*

METHOD

1. Centrifuge 1 liter of the cell culture of *E. coli* expressing the protein of interest at 5000*g* (5500 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C in preweighed centrifuge bottles.
   IMPORTANT *Perform Steps 2-4 at 4°C.*
2. Remove the supernatant and determine the weight of the *E. coli* pellet. For each gram (wet weight) of *E. coli*, add 3 ml of Cell lysis buffer I (15-8). Resuspend the pellet by gentle vortexing or by stirring with a polished glass rod.
3. For each gram of *E. coli*, add 4 µl of 100 mM PMSF and then 80 µl of 10 mg/ml lysozyme. Stir the suspension for 20 minutes.
4. Stirring continuously, add 4 mg of deoxycholic acid per gram of *E. coli*.
5. Store the suspension at 37°C and stir it occasionally with a glass rod. When the lysate becomes viscous, add 20 µl of 1 mg/ml DNase I per gram of *E. coli*.
6. Store the lysate at room temperature until it is no longer viscous (approx. 30 minutes).
7. Purify and wash the inclusion bodies using one of the following two methods.

Method 1: Recover inclusion bodies using Triton X-100

a. Centrifuge the cell lysate at maximum speed for 15 minutes at 4°C in a microfuge.
b. Decant the supernatant. Resuspend the pellet in 9 volumes of Cell lysis buffer II (15-8) at 4°C.
c. Store the suspension for 5 minutes at room temperature.
d. Centrifuge the tube at maximum speed for 15 minutes at 4°C in a microfuge.
e. Decant the supernatant and set it aside for the next step. Resuspend the pellet in 100 µl of H_2O.
f. Remove 10-µl samples of the supernatant and of the resuspended pellet. Mix each sample with 10 µl of 2x SDS gel-loading buffer and analyze the samples by SDS-polyacrylamide gel electrophoresis to determine which fraction contains the protein of interest.
g. If necessary, proceed with Step 8 to solubilize the inclusion bodies.

Method 2: Recover inclusion bodies using urea

a. Centrifuge the cell lysate at maximum speed for 15 minutes at 4°C in a microfuge.
   IMPORTANT *Perform Steps b, d, and f at 4°C.*
   The following steps involve washing and solubilization of inclusion bodies with buffers containing different concentrations of urea.
b. Decant the supernatant. Resuspend the pellet in 1 ml of H_2O per gram of *E. coli*. Transfer 100-µl aliquots to four microfuge tubes and store the remainder of the suspension at 4°C.
c. Centrifuge the 100-µl aliquots at maximum speed for 15 minutes at 4°C in a microfuge.
d. Discard the supernatants. Resuspend each pellet in 100 µl of 0.1 M Tris-Cl (pH 8.5) containing a different concentration of urea (e.g., 0.5, 1, 2, and 5 M).
e. Centrifuge the tubes at maximum speed for 15 minutes at 4°C in a microfuge.
f. Decant the supernatants and set them aside for the next step. Resuspend each pellet in 100 µl of H_2O.
g. Remove 10-µl samples of each supernatant and each resuspended pellet. Mix each sample and resuspended pellet with 10 µl of 2x SDS gel-loading buffer and analyze by SDS-polyacrylamide gel electrophoresis to determine which concentration of urea yields the best recovery of the inclusion bodies.
h. Use the appropriate concentration of urea, determined in Step g, to wash the remaining pellet (from Step b) as described in this method.

i. If necessary, proceed with Step 8 to solubilize the inclusion bodies.

8. Centrifuge the appropriate resuspended pellets from Step 7 at maximum speed for 15 minutes at 4°C in a microfuge, and suspend them in 100 µl of Inclusion-body solubilization buffer I containing 0.1 mM PMSF (freshly added).
9. Store the solution for 1 hour at room temperature.
10. Add this solution to 9 volumes of Inclusion-body solubilization buffer II and incubate the mixture for 30 minutes at room temperature. Check that the pH is maintained at 10.7 by spotting small aliquots onto pH paper. If necessary, readjust the pH to 10.7 with 10 N KOH.
11. Adjust the pH of the solution to 8.0 with 12 M HCl, and store the adjusted solution for at least 30 minutes at room temperature.
12. Centrifuge the solution at maximum speed for 15 minutes at room temperature in a microfuge.
13. Decant the supernatant and set it aside for the next step. Resuspend the pellet in 100 µl of 1x SDS gel-loading buffer.
14. Remove 10-µl samples of the supernatant and resuspended pellet. Mix the supernatant sample with 10 µl of 2x SDS gel-loading buffer. Analyze both samples by SDS-polyacrylamide gel electrophoresis to determine the degree of solubilization.

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Chapter 16 Introducing Cloned Genes into Cultured Mammalian Cells

Protocol 1: DNA Transfection Mediated by Lipofection

Because a large number of factors affect the efficiency of lipofection, this protocol should be viewed as a starting point for systematic optimization of transfection mediated by lipofecting agents. Once a positive signal has been obtained from a transfected plasmid carrying a standard reporter gene, optimal conditions for transfection can be established by systematic variation of parameters such as the initial cell density, the amount and purity of DNA, the media and serum, and the time of exposure of the cells to the cationic-lipid-DNA complex.

Protocol 2: Calcium-phosphate-mediated Transfection of Eukaryotic Cells with Plasmid DNAs

Calcium phosphate forms an insoluble precipitate with DNA, which attaches to the cell surface and is taken into the cells by endocytosis. This protocol is a modified version of a method published by Jordan et al. (1996) who rigorously optimized calcium-phosphate-based transfection methods for Chinese hamster ovary cells and the 293 line of human embryonic kidney cells. The protocol is easily adapted for use with other types of cells, both adherent and nonadherent.

Protocol 3: Calcium-phosphate-mediated Transfection of Cells with High-molecular-weight Genomic DNA

This protocol, as all others involving calcium phosphate transfection, is based on the venerable method of Graham and van der Eb (1973). The procedure is used chiefly to generate stable lines of cells carrying chromosomally integrated copies of the transfected DNA.

Protocol 4: Transfection Mediated by DEAE-Dextran: High-efficiency Method

DEAE-dextran is generally used to obtain a burst of transient expression of cloned genes after transfection of mammalian cells. Many variants of the technique have been described, all of which seek to maximize the uptake of DNA and to minimize the cytotoxic effects of DEAE-dextran. In the following protocol, cells are exposed briefly to a high concentration of DEAE-dextran-DNA and then to chloroquine diphosphate, which is a facilitator of transfection.

Protocol 5: DNA Transfection by Electroporation

Pulsed electrical fields can be used to introduce DNA into a wide variety of animal cells. Electroporation works well with cell lines that are refractive to other techniques, such as calcium phosphate-DNA coprecipitation. But, as with other transfection methods, the optimal conditions for electroporating DNA into untested cell lines must be determined experimentally.

Protocol 6: DNA Transfection by Biolistics

In this protocol, gold or tungsten particles are coated with DNA and then shot from a gun into monolayers of mammalian cells.

Protocol 7: DNA Transfection Using Polybrene

Polybrene and DMSO can be used to achieve stable transformation of several types of cells by plasmid DNA. The yield of transformants is up to 15-fold greater with Polybrene than with calcium phosphate-DNA coprecipitation. However, there is no difference between the two methods in the efficiency of transformation of cells by high-molecular-weight DNA.

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Protocol 1

DNA Transfection Mediated by Lipofection

Because a large number of factors affect the efficiency of lipofection, this protocol should be viewed as a starting point for systematic optimization of transfection mediated by lipofecting agents. Once a positive signal has been obtained from a transfected plasmid carrying a standard reporter gene, optimal conditions for transfection can be established by systematic variation of parameters such as the initial cell density, the amount and purity of DNA, the media and serum, and the time of exposure of the cells to the cationic-lipid-DNA complex.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Lipofection reagent
As illustrated in the table, several types of lipofection reagents are available. This protocol describes the use of two common lipids:

- *Lipofectin (DOTMA). This monocationic lipid mixed with a helper lipid is usually purchased at a concentration of 1 mg/ml.*
- *Transfectam (DOGS). Polycationic lipids such as DOGS may be substituted for Lipofectin in the protocol. DOGS can be purchased and reconstituted as directed (Promega). Polyamines, such as DOGS, do not require the use of polystyrene tubes; polypropylene tubes (i.e., standard microfuge tubes) can be safely used with these reagents. Polystyrene tubes must be used with DOTMA, because the lipid can bind nonspecifically to polypropylene.*

NaCl (5 M) (optional)
Use as the diluent for DOGS.

Sodium citrate (pH 5.5, 20 mM) containing 150 mM NaCl (optional)
Use instead of sterile H_2O as the diluent for the plasmid DNA if DOGS is the lipofection reagent.

Nucleic Acids and Oligonucleotides

Plasmid DNA
If carrying out lipofection for the first time or if using an unfamiliar cell line, obtain an expression plasmid encoding E. coli *β-galactosidase or green fluorescent protein. These can be purchased from several commercial manufacturers (e.g., pCMV-SPORT-β-gal, Life Technologies, or pEGFP-F, CLONTECH).*
Purify closed circular plasmid DNAs by column chromatography or ethidium bromide-CsCl gradient centrifugation as described in Chapter 1. Dissolve the DNAs in H_2O at 1 µg/µl.

Media

Cell culture growth medium (complete, serum-free, and [optional] selective)

Additional Reagents

Step 9 of this protocol may require the reagents listed in Chapter 17, Protocol 7.

Cells and Tissues

Exponentially growing cultures of mammalian cells

METHOD

1. Twenty-four hours before lipofection, harvest exponentially growing mammalian cells by trypsinization and replate them on 60-mm tissue culture dishes at a density of 10^5 cells/dish (or at 5 x 10^4 cells/35-mm dish). Add 5 ml (or 3 ml for 35-mm dish) of growth medium, and incubate the cultures for 20-24 hours at 37°C in a humidified incubator with an atmosphere of 5-7% CO_2.
   The cells should be 75% confluent at the time of lipofection.
2. For each 60-mm dish of cultured cells to be transfected, dilute 1-10 µg of plasmid DNA into 100 µl of sterile deionized H_2O (if using Lipofectin) or 20 mM sodium citrate containing 150 mM NaCl (pH 5.5) (if using Transfectam) in a polystyrene or polypropylene test tube. In a separate tube, dilute 2-50 µl of the lipid solution to a final volume of 100 µl with sterile deionized H_2O or 300 mM NaCl.
   IMPORTANT *When transfecting with Lipofectin, use polystyrene test tubes; do not use polypropylene tubes, because the cationic lipid DOTMA can bind nonspecifically to polypropylene. For other cationic lipids, use the tubes recommended by the manufacturer.*
3. Incubate the tubes for 10 minutes at room temperature.
4. Add the lipid solution to the DNA, and mix the solution by pipetting up and down several times. Incubate the mixture for 10 minutes at room temperature.
5. While the DNA-lipid solution is incubating, wash the cells to be transfected three times with serum-free medium. After the third rinse, add 0.5 ml of serum-free medium to each 60-mm dish and return the washed cells to a 37°C humidified incubator with an atmosphere of 5-7% CO_2.
   It is very important to rinse the cells free of serum before the addition of the lipid-DNA liposomes.
6. After the DNA-lipid solution has incubated for 10 minutes, add 900 µl of serum-free medium to each tube. Mix the solution by pipetting up and down several times. Incubate the tubes for 10 minutes at room temperature.
7. Transfer each tube of DNA-lipid-medium solution to a 60-mm dish of cells. Incubate the cells for 1-24 hours at 37°C in a humidified incubator with an atmosphere of 5-7% CO_2.
8. After the cells have been exposed to the DNA for the appropriate time, wash them three times with serum-free medium. Feed the cells with complete medium and return them to the incubator.
9. If the objective is stable transformation of the cells, proceed to Step 10. Examine the cells 24-96 hours after lipofection using one of the following assays.
   - If a plasmid DNA expressing *E. coli* β-galactosidase was used, follow the steps outlined in Chapter 17, Protocol 7 to measure enzyme activity in cell lysates.
   - If a green fluorescence protein expression vector was used, examine the cells with a microscope under 450-490-nm illumination.
   - For other gene products, newly synthesized protein may be analyzed by radioimmunoassay, by immunoblotting, by immunoprecipitation following in vivo metabolic labeling, or by assays of enzymatic activity in cell extracts.
10. To isolate stable transfectants: After the cells have incubated for 48-72 hours in complete medium, trypsinize the cells and replate them in the appropriate selective medium. Change this medium every 2-4 days for 2-3 weeks to remove the debris of dead cells and to allow colonies of resistant cells to grow. Thereafter, individual colonies may be cloned and propagated for assay.

TABLE: Some Lipids Used in Lipofection

Abbreviation	IUPAC Name	Type	Product Name	Cell Lines Commonly Used For Transfection
DOTMA	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N trimethylammonium chloride	monocationic	Lipofectin	AS52 H187 mouse L cells NIH-3T3 HeLa
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N trimethylammonium methyl sulfate	monocationic	DOTAP	HeLa
DMRIE	1,2-dimyristyloxypropyl-3-dimethyl hydroxyethylammonium bromide	monocationic	DMRIE-C	Jurkat CHO-K1 COS-7 BHK-21
DDAB	dimethyl dioctadecylammonium bromide	monocationic	LipofectACE	COS-7 CHO-K1 BHK-21 mouse L cells
Amidine	N-t-butyl-N'-tetradecyl-3-tetradecyl aminopropionamide	monocationic	CLONfectin	A-431 HEK293 BHK-21 HeLa L6 CV-1
DC-Cholesterol	3 β [N-(N',N'-dimethylaminoethane) carbamoyl]-cholesterol	monocationic	DC-Cholesterol	
DOSPER	1,3-dioleoyloxy-2-(6-carboxyspermyl) propylamide	dicationic	Tfx	CHO HeLa NIH-3T3
DOGS	spermine-5-carboxy-glycine dioctadecyl-amide	polycationic	Transfectam	293 HeLa HEPG2 HC11 NIH-3T3
DOSPA	2,3-dioleoyloxy-N-[2(sperminecarbox amido)ethyl]-N,N-dimethyl-1-propan-aminium trifluoroacetate	polycationic	LipofectAMINE	HT-29 BHK-21 keratinocytes MDCK NIH-3T3
TM-TPS	N,N',N'',N'''-tetramethyl-N, N',N'',N''' tetrapalmitylspermine	polycationic	CellFECTIN	CHO-K1 COS-7 BHK-21 Jurkat

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Protocol 2

Calcium-phosphate-mediated Transfection of Eukaryotic Cells with Plasmid DNAs

Calcium phosphate forms an insoluble precipitate with DNA, which attaches to the cell surface and is taken into the cells by endocytosis. This protocol is a modified version of a method published by Jordan et al. (1996) who rigorously optimized calcium-phosphate-based transfection methods for Chinese hamster ovary cells and the 293 line of human embryonic kidney cells. The protocol is easily adapted for use with other types of cells, both adherent and nonadherent.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- $CaCl_2$ (2.5 M)
- Chloroquine (100 mM) (optional)
  Dissolve 52 mg of chloroquine diphosphate in 1 ml of deionized distilled H_2O. Sterilize the solution by passing it through a 0.22-µm filter; store the filtrate in foil-wrapped tubes at -20°C. Please see Step 5.
- Giemsa stain (10% w/v)
  The Giemsa stain should be freshly prepared in PBS or H_2O and filtered through Whatman No. 1 filter paper before use.
- Glycerol (15% v/v) in 1x HEPES-buffered saline (optional)
  Add 15% (v/v) autoclaved glycerol to filter-sterilized HEPES-buffered saline solution just before use. Please see Step 5.
- 2x HEPES-buffered saline
- ⚠ Methanol
- PBS
  The solution should be sterilized by filtration before use and stored at room temperature.
- Sodium butyrate (500 mM) (optional)
  In a chemical fume hood, bring an aliquot of stock butyric acid solution to a pH of 7.0 with 10 N NaOH. Sterilize the solution by passing it through a 0.22-µm filter; store in 1-ml aliquots at -20°C. Please see Step 5.
- 0.1x TE (pH 7.6)

Nucleic Acids and Oligonucleotides

Plasmid DNA
Dissolve the DNA in 0.1x TE (pH 7.6) at a concentration of 25 µg/ml; 50 µl of plasmid solution is required per milliliter of medium.
To obtain the highest transformation efficiencies, plasmid DNAs should be purified by column chromatography (please see Chapter 1, Protocol 9) or by equilibrium centrifugation in CsCl-ethidium bromide density gradients (please see Chapter 1, Protocol 10). If the starting amount of plasmid DNA is limiting, then add carrier DNA to adjust the final concentration to 25 µg/ml. Eukaryotic carrier DNA prepared in the laboratory usually gives higher transfection efficiencies than commercially available DNA such as calf thymus or salmon sperm DNA. Sterilize the carrier DNA before use by ethanol precipitation or extraction with chloroform.

Media

Cell culture growth medium (complete and [optional] selective)

Additional Reagents

Step 6 of this protocol requires the reagents listed in Chapter 6, Protocol 10, and Chapter 7, Protocol 8.

Cells and Tissues

Exponentially growing cultures of mammalian cells

METHOD

1. Twenty-four hours before transfection, harvest exponentially growing cells by trypsinization and replate them at a density of 1 x 10^5 to 4 x 10^5 cells/cm^2 in 60-mm tissue culture dishes or 12-well plates in the appropriate complete medium. Incubate the cultures for 20-24 hours at 37°C in a humidified incubator with an atmosphere of 5-7% CO_2. Change the medium 1 hour before transfection.
   It is important to use exponentially growing cells.
2. Prepare the calcium phosphate-DNA coprecipitate as follows: Combine 100 µl of 2.5 M $CaCl_2$ with 25 µg of plasmid DNA in a sterile 5-ml plastic tube and, if necessary, bring the final volume to 1 ml with 0.1x TE (pH 7.6). Mix 1 volume of this 2x calcium-DNA solution with an equal volume of 2x HEPES-buffered saline at room temperature. Quickly tap the side of the tube to mix the ingredients and allow the solution to stand for 1 minute.
3. Immediately transfer the calcium phosphate-DNA suspension into the medium above the cell monolayer. Use 0.1 ml of suspension for each 1 ml of medium in a well or 60-mm dish. Rock the plate gently to mix the medium, which will become yellow-orange and turbid. Carry out this step as quickly as possible because the efficiency of transfection declines rapidly once the DNA precipitate is formed. If the cells will be treated with chloroquine, glycerol, and/or sodium butyrate, proceed directly to Step 5.
4. Transfected cells that will not be treated with transfection facilitators should be incubated at 37°C in a humidified incubator with an atmosphere of 5-7% CO_2. After 2-6 hours incubation, remove the medium and DNA precipitate by aspiration. Add 5 ml of warmed (37°C) complete growth medium and return the cells to the incubator for 1-6 days. Proceed to Step 6 to assay for transient expression of the transfected DNA, or proceed directly to Step 7 if the objective is stable transformation of the cells.
5. The uptake of DNA can be increased by treatment of the cells with chloroquine in the presence of the calcium phosphate-DNA coprecipitate or exposure to glycerol and sodium butyrate following removal of the coprecipitate solution from the medium.

   Treatment of cells with chloroquine

   a. Dilute 100 mM chloroquine diphosphate 1:1000 directly into the medium either before or after the addition of the calcium phosphate-DNA coprecipitate to the cells.
      The concentration of chloroquine added to the growth medium and the time of treatment are limited by the sensitivity of the cells to the toxic effect of the drug. The optimal concentration of chloroquine for the cell type used should be determined empirically.

   b. Incubate the cells for 3-5 hours at 37°C in a humidified incubator with an atmosphere of 5-7% CO_2.

c. After the treatment with DNA and chloroquine, remove the medium, wash the cells with phosphate-buffered saline, and add 5 ml of warmed complete growth medium. Return the cells to the incubator for 1-6 days. Proceed to Step 6 to assay for transient expression of the transfected DNA, or proceed directly to Step 7 if the objective is stable transformation of the cells.

Treatment of cells with glycerol

a. After cells have been exposed for 2-6 hours to the calcium phosphate-DNA coprecipitate in growth medium (± chloroquine), remove the medium by aspiration and wash the monolayer once with phosphate-buffered saline. *This procedure may be used following treatment with chloroquine. Because cells vary widely in their sensitivity to the toxic effects of glycerol, each cell type must be tested in advance to determine the optimum time (30 seconds to 3 minutes) of treatment.*

b. Add 1.5 ml of 15% glycerol in 1x HEPES-buffered saline to each monolayer, and incubate the cells for the predetermined optimum length of time at 37°C.

c. Remove the glycerol by aspiration, and wash the monolayers once with phosphate-buffered saline.

d. Add 5 ml of warmed complete growth medium, and incubate the cells for 1-6 days. Proceed to Step 6 to assay for transient expression of the transfected DNA, or proceed directly to Step 7 if the objective is stable transformation of the cells.

Treatment of cells with sodium butyrate

a. Following the glycerol shock, dilute 500 mM sodium butyrate directly into the growth medium (Step d, treatment of cells with glycerol). Different concentrations of sodium butyrate are used depending on the cell type. For example:

CV-1	10 mM
NIH-3T3	7 mM
HeLa	5 mM
CHO	2 mM

The correct amount for other cell lines that may be transfected should be determined empirically.

b. Incubate the cells for 1-6 days. Proceed to Step 6 to assay for transient expression of the transfected DNA, or proceed directly to Step 7 if the objective is stable transformation of the cells.

6. To assay the transfected cells for transient expression of the introduced DNA, harvest the cells 1-6 days after transfection. Analyze RNA or DNA using hybridization. Analyze newly synthesized protein by radioimmunoassay, by immunoblotting, by immunoprecipitation following in vivo metabolic labeling, or by assays of enzymatic activity in cell extracts.
7. To isolate stable transfectants:
   a. Incubate the cells for 24-48 hours in nonselective medium to allow time for expression of the transferred gene(s).
   b. Either trypsinize and replate the cells in the appropriate selective medium or add the selective medium directly to the cells without further manipulation.
   c. Change the selective medium with care every 2-4 days for 2-3 weeks to remove the debris of dead cells and to allow colonies of resistant cells to grow.
   d. Clone individual colonies and propagate for appropriate assay.
   e. Obtain a permanent record of the numbers of colonies by fixing the remaining cells with ice-cold methanol for 15 minutes followed by staining with 10% Giemsa for 15 minutes at room temperature before rinsing in tap water.

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Protocol 3

Calcium-phosphate-mediated Transfection of Cells with High-molecular-weight Genomic DNA

This protocol, as all others involving calcium phosphate transfection, is based on the venerable method of Graham and van der Eb (1973). The procedure is used chiefly to generate stable lines of cells carrying chromosomally integrated copies of the transfected DNA.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- $CaCl_2$ (2 M)
  Sterilize by filtration, and store frozen as 5-ml aliquots.
- Glycerol (15% v/v) in 1x HEPES-buffered saline
  Add 15% (v/v) autoclaved glycerol to filter-sterilized HEPES-buffered saline solution just before use. Please see Step 10.
- HEPES-buffered saline (16-3)
- Isopropanol
- NaCl (3 M)
  Sterilize by filtration, and store at room temperature.

Nucleic Acids and Oligonucleotides

Genomic DNA
Prepare high-molecular-weight DNA in TE from appropriate cells as described in Chapter 6, Protocol 3. Dilute the DNA to 100 µg/ml in TE (pH 7.6). Approx. 20-25 µg of genomic DNA is required to transfect each 90-mm plate of cultured cells.
The genomic DNA must be sheared to a size range of 45-60 kb before using it to transfect cells (please see Steps 2 and 3).

Plasmid with selectable marker
Optional, please see Steps 3 and 12.

Media

Cell culture growth medium (complete and selective)

Cells and Tissues

Exponentially growing cultures of mammalian cells

METHOD

1. On day 1 of the experiment, plate exponentially growing cells (e.g., CHO cells) at a density of 5 x 10^5 cells per 90-mm culture dish in appropriate growth medium containing serum. Incubate the cultures for approx. 16 hours at 37°C in a humidified incubator with an atmosphere of 5% CO_2.
2. On day 2, shear an appropriate amount of high-molecular-weight DNA into fragments ranging in size from 45 kb to 60 kb, by passing it through a 22-gauge needle for the predetermined number of times.
   Cells should be transfected with 20-25 µg of genomic DNA per 90-mm dish.
3. Precipitate the sheared DNA by adding 0.1 volume of 3 M NaCl and 1 volume of isopropanol. Collect the DNA on a Shepherd's crook. Drain the precipitate briefly against the side of the tube and transfer it to a second tube containing HEPES-buffered saline (16-3) (1 ml per 12-15 µg of DNA). Redissolve the DNA by gentle rotation for 2 hours at 37°C. Make sure that all of the DNA has dissolved before proceeding.
4. Transfer 3-ml aliquots of sheared genomic DNA into 12-ml polyethylene tubes (one aliquot per two dishes to be transfected).
5. To form the calcium phosphate-DNA coprecipitate, gently vortex an aliquot of sheared genomic DNA, and add 120 µl of 2 M $CaCl_2$ in a dropwise fashion. Incubate the tube for 15-20 minutes at room temperature.
   The solution should turn hazy, but it should not form visible clumps of precipitate.
6. Aspirate the medium from two dishes of cells (from Step 1) and gently add 1.5 ml of the calcium phosphate-DNA coprecipitate to each dish. Carefully rotate the dishes to swirl the medium and spread the precipitate over the monolayer of cells. Incubate the cells for 20 minutes at room temperature, rotating the dishes once during the incubation.
7. Gently add 10 ml of warmed (37°C) growth medium to each dish and incubate for 6 hours at 37°C in a humidified incubator with an atmosphere of 5% CO_2.
8. Repeat Steps 5-7 until all of the dishes of cells contain the calcium phosphate-DNA precipitate.
9. After 6 hours of incubation, examine each dish under a light microscope. A "peppery" precipitate should be seen adhering to the cells. The precipitate should be neither too fine nor clumpy.
10. In most cases, treatment with glycerol at this step will enhance the transfection frequency. To shock the cells with glycerol:
    a. Aspirate the medium containing the calcium phosphate-DNA coprecipitate.
    b. To each dish of cells, add 3 ml of 15% glycerol in 1x HEPES-buffered saline that has been warmed to 37°C. Incubate for *no longer than 3 minutes* at room temperature.
       It is important that the glycerol in the HEPES-buffered saline not be left in contact with the cells for too long. The optimum time period usually spans a narrow range and varies from one cell line to another and from one laboratory to the next. For these reasons, treat only a few dishes at a time and take into account the length of time to aspirate the glycerol in the HEPES-buffered saline. Do not exceed the optimum incubation period. Seconds can count!
    c. Aspirate the glycerol in the HEPES-buffered saline and rapidly wash the dishes twice with 10 ml of warmed growth medium.
    d. Add 10 ml warmed growth medium and incubate the cultures for 12-15 hours at 37°C in a humidified incubator with an atmosphere of 5% CO_2.
11. Replace the medium with 10 ml of fresh growth medium. Continue the incubation overnight at 37°C in a humidified incubator with an atmosphere of 5% CO_2.
12. Microscopic examination of cells at this point (day 4) should reveal a normal morphology. Cells can be trypsinized and replated in selective medium on day 4. Continue the incubation for 2-3 weeks to allow growth of complemented and/or resistant colonies. Change the medium every 2-3 days.
13. Thereafter, clone individual colonies and propagate them for the appropriate assay.

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Protocol 4

Transfection Mediated by DEAE-Dextran: High-efficiency Method

DEAE-dextran is generally used to obtain a burst of transient expression of cloned genes after transfection of mammalian cells. Many variants of the technique have been described, all of which seek to maximize the uptake of DNA and to minimize the cytotoxic effects of DEAE-dextran. In the following protocol, cells are exposed briefly to a high concentration of DEAE-dextran-DNA and then to chloroquine diphosphate, which is a facilitator of transfection.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Chloroquine diphosphate (100 mM)
  Dissolve 60 mg of chloroquine diphosphate in 1 ml of deionized distilled H_2O. Sterilize the solution by passing it through a 0.22-µm filter. Store the filtrate in foil-wrapped tubes at -20°C.
- DEAE-dextran (50 mg/ml)
  Dissolve 100 mg of DEAE-dextran (M_r = 500,000; Pharmacia) in 2 ml of distilled H_2O. Sterilize the solution by autoclaving for 20 minutes at 15 psi (1.05 kg/cm^2) on liquid cycle. Autoclaving also assists dissolution of the polymer.
  The molecular weight of the DEAE-dextran originally used for transfection was >2 x 10^6. Although this material is no longer available commercially, it is still occasionally found in chemical storerooms. The older batches of higher-molecular-weight DEAE-dextran are more efficient for transfection than the lower-molecular-weight polymers currently available.
- PBS
  Sterilize the solution by filtration before use and store it at room temperature.
- TBS-D (Tris-buffered saline with dextrose)
  Immediately before use, add 20% (w/v) dextrose (prepared in H_2O and sterilized by autoclaving or filtration) to the TBS solution. The final dextrose concentration should be 0.1% (v/v).

Nucleic Acids and Oligonucleotides

Plasmid DNA
To obtain the highest transformation efficiencies, purify the plasmid DNAs by column chromatography (please see Chapter 1, Protocol 9) or by equilibrium centrifugation in CsCl-ethidium bromide density gradients (please see Chapter 1, Protocol 10).

Media

Cell culture growth medium (complete and serum-free)

Additional Reagents

Step 8 of this protocol requires the reagents listed in Chapter 6, Protocol 1 and Chapter 7, Protocol 8.

Cells and Tissues

Exponentially growing cultures of mammalian cells

METHOD

1. Twenty-four hours before transfection, harvest exponentially growing cells by trypsinization and transfer them to 60-mm tissue culture dishes at a density of 10^5 cells/dish (or 35-mm dishes at a density of 5 x 10^4 cells/dish). Add 5 ml (or 3 ml for 35-mm dish) of complete growth medium, and incubate the cultures for 20-24 hours at 37°C in a humidified incubator with an atmosphere of 5-7% CO_2.
   The cells should be 75% confluent at the time of transfection.
2. Prepare the DNA/DEAE-dextran/TBS-D solution by mixing 0.1-4 µg of supercoiled or circular plasmid DNA into 1 mg/ml DEAE-dextran in TBS-D.
   0.25 ml of the solution is required for each 60-mm dish; 0.15 ml is required for each 35-mm dish.
3. Remove the medium from the cell culture dishes by aspiration, and wash the monolayers twice with warmed (37°C) PBS and once with warmed TBS-D.
4. Add the DNA/DEAE-dextran/TBS-D solution (250 µl per 60-mm dish, 150 µl per 35-mm dish). Rock the dishes gently to spread the solution evenly across the monolayer of cells. Return the cultures to the incubator for 30-90 minutes (the time will depend on the sensitivity of each batch of cells to the DNA/DEAE-dextran/TBS-D solution). At 15-20-minute intervals, remove the dishes from the incubator, swirl them gently, and check the appearance of the cells under the microscope. If the cells are still firmly attached to the substratum, continue the incubation. Stop the incubation when the cells begin to shrink and round up.
5. Remove the DNA/DEAE-dextran/TBS-D solution by aspiration. Gently wash the monolayers once with warmed TBS-D and then once with warmed PBS, taking care not to dislodge the transfected cells.
6. Add 5 ml (per 60-mm dish) or 3 ml (per 35-mm dish) of warmed medium supplemented with serum and chloroquine (100 µM final concentration), and incubate the cultures for 3-5 hours at 37°C in a humidified incubator with an atmosphere of 5-7% CO_2.
7. Remove the medium by aspiration, and wash the monolayers three times with serum-free medium. Add to the cells 5 ml (per 60-mm dish) or 3 ml (per 35-mm dish) of medium supplemented with serum, and incubate the cultures for 36-60 hours at 37°C in a humidified incubator with an atmosphere of 5-7% CO_2 before assaying for transient expression of the transfected DNA.
   The time of incubation should be optimized for the particular conditions.
8. To assay the transfected cells for transient expression of the introduced DNA, harvest the cells 36-60 hours after transfection. Analyze RNA or DNA using hybridization. Analyze newly synthesized protein by radioimmunoassay, by immunoblotting, by immunoprecipitation following in vivo metabolic labeling, or by assays of enzymatic activity in cell extracts.

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Protocol 5

DNA Transfection by Electroporation

Pulsed electrical fields can be used to introduce DNA into a wide variety of animal cells. Electroporation works well with cell lines that are refractive to other techniques, such as calcium phosphate-DNA coprecipitation. But, as with other transfection methods, the optimal conditions for electroporating DNA into untested cell lines must be determined experimentally.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Giemsa stain (10% w/v)
  The Giemsa stain should be freshly prepared in PBS or H_2O and filtered through Whatman No. 1 filter paper before use.
- ⚠ Methanol
- PBS
  Sterilize the solution by filtration before use and store it at room temperature.
- Sodium butyrate (500 mM) (optional)

Nucleic Acids and Oligonucleotides

Carrier DNA (10 mg/ml; e.g., sonicated salmon sperm DNA) (optional)

Linearized or circular plasmid DNA (1 µg/µl in sterile deionized H_2O)

Media

Cell culture growth medium (complete and [optional] selective)

Additional Reagents

Step 10 of this protocol may require the reagents listed in Chapter 17, Protocol 7.

Cells and Tissues

Exponentially growing cultures of mammalian cells

METHOD

1. Harvest the cells to be transfected from cultures in the mid- to late-logarithmic phase of growth. Use either a rubber policeman or trypsin to release adherent cells. Centrifuge at 500*g* (1500 rpm in a Sorvall H1000B rotor) for 5 minutes at 4°C.
2. Resuspend the cell pellet in 0.5x volume of the original growth medium and measure the cell number using a hemocytometer.
3. Collect the cells by centrifugation as described in Step 1, and resuspend them in growth medium or phosphate-buffered saline at room temperature at a concentration of 2.5 x 10^6 to 2.5 x 10^7 cells/ml.
4. Transfer 400-µl aliquots of the cell suspension (10^6 to 10^7 cells) into as many labeled electroporation cuvettes as needed. Place the loaded cuvettes on ice.
5. Set the parameters on the electroporation device. A typical capacitance value is 1050 µF. Voltages range from 200 to 350 V, depending on the cell line, but generally average 260 V. Use an infinite internal resistance value. Discharge a blank cuvette containing phosphate-buffered saline at least twice before beginning electroporation of cells.
6. Add 10-30 µg of plasmid DNA in a volume of up to 40 µl to each cuvette containing cells. (Some investigators add carrier DNA [e.g., salmon sperm DNA] to bring the total amount of DNA to 120 µg.) Gently mix the cells and DNA by pipetting the solution up and down. Proceed to Step 7 without delay.
   IMPORTANT *Do not introduce air bubbles into the suspension during the mixing step.*
7. Immediately transfer the cuvette to the electroporator and discharge the device. After 1-2 minutes, remove the cuvette, place it on ice, and proceed immediately to the next step.
8. Transfer the electroporated cells to a 35-mm culture dish using a micropipettor equipped with a sterile tip. Rinse out the cuvette with a fresh aliquot of growth medium, and add the washings to the culture dish. Transfer the dish to a humidified incubator at 37°C with an atmosphere of 5-7% CO_2.
9. Repeat Steps 6-8 until all of the DNA cell samples in cuvettes are shocked. Record the actual pulse time for each cuvette to facilitate comparisons between experiments.
10. If the objective is stable transformation of the cells, proceed directly to Step 11. For transient expression, examine the cells 24-96 hours after electroporation using one of the following assays:
    - If a plasmid DNA expressing *E. coli* β-galactosidase was used, follow the steps outlined in Chapter 17, Protocol 7 to measure enzyme activity in cell lysates.
    - If a green fluorescence protein expression vector was used, examine the cells with a microscope under 450-490-nm illumination.
    - For other gene products, analyze the newly synthesized protein by radioimmunoassay, by immunoblotting, by immunoprecipitation following in vivo metabolic labeling, or by assays of appropriate enzymatic activity in cell extracts.
11. To isolate stable transfectants: After incubation for 48-72 hours in complete medium, trypsinize the cells and replate them in the appropriate selective medium. The selective medium should be changed every 2-4 days for 2-3 weeks to remove the debris of dead cells and to allow colonies of resistant cells to grow. Thereafter, clone individual colonies and propagate for the appropriate assay.

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Protocol 6

DNA Transfection by Biolistics

In this protocol, gold or tungsten particles are coated with DNA and then shot from a gun into monolayers of mammalian cells.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

$CaCl_2$ (2.5 M)

Ethanol
Use a fresh bottle of absolute ethanol that has not been opened previously. Ethanol is hygroscopic and with exposure to air picks up small amounts of water. In the method described below, the presence of water in the ethanol washes of Steps 1, 2, and 3 can interfere with effective bombardment of cells and tissues.

Glycerol (50% in H_2O)
Sterilize the solution by autoclaving.

Spermidine (0.1 M)

Nucleic Acids and Oligonucleotides

Plasmid DNA
When carrying out a gene gun experiment for the first time or if a new cell line or tissue is to be transfected, obtain an expression plasmid encoding an appropriate marker gene for use in optimizing delivery. Examples include vectors that express E. coli *β-galactosidase, green fluorescent protein, and β-glucuronidase (for plants) or selectable markers such as neomycin resistance. Plasmids expressing a gene or cDNA of interest can be used after the process has been optimized.*

Media

Cell culture growth medium (complete and [optional] selective)

Additional Reagents

Step 8 of this protocol may require the reagents listed in Chapter 17, Protocol 7.

Cells and Tissues

Cells or tissue to be transfected
Adherent cultured cells from various species should be bombarded at 50-80% confluency. Collect plant cells grown in suspension by sterile filtration onto Whatman No. 1 filter papers (7-cm diameter) using a Buchner funnel, and place them on sterile filter papers soaked with culture medium of high osmolarity before bombardment (for details, please see Sanford et al. 1993).
Freshly dissect the mammalian tissue, section at approx. 400 µm, and maintain the slices in culture dishes.
Culture bacteria and yeast to mid- to late-logarithmic growth depending on the species and strain, collect by centrifugation, resuspend in a small volume of culture medium of high osmolarity, and plate (1×10^8 to 2×10^9 cells) on a thin layer of agar atop a piece of filter paper in a Petri dish before being shot.

METHOD

1. Prepare tungsten or gold particles.
   a. Weigh 60 mg of gold or tungsten particles into a 1.5-ml microfuge tube.
   b. Add 1 ml of 70% ethanol to the particles and vortex the tube continuously for 5 minutes at room temperature. Store the tube on the bench top for 15 minutes.
   c. Collect the particles by centrifugation at maximum speed for 5 seconds in a microfuge.
   d. Gently remove the supernatant. Resuspend the metal particles in 1-ml of sterile H_2O and vortex the suspension for 1 minute. Store the tube on the bench top for 1 minute.
   e. Collect the metal particles by centrifugation at maximum speed for 5 seconds in a microfuge.
   f. Repeat the H_2O wash (Steps d and e) three more times.
   g. Remove the supernatant after the fourth H_2O wash. Resuspend the particles in 1 ml of sterile 50% glycerol.
2. For every six dishes of cells or slices of tissue to be shot, prepare an aliquot of DNA-coated particles as follows:
   a. While continuously vortexing the stock solution of microcarrier particles, remove a 50-µl aliquot (approx. 3 mg).
   b. Transfer the aliquot to a fresh microfuge tube, and while vortexing, add the following to the tube:

plasmid DNA (approx. 2.5 µg)	2.5 µl
2.5 M $CaCl_2$	50 µl
0.1 M spermidine	20 µl

   After all ingredients are added, continue vortexing the tube for an additional 3 minutes.
   It is very important that the microfuge tube be continuously vortexed during this procedure to ensure uniform coating of the particles with plasmid DNA.
   c. Stand the tube on the bench for 1 minute to allow the particles to settle, and then collect them by centrifugation at maximum speed for 2 seconds in a microfuge.
   d. Remove the supernatant and carefully layer 140 µl of 70% ethanol over the pelleted particles. Remove the 70% ethanol, and add 140 µl of 100% ethanol, again without disturbing the particles. Remove the supernatant and replace with 50 µl of ethanol.
   e. Resuspend the particle pellet by tapping the side of the tube, followed by gentle vortexing for 2-3 seconds.
3. Place a macrocarrier in the metal holder of the gene gun apparatus using the seating device supplied by the manufacturer. Wash the sheet twice with 6-µl aliquots of ethanol. Between washes, blot the sheet dry with lens paper.
4. Vortex the pellet sample from Step 2e for 1 minute. While vortexing, withdraw 6 µl of the pellet slurry (approx. 500 µg of particles) and, as quickly as possible, spread the aliquot around the central 1 cm of the macrocarrier.
5. Repeat Steps 3 and 4 until the desired number of loaded macrocarriers has been prepared. Allow the ethanol solution containing the DNA-coated particles to dry on the macrocarrier.
6. Load a macrocarrier into the gene gun, and following the manufacturer's directions, shoot a plate of cells or tissue slice.
7. After the vacuum has returned to atmospheric pressure, remove the wounded cells or tissue and place in appropriate culture conditions. Remove the ruptured macrocarrier and repeat Steps 6 and 7 until all plates are shot.
8. If the objective is stable transformation of the cells, proceed directly to Step 9. For transient expression, examine the cells 24-96 hours after shooting, using one of the following assays.

- If a plasmid DNA expressing *E. coli* β-galactosidase was used, follow the steps outlined in Chapter 17, Protocol 7, to measure enzyme activity in cell lysates. Alternatively, carry out a histochemical staining assay as detailed in the additional protocol in Chapter 16, Protocol 1.
- If a green fluorescence protein expression vector was used, examine the cells with a microscope under 450-490-nm illumination.
- If a plasmid DNA expressing β-glucuronidase was used, assay for β-glucuronidase activity.
- For other gene products, analyze the newly synthesized protein by radioimmunoassay, by immunoblotting, by immunoprecipitation following in vivo metabolic labeling, or by assays of appropriate enzymatic activity in cell extracts.

9. To isolate stable transfectants: After the cells have incubated for 48-72 hours in complete medium, transfer the bombarded cells to selective medium. The concentration of selective agent and the culture conditions will vary depending on the cell type.

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Protocol 7

DNA Transfection Using Polybrene

Polybrene and DMSO can be used to achieve stable transformation of several types of cells by plasmid DNA. The yield of transformants is up to 15-fold greater with Polybrene than with calcium phosphate-DNA coprecipitation. However, there is no difference between the two methods in the efficiency of transformation of cells by high-molecular-weight DNA.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ DMSO (30%) in serum-containing medium
Dilute high-performance liquid chromatography (HPLC)-grade or tissue-culture-grade DMSO to a final concentration of 30% (v/v) in the cell growth medium containing serum just before use in Step 3.

Giemsa stain (10% w/v)
The Giemsa stain should be freshly prepared in PBS or H_2O and filtered through Whatman No. 1 filter paper before use.

⚠ Methanol

Polybrene (10 mg/ml)
Dissolve Polybrene (Aldrich) at a concentration of 10 mg/ml in H_2O and sterilize the solution by passing it through a 0.22-µm filter. Store the solution as small aliquots (0.25-ml) at -20°C until needed. Discard aliquots after use.

Sodium butyrate (500 mM) (optional)

Nucleic Acids and Oligonucleotides

DNA to be transformed, e.g., plasmid DNA (1 µg/µl) in H_2O

Media

Minimum essential medium (MEM)-α (containing 10% fetal calf serum, serum-free, and [optional] selective agents)

Additional Reagents

Step 6 of this protocol may require the reagents listed in Chapter 17, Protocol 7.

Cells and Tissues

Exponentially growing cultures of mammalian cells

METHOD

1. Harvest exponentially growing cells (e.g., CHO cells) by trypsinization, and replate them at a density of 5 x 10^5 cells per 90-mm tissue culture dish in 10 ml of MEM-α containing 10% fetal calf serum. Incubate the cultures for 18-20 hours at 37°C in a humidified incubator in an atmosphere of 5-7% CO_2.
2. Replace the medium with 3 ml of warmed (37°C) medium containing serum, DNA (5 ng to 40 µg; no carrier DNA), and 30 µg of Polybrene. Mix the DNA with the medium before adding the 10 mg/ml Polybrene. Return the cells to the incubator for 6-16 hours. Gently rock the dishes every 90 minutes during the early stages of this incubation to ensure even exposure of the cells to the DNA-Polybrene mixture.
3. Remove the medium containing the DNA and Polybrene by aspiration. Add 5 ml of 30% DMSO in serum-containing medium. Gently swirl the DMSO medium around the dish to ensure even exposure of the cells to the solvent and place the dishes in the incubator.
4. After 4 minutes of incubation, remove the dishes from the incubator and immediately aspirate the DMSO solution. Wash the cells once or twice with warmed (37°C) serum-free medium, and add 10 ml of complete medium containing 10% fetal calf serum. If a sodium butyrate boost is to be included, then proceed to Step 5. If not, incubate the cultures for 48 hours at 37°C in a humidified incubator with an atmosphere of 5-7% CO_2. Then proceed directly to either Step 6 (to assay for transient expression) or Step 7 (to establish stable transformants).
5. (*Optional*) To facilitate the transfection of cells treated with DMSO and Polybrene:
   a. Add 500 mM sodium butyrate directly to the growth medium to a final concentration of 2.5-10 mM.
   b. Incubate the cells for 20-24 hours at 37°C in a humidified incubator with an atmosphere of 5-7% CO_2.
   c. Remove the medium containing sodium butyrate, and replace it with butyrate-free medium containing 10% fetal bovine serum. Return the cells to the incubator.
6. If the objective is stable transformation of the cells, proceed directly to Step 7. For transient expression, examine the cells 1-2 days after transfection using one of the following assays:
   - If a plasmid DNA expressing *E. coli* β-galactosidase was used, follow the steps outlined in Chapter 17, Protocol 7, to measure enzyme activity in cell lysates. Alternatively, carry out a histochemical staining assay as detailed in the additional protocol in Chapter 16, Protocol 1.
   - If a green fluorescence protein expression vector was used, examine the cells with a microscope under 450-490-nm illumination.
   - For other gene products, analyze the newly synthesized protein by radioimmunoassay, by immunoblotting, by immunoprecipitation following in vivo metabolic labeling, or by assays of enzymatic activity in cell extracts.
7. To isolate stable transfectants: After the cells have incubated for 48 hours in nonselective medium (to allow expression of the transferred gene[s] to occur [Step 4]), either trypsinize or replate the cells in the appropriate selective medium or add the selective medium directly to the cells without further manipulation. Change this medium every 2-4 days for 2-3 weeks to remove the debris of dead cells and to allow colonies of resistant cells to grow.
8. Thereafter, clone individual colonies and propagate for the appropriate assay.

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Chapter 17 Analysis of Gene Expression in Cultured Mammalian Cells

Protocol 1: Mapping Protein-binding Sites on DNA by DNase I Footprinting

In this protocol, DNase I is used to fragment a radiolabeled target DNA in the presence and absence of a nuclear extract. A "footprint" is generated when a protein binds to the target and protects a specific segment of DNA from the nucleolytic activity of DNase I. By comparing the electrophoretic mobility of the DNase I cleavage products to those of a sequence ladder derived from the same DNA fragment, the position(s) of the DNA sequences recognized by DNA-binding proteins can be determined.

Protocol 2: Gel Retardation Assays for DNA-binding Proteins

This protocol exploits differences in electrophoretic mobility through a nondenaturing polyacrylamide gel between a rapidly migrating target DNA and a more slowly migrating DNA-protein complex.

Protocol 3: Mapping DNase-I-hypersensitive Sites

In this protocol, isolated nuclei are incubated with varying amounts of DNase I. Genomic DNA is then isolated from the nuclei and digested with a restriction enzyme, analyzed by gel electrophoresis, and probed by Southern hybridization. If the probe corresponds to the 5´ end of the gene, intact restriction fragments arising from that region will be detected in DNA isolated from control nuclei not treated with DNase I. If DNase-I-hypersensitive sites exist in one or more of the fragments recognized by the probe, then shorter DNAs will be detected on the Southern blot.

Protocol 4: Transcriptional Run-on Assays

In this protocol, nuclei isolated from cells expressing the gene of interest are incubated with radiolabeled UTP, which is incorporated into nascent RNA transcripts by RNA polymerase molecules that were actively transcribing at the time the cells were harvested. Because very little de novo initiation of RNA synthesis occurs in isolated nuclei, transcription of the target gene can be measured by hybridizing the radiolabeled RNA to an excess of the target gene immobilized on a nitrocellulose or nylon membrane. The fraction of the RNA that hybridizes to the immobilized DNA reflects the contribution of the target gene to the total transcriptional activity of the cell. All test tubes and solutions must be prepared RNase-free.

Protocol 5: Measurement of Chloramphenicol Acetyltransferase in Extracts of Mammalian Cells Using Thin-layer Chromatography

In this protocol, extracts prepared from cells transfected with a chloramphenicol acetyltransferase (CAT) reporter plasmid are incubated with radiolabeled chloramphenicol. The acetylated products generated by the action of CAT are separated from the unmodified drug by thin-layer chromatography and quantitated by scraping the spots from the thin-layer plates and counting them by scintillation spectroscopy. Other methods of measuring the activity of CAT are described on pages 17.40, 17.41, and 17.95 of the print version of the manual.

Protocol 6: Assay for Luciferase in Extracts of Mammalian Cells

In this protocol, cells transfected with a luciferase reporter plasmid are lysed in a detergent-containing buffer. Luciferase in the extract catalyzes an oxidation reaction in which D-luciferin is converted to oxyluciferin, with production of light at 556 nm that can be quantified in a luminometer.

Protocol 7: Assay for β-galactosidase in Extracts of Mammalian Cells

The assay for β-galactosidase relies on the ability of the enzyme to catalyze the hydrolysis of ONPG (*o*-nitrophenyl-β-D- galactopyranoside) to free *o*-nitrophenol, which absorbs light at 420 nm. In this protocol, extracts of cells transfected with a β-galactosidase reporter plasmid are incubated with ONPG. When the substrate is in excess, the OD_{420} of the assay solution increases with time and is proportional to the enzyme concentration.

Protocol 8: Tetracycline as Regulator of Inducible Gene Expression Stage 1: Stable Transfection of Fibroblasts with pTet-tTAk

The following protocol uses an autoregulatory system in which the transcriptional *trans*-activator tTA drives its own expression and that of a target gene. The first stage of the procedure describes how to generate stable lines of NIH-3T3 cells that express either tTA alone or tTA and the tetracycline-regulated target gene.

Protocol 9: Tetracycline as Regulator of Inducible Gene Expression Stage 2: Stable Transfection of Inducible tTA-expressing NIH-3T3 Cells with Tetracycline-regulated Target Genes

This stage of the procedure describes the transfection with target genes of cell lines already expressing inducible tTA. In this example, the target genes are transfected on a plasmid that carries puromycin resistance as a selectable marker.

Protocol 10: Tetracycline as Regulator of Inducible Gene Expression Stage 3: Analysis of Protein Expression in Transfected Cells

Stably transfected cells, generated in the first two stages of the procedure, are induced for expression of the target gene. After harvesting and lysis, the lysates are analyzed by SDS-PAGE and immunoblotting. For further details on expression using this system, please see http://www.clontech.com/tet/Refs/index.html.

Protocol 11: Ecdysone as Regulator of Inducible Gene Expression in Mammalian Cells

This protocol is adapted from the information supplied by Invitrogen as part of their Edysone-inducible Mammalian Expression System.

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Protocol 1

Mapping Protein-binding Sites on DNA by DNase I Footprinting

In this protocol, DNase I is used to fragment a radiolabeled target DNA in the presence and absence of a nuclear extract. A "footprint" is generated when a protein binds to the target and protects a specific segment of DNA from the nucleolytic activity of DNase I. By comparing the electrophoretic mobility of the DNase I cleavage products to those of a sequence ladder derived from the same DNA fragment, the position(s) of the DNA sequences recognized by DNA-binding proteins can be determined.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Cell homogenization buffer
- Cell homogenization buffer containing 0.05% (v/v) Nonidet P-40
- Cell resuspension buffer (17-1)
- Cell rinse buffer

  Ethanol
- Ficoll 400 (20% w/v)
  Dissolve the Ficoll in sterile H_2O and store the solution frozen in 100-µl aliquots at -20°C.
- ⚠ Formamide dye mix
- $MgCl_2$-$CaCl_2$ solution (17-1)
- NaCl (5 M)

  Nonidet P-40 (0.05% v/v)
- PBS without $CaCl_2$ and $MgCl_2$ salts
- ⚠ Phenol:chloroform
- Polyvinyl alcohol (10% w/v)
  Dissolve the polyvinyl alcohol in sterile H_2O and store the solution frozen in 100-µl aliquots at -20°C.
- Stop mix (17-1)
- Tissue homogenization buffer
- Tissue resuspension buffer
- Trypan Blue dye (0.4% w/v)

Enzymes and Buffers

DNase I (1 mg/ml)
Dissolve the enzyme in 10 mM Tris-Cl (pH 8.0). Store the solution frozen in small aliquots at -20°C. Dilute the solution 1:100 in ice-cold 10 mM Tris-Cl (pH 8.0) just before Step 3.

Nucleic Acids and Oligonucleotides

Poly(dI-dC) (1 mg/ml)
Dissolve an appropriate amount of poly(dI-dC) in sterile H_2O and store the solution in 100-µl aliquots at -20°C. The nucleic acid copolymer is added to decrease nonspecific binding of proteins to the radiolabeled DNA fragment. The optimum concentration (usually between 0 and 100 µg/ml) of poly(dI-dC) in a binding reaction should be determined empirically. Other nucleic acids that can be used to decrease nonspecific binding include sheared genomic DNA (e.g., from E. coli*, salmon sperm, or calf thymus), tRNAs, sheared or restricted plasmid DNA, poly(dA-dT), and poly(dG-dC).*

Sequencing gel size standards
These typically consist of the (A+G) reactions of a Maxam and Gilbert sequencing experiment derived from the target DNA fragment. For a method to perform chemical sequencing reactions, please see Chapter 12, Protocol 7. Alternatively, use a dideoxy terminator sequencing reaction (Chapter 12, Protocol 3), of a DNA whose 5' end is identical to the radiolabeled end of the DNA fragment digested with DNase I.

Radioactive Compounds

⚠ ^{32}P-end-labeled DNA (200-500 bp in length, specific activity ≥2.5 x 10^7 cpm/µg [≥5000 cpm/fmole])
End labeling of the DNA fragment can be accomplished by phosphorylation (Chapter 9, Protocol 13 or Chapter 10, Protocol 2), by filling in of a 3'-recessed end using a DNA polymerase (Chapter 9, Protocol 10 or Chapter 10, Protocol 7), or by using an end-labeled primer in a polymerase chain reaction (PCR; Chapter 8, Protocol 1). Purify the DNA fragment by electrophoresis through an agarose or polyacrylamide gel before use in the footprinting reaction. The DNA fragment used in the reaction should be 200-500 bp in length, with the binding site of interest at least 30 bp from the radiolabeled end.

Additional Reagents

Steps 7-10 of this protocol require the reagents listed in Chapter 12, Protocol 8, Chapter 12, Protocol 11, and Chapter 12, Protocol 12.

Cells and Tissues

Fresh tissue, Cultured cells, or Protein fractions derived from cells or tissues

METHOD

1. Prepare nuclear extracts using one of the following three methods. Alternatively, fractions derived from purification of cellular proteins can be used directly in Step 2.

   Preparation of nuclear extracts from tissue

   a. Dissect and mince 10-15 g of tissue. Adjust the volume of minced tissue to 30 ml with ice-cold tissue homogenization buffer. Homogenize in a tight-fitting Dounce homogenizer until >80-90% of the cells are broken as determined by microscopy.

   b. To monitor lysis, mix 10 µl of the cell suspension with an equal volume of 0.4% Trypan Blue dye and examine the solution under a microscope equipped with a 20x objective. Lysed cells take up the dye and stain blue, whereas intact cells exclude dye and remain translucent. Continue to homogenize the tissue until >80-90% of the cells are broken.

c. Dilute the homogenate to 85 ml with ice-cold tissue homogenization buffer. Layer 27-ml aliquots over 10-ml cushions of ice-cold tissue homogenization buffer in ultraclear or polyallomer swinging-bucket centrifuge tubes. Centrifuge the tubes at 103,900*g* (24,000 rpm in a Beckman SW28 rotor) for 40 minutes at 4°C.

d. Decant the supernatant and allow the tubes to drain in an inverted position for 1-2 minutes. Place the tubes on ice. *(Optional) Use a razor blade to cut off the top two thirds of the tube and place the bottom one third containing the nuclei on ice.*

e. Resuspend the pellet of nuclei in 2 ml of ice-cold tissue resuspension buffer. Accurately measure the volume of the resuspended nuclei and add ice-cold 5 M NaCl to a final concentration of 300 mM. Mix the suspension gently. Incubate the suspension for 30 minutes on ice.

f. Recover the nuclei by centrifugation at 103,900*g* (24,000 rpm in a Beckman SW28 rotor) for 20 minutes at 4°C. Carefully transfer the supernatant to a fresh tube. Divide the supernatant into aliquots of 100-200 µl. Reserve an aliquot for protein concentration determination. Snap-freeze the remainder of the aliquots in liquid nitrogen, and store them in liquid nitrogen.

g. Determine the protein concentration of the supernatant by the Bradford method.

Preparation of nuclear extracts from small numbers of cultured mammalian cells

a. Harvest 0.5 x 10^8 to 1 x 10^8 cells from their culture flasks, plates, or wells. Collect the cells by centrifugation at 250*g* (1100 rpm in a Sorvall H1000B rotor) for 10 minutes at room temperature. Rinse the cells several times with phosphate-buffered saline without calcium and magnesium salts.

b. Resuspend the cell pellet in 5 volumes of ice-cold cell homogenization buffer. Incubate the cells for 10 minutes on ice, and then collect them by centrifugation as before.

c. Resuspend the cell pellet in 3 volumes of ice-cold cell homogenization buffer containing 0.05% (v/v) Nonidet P-40, and homogenize the cells with 20 strokes of a tight-fitting Dounce homogenizer. The body of the homogenizer should be buried in ice during the homogenization process, during which the swollen cells lyse and release intact nuclei.

d. Collect the nuclei by centrifugation at 250*g* (1100 rpm in a Sorvall H1000B rotor) for 10 minutes at 4°C. Remove the supernatant, and resuspend the pellet of nuclei in 1 ml of cell resuspension buffer (17-1). Accurately measure the volume of the resuspended nuclei and add 5 M NaCl to a final concentration of 300 mM. Mix the suspension gently and incubate for 30 minutes on ice.

e. Recover the nuclei by centrifugation at 103,900*g* (24,000 rpm in a Beckman SW28 rotor) for 20 minutes at 4°C. Carefully transfer the supernatant to a chilled, fresh tube. Divide the supernatant into aliquots of 100-200 µl. Reserve an aliquot for protein concentration determination. Quick freeze the aliquots in liquid nitrogen, and store them in liquid nitrogen. Determine the protein concentration of the supernatant by the Bradford method.

Preparation of nuclear extracts from small numbers of cultured mammalian cells

a. Rinse the cells with several changes of cell rinse buffer. Add 1 ml of the cell rinse buffer to each dish, and scrape the cells into the buffer using a rubber policeman.
This procedure is suitable for cells transfected with plasmids expressing cDNAs encoding transcription factors.

b. Transfer the cell suspension to a 1.5-ml microfuge tube, and pellet the cells by centrifuging at maximum speed for 2 minutes at room temperature in a microfuge.

c. Resuspend the cell pellet in 300 µl of Cell resuspension buffer (17-1) per 150-mm dish of original cells. Subject the resuspended cells to three cycles of freezing and thawing.

d. Remove the cellular debris by centrifuging the tubes at maximum speed for 5 minutes at 4°C in a microfuge. Store the supernatant (i.e., the cell lysate) in small aliquots at -70°C.

2. To an appropriate number of 1.5-ml microfuge tubes add:

nuclear extract or protein fraction	1-23 µl
^{32}P-end-labeled DNA	1-10 fmoles
1 mg/ml poly(dI-dC)	1 µl
H_2O	to 25 µl
Optional additions:	
20% Ficoll 400	12 µl
or	
10% polyvinyl alcohol	10 µl

Centrifuge the tubes for 5 seconds at 4°C in a microfuge to deposit the reaction mixtures at the bottom of the tubes. Incubate the reaction mixtures for 10-30 minutes on ice.
For each DNA fragment or fraction to be assayed, set up two control reactions. One control without the nuclear extract, the other without addition of DNase I in Step 3.

3. Add 50 µl of $MgCl_2$-$CaCl_2$ solution (17-1) at room temperature and mix gently. Incubate the reactions for 1 minute at room temperature. Add 1-8 µl of diluted DNase I solution to the microfuge tubes, mix gently, and incubate the reactions for 1 minute at room temperature.
4. Stop the reactions by adding 75 µl of stop mix. Vortex briefly and extract the reactions with an equal volume of phenol:chloroform.
5. Transfer the aqueous phases to fresh microfuge tubes, and precipitate the nucleic acids with 2.5 volumes of ethanol. Chill the ethanolic solution for 15 minutes at -70°C, and collect precipitates by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge. Rinse the pellets with 1 ml of 70% ethanol, centrifuge again, and dry in the air to remove the last traces of ethanol.
6. Solubilize the DNA pellets in 5-10 µl of formamide dye mix by vigorous vortexing. Denature the DNA solutions by boiling for 3-5 minutes.
7. Set up a denaturing 6% or 8% polyacrylamide sequencing gel and run the gel for at least 30 minutes before loading the DNA samples.
8. Load the DNA samples in the following order:
sequence ladder
control DNA digested with DNase I in the absence of nuclear extract
target DNA from reactions digested with DNase I in the presence of nuclear extract
target DNA incubated with nuclear extract and no DNase I
9. Run the gel at sufficient constant power to maintain a temperature of 45-50°C.
The time required to achieve optimal resolution of the sequence of interest must be determined empirically.
10. After electrophoresis is complete, pry the glass plates apart and transfer the gel to a piece of thick blotting paper. Dry the gel under vacuum for approx. 1 hour, and expose it to X-ray film without an intensifying screen for 12-16 hours at -20°C. Alternatively, subject the dried gel to phosphorimage analysis for 1-3 hours.

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Protocol 2

Gel Retardation Assays for DNA-binding Proteins

This protocol exploits differences in electrophoretic mobility through a nondenaturing polyacrylamide gel between a rapidly migrating target DNA and a more slowly migrating DNA-protein complex.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Ficoll 400 (20% w/v)
- 6x Gel-loading buffer I
- Polyvinyl alcohol (10% w/v)
- 10x Tris-glycine buffer
  TBE buffer may be used in place of Tris-glycine.

Nucleic Acids and Oligonucleotides

Poly(dI-dC) (1 mg/ml)
Dissolve an appropriate amount of poly(dI-dC) in sterile H_2O and store the solution in 100-µl aliquots at -20°C.

Radioactive Compounds

⚠ ^{32}P-labeled control DNA

⚠ ^{32}P-labeled target DNA of >20 bp (specific activity ≥ 2 x 10^7 cpm/µg [≥ 5000 cpm/fmole])
Labeling of the DNA fragment can be accomplished by phosphorylation (Chapter 9, Protocol 13 or Chapter 10, Protocol 2), filling in of a 3'-recessed end using a DNA polymerase (Chapter 9, Protocol 10 or Chapter 10, Protocol 7), or by using PCR to incorporate radiolabeled nucleotides into the body of the probe (Chapter 8, Protocol 1).

Cells and Tissues

Control nuclear extract or protein fraction

Nuclear extract or Protein fraction(s)
Prepare the extract by one of the methods described in Chapter 17, Protocol 1.

METHOD

1. To a sterile 1.5-ml microfuge tube add:

^{32}P-labeled target DNA	1 ng (1-10 fmoles)
1 mg/ml poly(dI-dC)	1 µl
nuclear extract (5-10 µg)	≤ 10 µl
or	
protein fraction	≤ 10 µl
20% Ficoll 400	5 µl
or	
10% polyvinyl alcohol	4 µl
H_2O	to 20 µl

   Include control reactions with every experiment. Positive control reactions contain a nuclear extract (or protein fraction) and a radiolabeled DNA fragment carrying a sequence recognized by a DNA-binding protein that is abundant in the extract and has high affinity for the DNA sequence. Examples are a DNA fragment containing an Sp1, C/EBP, or NF-1 site and mammalian cell nuclear extract, or a lacI *recognition site and extract derived from a* $lacI^q$ *strain of* E. coli*. The negative control reactions contain the radiolabeled target DNA fragment, but no nuclear extract.*
2. Centrifuge the reaction tubes for several seconds in a microfuge to deposit the reaction mixtures at the bottom of the tubes. Incubate the reactions for 10-30 minutes on ice.
3. Add 3 µl of 6x gel-loading buffer I to each tube. Load the samples into the slots of a neutral 4-7% polyacrylamide gel.
4. Run the gel in either 0.5x Tris-glycine buffer or 0.5x TBE buffer at 200-250 V and 20 mA for ≥ 2 hours.
   Depending on the lability of the binding protein(s) and the affinity of the binding reaction(s), it may be necessary to run the gel at 4°C.
5. After electrophoresis is complete, pry the gel plates apart, transfer the gel to a piece of sturdy blotting paper, and dry the gel for approx. 1 hour on a gel dryer.
6. Expose the dried gel to X-ray film for ≥ 1 hour at -20°C to visualize radiolabeled DNA fragments. Less abundant DNA-protein complexes can be detected after 1-3-hours on a phosphorimager.

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1. Fried M.G. and Bromberg J.L. 1987. Factors that affect the stability of protein-DNA complexes during gel electrophoresis. *Electrophoresis* 18:6-11.
2. Fried M.G. and Crothers D.M. 1981. Equilibria and kinetics of *lac* repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* 9:6505-6524.

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Protocol 3

Mapping DNase-I-hypersensitive Sites

In this protocol, isolated nuclei are incubated with varying amounts of DNase I. Genomic DNA is then isolated from the nuclei and digested with a restriction enzyme, analyzed by gel electrophoresis, and probed by Southern hybridization. If the probe corresponds to the 5′ end of the gene, intact restriction fragments arising from that region will be detected in DNA isolated from control nuclei not treated with DNase I. If DNase-I-hypersensitive sites exist in one or more of the fragments recognized by the probe, then shorter DNAs will be detected on the Southern blot.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Buffer A
- EDTA (0.5 M, pH 8.0)
- Ethanol
- Lysis buffer (17-3)
- PBS without $CaCl_2$ and $MgCl_2$ salts
- ⚠ Phenol:chloroform
- SDS buffer
- TE
- Trypan Blue dye (0.4% w/v)

Enzymes and Buffers

- DNase I dilution buffer (17-3)
- DNase I solution
- Proteinase K solution (17-3)
- RNase solution

Additional Reagents

Step 18 of this protocol requires the reagents listed in Chapter 6, Protocol 8 and Chapter 6, Protocol 10.

Cells and Tissues

Eukaryotic cells

Approximately 10^8 cells are required per DNase I hypersensitivity mapping experiment.

METHOD

1. Harvest approx. 10^8 cells from spinner cultures, flasks, or dishes, and wash the cells twice with 25-ml aliquots of ice-cold PBS without calcium and magnesium salts.
   Alternatively, if starting with fresh tissue, isolate the nuclei as described in Chapter 17, Protocol 1, Step 1.
2. Resuspend the cell pellet from the final wash in 1.5 ml of ice-cold lysis buffer (17-3). Incubate the cells for 10 minutes on ice to allow cell lysis to occur.
3. Mix a 10-µl aliquot of cell lysate with an equal volume of 0.4% Trypan Blue dye and examine the solution under a microscope equipped with a 20x objective. Lysed cells and nuclei take up the dye and appear blue, whereas unlysed cells are impermeable to the dye and remain translucent. Continue the incubation on ice until >80% of cells are lysed.
4. Recover nuclei from the lysed cells by centrifugation of the suspension at 1300*g* (2500 rpm in a Sorvall H1000B rotor) for 15 minutes at 4°C.
5. Carefully remove the supernatant and resuspend the pellet of nuclei in 1.5 ml of ice-cold Buffer A. Collect the nuclei by centrifugation as described in Step 4.
6. Resuspend the nuclear pellet in 4 ml of ice-cold Buffer A.
7. Set up a series of dilutions of the standard DNase I solution (1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, and 1/2560 in DNase I dilution buffer [17-3]). Store the dilutions on ice.
8. Label a series of tubes 1 through 9 and add 180 µl of resuspended nuclei from Step 6 to each tube.
9. To Tube 1, add 20 µl of DNase I dilution buffer (17-3) containing no DNase I and store the tube on ice until Step 12, below. To Tube 2, add 20 µl of DNase I dilution buffer (17-3) containing no DNase and incubate as described in Step 11, below. Tubes 1 and 2 are controls.
10. To Tubes 3 through 9, add 20 µl of each of the progressive dilutions, i.e., to Tube 3, add 20 µl of the 1/2560 dilution, to Tube 4, add 20 µl of the 1/1280 dilution, etc.
11. Incubate Tubes 2-9 for 20 minutes at 37°C.
12. Terminate the reactions by adding three individual aliquots of 16.6 µl of 0.5 M EDTA to each tube, with vortexing between additions. When all of the tubes have been treated, add 12 µl of RNase solution to each tube. Incubate the reactions for 30 minutes at 37°C to allow digestion of nuclear RNA.
13. Digest nuclear proteins by adding 40 µl of proteinase K solution (17-3) to each tube. Mix the solution gently by pipetting the mixture up and down. Add 100 µl of SDS buffer to each tube and mix once more. Incubate the tubes for 16 hours at 50°C with rotation or rocking.
14. Add an additional aliquot of 100 µl of proteinase K solution and continue the digestion for a further 2-3 hours at 50°C.
15. Extract the digestion mixtures three times with phenol:chloroform. Be gentle. Precipitate the DNA with the addition of 3 volumes of ice-cold ethanol, incubate for 30 minutes on ice, and collect the DNA precipitates by centrifugation at 1200*g* (2400 rpm in a Sorvall H1000B rotor) in a benchtop centrifuge. Decant the supernatant and drain the last dregs of ethanol from the tubes on a paper towel.
16. Add 200 µl of TE to each tube and allow the DNA to redissolve with rocking or rotation overnight at 55°C.
    IMPORTANT *An extended incubation is required for complete solubilization and recovery of the DNase-treated DNA.*
17. Determine the A_{260} of the resuspended DNA and estimate the concentration.
18. Digest the DNA with a restriction enzyme(s), followed by Southern blotting and hybridization as described in Chapter 6, Protocol 8 and Chapter 6, Protocol 10. Load 15-30 µg of restricted genomic DNA per lane on the agarose gel.
    It is crucial to use high-specific-activity radioactive probes when mapping hypersensitive sites. The specific activity of the probe should be >5 x 10^8 cpm/µg. Single-stranded DNA probes derived from bacteriophage M13 templates (Chapter 3, Protocol 4) are ideal for high-resolution hypersensitivity mapping experiments.

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Protocol 4

Transcriptional Run-on Assays

In this protocol, nuclei isolated from cells expressing the gene of interest are incubated with radiolabeled UTP, which is incorporated into nascent RNA transcripts by RNA polymerase molecules that were actively transcribing at the time the cells were harvested. Because very little de novo initiation of RNA synthesis occurs in isolated nuclei, transcription of the target gene can be measured by hybridizing the radiolabeled RNA to an excess of the target gene immobilized on a nitrocellulose or nylon membrane. The fraction of the RNA that hybridizes to the immobilized DNA reflects the contribution of the target gene to the total transcriptional activity of the cell. All test tubes and solutions must be prepared RNase-free.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Chloroform

⚠ Chloroform:isoamyl alcohol

DNA denaturation solution (17-4)

Ethanol

Glycerol storage buffer

HSB buffer

LiCl (5 M)

Lysis buffer (17-4)

NaCl (2 M)

⚠ NaOH (0.1 M)

Nonidet P-40 (5% v/v)

Nuclei labeling buffer

Nuclei wash buffer

PBS

⚠ Phenol

⚠ Prehybridization/Hybridization solution
Hybridization buffer containing formamide is generally used in nuclear run-on assays, e.g., 50% (v/v) formamide, 6x SSC, 5 mM sodium pyrophosphate, 2x Denhardt's solution, 0.5% (w/v) SDS, 10 µg/ml poly(A), and 100 µg/ml salmon sperm DNA (please see Chapter 6, Protocol 10).

2x Reaction buffer (17-4)

RNasin

⚠ SDS (0.5% w/v)

6x SSC

Stop buffer (17-4)

Tissue homogenization buffer

Trypan Blue dye (0.4% w/v)

Enzymes and Buffers

DNase solution

Proteinase K (optional)

Restriction enzyme(s)
Please see Step 9.

Radioactive Compounds

⚠ [α-^{32}P]UTP (500-5000 µCi/ml)
Use 100 µCi for each sample of nuclei to be radiolabeled.

Additional Reagents

Step 11 of this protocol requires the reagents and equipment listed in Chapter 7, Protocol 9.

Step 13 of this protocol requires the reagents listed in Chapter 6, Protocol 8.

Steps 14-16 of this protocol require the reagents listed in Chapter 6, Protocol 8 and Chapter 6, Protocol 10.

Vectors and Bacterial Strains

Nonrecombinant plasmid vector

Recombinant plasmid containing the cDNA or gene of interest

Cells and Tissues

Cultured cells or Fresh tissue

METHOD

1. Isolate nuclei from either cultured cells or fresh tissue.
Isolation of nuclei from cultured cells
   a. Scrape the cells from culture dishes and wash them twice with ice-cold PBS. Resuspend 1 x 10^7 to 1 x 10^8 cells in 1 ml of ice-cold lysis buffer (17-4) in a 17 x 100-mm polypropylene tube. Add 2-4 µl of 5% Nonidet P-40 and incubate the suspension for 10 minutes on ice.
   b. Mix 10 µl of the suspension with an equal volume of 0.4% Trypan Blue dye and examine the solution under a microscope equipped with a 20x objective. Lysed cells take up the dye and appear blue, whereas unlysed cells are impermeable to the dye and remain translucent. Continue adding 2-µl aliquots of 5% Nonidet P-40 and check cell lysis until >80% of the cells are lysed.
   c. Recover the nuclei by centrifugation at 1300*g* (2500 rpm in a Sorvall H1000B rotor) for 1 minute in a benchtop centrifuge. Remove and discard the supernatant. Wash the pellet of nuclei twice in 1-ml of ice-cold nuclei wash buffer. Proceed to Step 2.

Isolation of nuclei from tissue

a. Dissect and mince 10-15 g of tissue. Adjust the volume of minced tissue to 30 ml with ice-cold tissue homogenization buffer, and homogenize in a tight-fitting Dounce homogenizer.

b. To monitor lysis, mix 10 µl of the cell suspension with an equal volume of 0.4% Trypan Blue dye and examine the solution under a microscope equipped with a 20x objective. Lysed cells take up the dye and stain blue, whereas intact cells exclude dye and remain translucent. Continue to homogenize the tissue until >80-90% of the cells are broken.

c. Dilute the homogenate to 85 ml with ice-cold tissue homogenization buffer. Layer 27-ml aliquots over 10-ml cushions of ice-cold homogenization buffer in ultraclear or polyallomer swinging bucket centrifuge tubes. Centrifuge the tubes at 103,900*g* (24,000 rpm in a Beckman SW28 rotor) for 40 minutes at 4°C.

d. Decant the supernatant and allow the tubes to drain in an inverted position for 1-2 minutes. Place the tubes on ice. Resuspend each pellet in 2 ml of glycerol storage buffer by pipetting the mixture up and down.

e. Mix 10 µl of resuspended nuclei with 990 µl of 0.5% SDS. Measure the OD_{260} in a UV spectrophotometer and dilute resuspended nuclei with glycerol storage buffer to a final concentration of 50 OD_{260}/ml. Divide the preparation of nuclei into 200-µl aliquots in 1.5-ml microfuge tubes, snap freeze the aliquots in liquid nitrogen, and store them at -70°C. Proceed to Step 2.

2. Radiolabel the nascent RNA transcripts in the isolated nuclei.

Radiolabeling of the transcripts in nuclei isolated from cultured cells

a. Remove as much supernatant as possible from the last wash (Step 1c), and resuspend the nuclei in 50-100 µl of nuclei labeling buffer. Incubate the nuclei for 15-20 minutes at 30°C in a shaking water bath.

b. Pellet the nuclei by centrifugation at 800*g* (1960 rpm in a Sorvall H1000B rotor) for 5 minutes in a benchtop centrifuge, and carefully discard the supernatant as radioactive waste. Proceed to Step 3.

Radiolabeling of the transcripts in nuclei isolated from tissue

a. Transfer an appropriate number of aliquots of the nuclear preparation from -70°C to an ice bucket. When the aliquots have thawed, add 400 units of RNasin to each tube. Add 200 µl of 2x reaction buffer (17-4) supplemented with nucleotides and dithiothreitol to each tube of nuclei. Add 100 µCi of [α-^{32}P]UTP.

b. Incubate the nuclei for 20 minutes in a 30°C shaking water bath. Recover the nuclei by centrifugation at 2000 rpm for 1-2 minutes. Carefully discard the supernatant as radioactive waste. Proceed to Step 3.

3. Resuspend the nuclear pellet in 1 ml of ice-cold HSB buffer, and add 10 µl of DNase solution. Pipette the nuclei up and down until they are resuspended and the viscosity of the solution is reduced (1-5 minutes). Add 2 ml of stop buffer.
4. (*Optional*) To increase the yield of radiolabeled RNA, add proteinase K after the DNase step. Following the addition of 2 ml of stop buffer (17-4), add proteinase K to a final concentration of 100 µg/ml. Incubate the solution for 30 minutes at 42°C. Proceed to Step 5.
5. Add 3 ml of phenol and incubate the mixture for 15 minutes at 65°C with vortexing every 5 minutes. Add 3 ml of chloroform:isoamyl alcohol, vortex, and separate the organic and aqueous phases by centrifugation at 1900*g* (3000 rpm in a Sorvall H1000B rotor). Extract the aqueous layer again with 3 ml of chloroform, centrifuge as before, and transfer the aqueous layer to a fresh tube.
6. Add 0.3 ml of 5 M LiCl and 2.5 volumes of ethanol, and mix well. Collect precipitated nucleic acids by centrifugation at 12,000*g* (10,000 rpm in a Sorvall SS-34 rotor) for 10 minutes.
7. Resuspend the pellet in 0.4 ml of H_2O and transfer it to a 1.5-ml microfuge tube. Add 40 µl of 5 M LiCl and 2.5 volumes of ethanol. Centrifuge the solution for 10 minutes at maximum speed in a microfuge.
8. Resuspend the pellet in 100 µl of H_2O and measure the cpm/µl in a liquid scintillation counter.
9. Linearize 10 µg of recombinant plasmid DNA containing the cDNA or gene of interest and 10 µg of empty plasmid vector using a restriction enzyme whose sites of cleavage are present in the vector sequences.
10. Recover the cleaved DNAs using standard ethanol precipitation, and resuspend each of the pellets separately in 20 µl of DNA denaturation solution (17-4). Boil the resuspended DNAs for 2 minutes, and then add 180 µl of 6x SSC to each tube.
11. Cut a piece of nylon or nitrocellulose membrane to the appropriate size for use in a dot- or slot-blotting apparatus. Wet the membrane in H_2O and then soak it for 5-10 minutes in 6x SSC. Clamp the wet membrane in the blotting apparatus, attach a vacuum line, and apply suction to the device (please see Chapter 7, Protocol 9).
12. Filter the denatured DNAs through separate slots and wash each filter with 200 µl of 6x SSC.
13. Dismantle the device, dry the membrane in the air, and fix the DNA to the membrane by baking or by exposure to UV light.
14. Place the membrane in prehybridization solution and incubate it for at least 16 hours at an appropriate temperature (e.g., 42°C for solvents containing 50% formamide).
15. Add the radiolabeled probe (2 x 10^6 to 4 x 10^6 cpm/ml of ^{32}P-labeled RNA from Step 8) directly to the prehybridization solution, and incubate the filter for a further 72 hours.
16. Wash the membrane at high stringency and expose it to X-ray film or a phosphorimager plate. Typical exposure times are 24-72 hours for X-ray film and 4-24 hours for a phosphorimager plate.

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Protocol 5

Measurement of Chloramphenicol Acetyltransferase in Extracts of Mammalian Cells Using Thin-layer Chromatography

In this protocol, extracts prepared from cells transfected with a chloramphenicol acetyltransferase (CAT) reporter plasmid are incubated with radiolabeled chloramphenicol. The acetylated products generated by the action of CAT are separated from the unmodified drug by thin-layer chromatography and quantitated by scraping the spots from the thin-layer plates and counting them by scintillation spectroscopy. Other methods of measuring the activity of CAT are described on pages 17.40, 17.41, and 17.95 of the print version of the manual.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- CAT lysis buffer
- CAT reaction mixture 1
- ⚠ Ethyl acetate
- PBS without $CaCl_2$ and $MgCl_2$ salts
- TLC solvent (thin-layer chromatography)
- Tris-Cl (1 M, pH 7.8)

Radioactive Compounds

- ⚠ Radioactive ink
  Use to mark adhesive dot labels as guides for orienting the autoradiogram with the TLC plate.

Cells and Tissues

Cultured mammalian cells transfected with pCAT vectors carrying the DNA of interest
The cells should be transfected (using one of the transfection protocols in Chapter 16) with a CAT reporter construct (e.g., the pCAT3 series) and an expression plasmid containing the β-galactosidase gene (pCMV-SPORT-β-gal) or another reporter gene suitable for normalizing the results of CAT assay.

METHOD

1. Use gentle aspiration to remove the medium from transfected monolayers of cells growing in 90-mm tissue culture dishes. Wash the monolayers three times with 5 ml of PBS without calcium and magnesium salts.
2. Stand the dishes at an angle for 2-3 minutes to allow the last traces of PBS to drain to one side. Remove the last traces of PBS by aspiration. Add 1 ml of PBS to each plate, and use a rubber policeman to scrape the cells into microfuge tubes. Store the tubes in ice until all of the plates have been processed.
3. Recover the cells by centrifugation at maximum speed for 10 seconds at room temperature in a microfuge. Gently resuspend the cell pellets in 1 ml of ice-cold PBS, and again recover the cells by centrifugation. Remove the last traces of PBS from the cell pellets and from the walls of the tubes. Store the cell pellets at -20°C for future analysis or prepare cell extracts by either of the methods in Step 4.
4. Lyse the cells either by repeated cycles of freezing and thawing or by incubating the cells in detergent-containing buffers. The latter is a quicker and easier method of cell lysis that permits CAT, β-galactosidase, and other marker gene assays to be adapted to a 96-well microtiter plate format (Chapter 17, Protocol 6).

   Lysis of cells by repeated freezing and thawing

   a. Resuspend the cell pellet from one 90-mm dish in 100 µl of 0.25 M Tris-Cl (pH 7.8). Vortex the suspension vigorously to break up clumps of cells.
   b. Disrupt the cells by three cycles of freezing in a dry ice/ethanol bath and thawing at 37°C. Make sure that the tubes have been marked with ethanol-insoluble ink.
   c. Centrifuge the suspension of disrupted cells at maximum speed for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh microfuge tube. Set aside 50 µl of this supernatant for the CAT assay, and store the remainder of the extract at -20°C.

   Lysis of cells using detergent-containing buffers

   a. To lyse cells with detergent, resuspend the cell pellets from Step 3 in 500 µl of CAT lysis buffer. Incubate the mixture for 15 minutes at 37°C.
      Use 100 µl of this lysis buffer per cell pellet for extracts prepared from cells grown in 35-mm dishes.
   b. Remove the cellular debris by centrifuging the tubes at maximum speed for 10 minutes in a microfuge. Recover the supernatant. Assay CAT activity using one of the methods described in this protocol. Snap freeze the remainder of the cleared lysates in liquid nitrogen and store them at -70°C.
5. Incubate a 50-µl aliquot of the cell extract for 10 minutes at 65°C to inactivate endogenous deacetylases. If the extract is cloudy or opaque at this stage, remove the particulate material by centrifugation at maximum speed for 2 minutes at 4°C in a microfuge.
6. Mix each of the samples to be assayed with 80 µl of CAT reaction mixture 1, and incubate the reactions at 37°C. The length of the incubation depends on the concentration of CAT in the cell extract, which in turn depends on the strength of the promoter and the cell type under investigation. In most cases, incubation for 30 minutes to 2 hours is sufficient.
7. Add 1 ml of ethyl acetate to each sample, and mix the solutions thoroughly by vortexing for three periods of 10 seconds. Centrifuge the mixtures at maximum speed for 5 minutes at room temperature in a microfuge.
8. Use a pipette to transfer exactly 900 µl of the upper phase to a fresh tube, carefully avoiding the lower phase and the interface. Discard the tube containing the lower phase in the radioactive waste.
9. Evaporate the ethyl acetate under vacuum by placing the tubes in a rotary evaporator (e.g., Savant SpeedVac) for approx. 1 hour.
10. Add 25 µl of ethyl acetate to each tube and dissolve the reaction products by gentle vortexing.
11. Apply 10-15 µl of the dissolved reaction products to the origin of a 25-mm silica gel TLC plate. The origin on the plate can be marked with a soft-lead pencil. Apply 5 µl at a time, and evaporate the sample to dryness with a hair dryer after each application.
12. Prepare a TLC tank containing 200 ml of TLC solvent. Place the TLC plate in the tank, close the chamber, and allow the solvent front to move approx. 75% of the distance to the top of the plate.
13. Remove the TLC plate from the tank and allow it to dry at room temperature. Place adhesive dot labels marked with radioactive ink on the TLC plate to align the plate with the film, and then expose the plate to X-ray film. Alternatively, enclose the plate in a phosphorimaging cassette. Store the cassette at room temperature for an appropriate period of time.
    Do not cover the TLC plate with Saran Wrap, as this coverage will block the relatively weak radiation emitted by the ^{14}C isotope.
14. Develop the X-ray film and align it with the plate. Alternatively, expose the chromatogram to the imager plate of a

phosphorimager device or subject the plate to scanning.

15. To quantitate CAT activity, cut the radioactive spots from the TLC plate and measure the amount of radioactivity they contain in a liquid scintillation counter. Use another aliquot of the cell extract (from Step 3 above) to determine the concentration of protein in the extract, using a rapid colorimetric assay, such as the Bradford assay. Reduce the concentration of Triton X-100 to $\leq$0.1% by dilution before determining the concentration of protein to prevent interference with the assay. Express the CAT activity as pmoles of acetylated product formed per unit time per milligram of cell extract protein.

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Protocol 6

Assay for Luciferase in Extracts of Mammalian Cells

In this protocol, cells transfected with a luciferase reporter plasmid are lysed in a detergent-containing buffer. Luciferase in the extract catalyzes an oxidation reaction in which D-luciferin is converted to oxyluciferin, with production of light at 556 nm that can be quantified in a luminometer.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- Luciferase assay buffer
- Luciferase cell lysis buffer
- Luciferin solution
- PBS without $CaCl_2$ and $MgCl_2$ salts

Cells and Tissues

Cultured mammalian cells transfected with the DNA of interest
The cells should be transfected (using one of the transfection protocols in Chapter 16) with a luciferase reporter construct and an expression plasmid containing the β-galactosidase gene (pCMV-SPORT-β-gal) or another reporter gene suitable for normalizing the results of luciferase assay.

METHOD

1. Between 24 and 72 hours after transfection, wash the cells three times at room temperature with PBS without calcium and magnesium salts. Add and remove the PBS gently, because some mammalian cells (e.g., human embryonic kidney 293 cells) can be easily displaced from the dish by vigorous pipetting.
2. Add 1 ml of ice-cold Luciferase cell lysis buffer per 100-mm dish of transfected cells. Swirl the buffer gently and scrape the lysed cells from the dish using a rubber policeman. Transfer the cell lysate to a 1.5-ml microfuge tube.
3. Centrifuge the cell lysate at maximum speed for 5 minutes at 4°C in a microfuge. Carefully transfer the supernatant to a fresh 1.5-ml microfuge tube.
4. Determine the concentration of protein in the lysate using a rapid colorimetric assay, such as the Bradford assay. Reduce the concentration of Triton X-100 to ≤0.1% by dilution before determining the concentration of protein to prevent interference with the assay.
5. Tap the side of the tube containing the lysate to gently mix the contents. Add 5-200-µl aliquots of cell lysate to individual luminometer tubes containing 360 µl of luciferase assay buffer at room temperature. Place a tube in the luminometer.
6. To start the assay, inject 200 µl of luciferin solution into the luminometer tube and measure the light output for a period of 2-60 seconds at room temperature.
   The optimal time of light collection must be determined empirically.
7. Measure the relative light units generated in each tube and determine the linear range of the assay. Use the amount of cell lysate protein that produces a response in the middle of the linear range in subsequent assays. This amount will vary depending on the strength of the promoter being studied, and to a lesser extent, on the efficiency of transfection in individual experiments. Express luciferase activity as relative light units/mg of protein in the cell lysate.

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Protocol 7

Assay for β-galactosidase in Extracts of Mammalian Cells

The assay for β-galactosidase relies on the ability of the enzyme to catalyze the hydrolysis of ONPG (*o*-nitrophenyl- β-D- galactopyranoside) to free *o*-nitrophenol, which absorbs light at 420 nm. In this protocol, extracts of cells transfected with a β-galactosidase reporter plasmid are incubated with ONPG. When the substrate is in excess, the OD_{420} of the assay solution increases with time and is proportional to the enzyme concentration.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- 100x Mg^{2+} solution (17-7)
- Na_2CO_3 (1 M)
- 1x ONPG
- Sodium phosphate (0.1 M, pH 7.5)
- Tris-Cl (1 M, pH 7.8)

Enzymes and Buffers

E. coli β-galactosidase
The enzyme is commercially available (e.g., Sigma).

Additional Reagents

Step 1 of this protocol requires the reagents listed in Chapter 17, Protocol 5.

Cells and Tissues

Cultured mammalian cells transfected with the DNA of interest
Use one of the transfection protocols in Chapter 16 to transfect the cells with a plasmid containing a β-galactosidase reporter gene (e.g., the pβ-gal reporter series from CLONTECH).

METHOD

1. Prepare cell extracts from the transfected cells as described in Chapter 17, Protocol 5, Steps 1-4. Set aside approx. 30 µl of the extract for the β-galactosidase assay. The exact amount of extract required will depend on the strength of the promoter driving the expression of the β-galactosidase gene, the efficiency of transfection, and the incubation time of the assay. If a heat treatment is to be used to inactivate endogenous β-galactosidases, incubate the cell lysates for 45-60 minutes at 50°C before assay. Luciferase activity is also inactivated by preheating; assay luciferase and β-galactosidase activities in separate aliquots of cell lysate if a preheating step has been used.
2. For each sample of transfected cell lysate to be assayed, mix:

100x Mg^{2+} solution (17-7)	3 µl
1x ONPG	66 µl
cell extract	30 µl
0.1 M sodium phosphate (pH 7.5)	201 µl

It is essential to include positive and negative controls. These assays check for the presence of endogenous inhibitors and β-galactosidase, respectively. All of the controls should contain 30 µl of cell extract from mock-transfected cells. In addition, the positive controls should include 1 µl of a commercial preparation of E. coli *β-galactosidase (50 units/ml). The commercial enzyme preparation should be dissolved at a concentration of 3000 units/ml in 0.1 M sodium phosphate (pH 7.5). Just before use, transfer 1 µl of the stock solution of β-galactosidase into 60 µl of 0.1 M sodium phosphate (pH 7.5) to make a working stock of the enzyme containing 50 units/ml. One unit of* E. coli *β-galactosidase is defined as the amount of enzyme that will hydrolyze 1 µmole of ONPG substrate in 1 minute at 37°C.*

3. Incubate the reactions for 30 minutes at 37°C or until a faint yellow color has developed. In most cell types, the background of endogenous β-galactosidase activity is very low, allowing incubation times as long as 4-6 hours to be used.
4. Stop the reactions by adding 500 µl of 1 M Na_2CO_3 to each tube. Read the optical density of the solutions at a wavelength of 420 nm in a spectrophotometer.

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Protocol 8

Tetracycline as Regulator of Inducible Gene Expression
Stage 1: Stable Transfection of Fibroblasts with pTet-tTAk

The following protocol uses an autoregulatory system in which the transcriptional *trans*-activator tTA drives its own expression and that of a target gene. The first stage of the procedure describes how to generate stable lines of NIH-3T3 cells that express either tTA alone or tTA and the tetracycline-regulated target gene.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- $CaCl_2$ (2 M)
  Sterilize the solution by filtration, and store the filtrate in 5-ml aliquots at -20°C.
- Calf serum (10%)
- Chloroquine (10 mg/ml)
  Optional, please see Step 7.
  Prepare the chloroquine in H_2O, sterilize the solution by filtration, and store the filtrate at -20°C. Chloroquine at 10 mg/ml is equivalent to 19 mM.
- Glycerol (15%) in HEPES-buffered saline
- HEPES-buffered saline
- PBS
- Trypsin-EDTA

Enzymes and Buffers

Appropriate restriction endonuclease(s)
Please see Step 2.

Media

- DMEM complete (Dulbecco's modified Eagle's medium complete)
- DMEM complete containing 0.5 µg/ml tetracycline-HCl
  Prepare a 10 mg/ml stock of tetracycline-HCl in 70% ethanol and store at -20°C. All DMEM complete media used in this protocol (with or without the selection reagents) that contain 0.5 µg/ml tetracycline-HCl may be stored protected from the light for approx. 1 month at 4°C.
- DMEM selection medium containing L-histidinol and 0.5 µg/ml tetracycline

Additional Reagents

Step 23 requires the reagents listed in Chapter 7, Protocol 8.

Vectors and Bacterial Strains

pSV2-His
Purify all plasmids by equilibrium centrifugation in CsCl-ethidium bromide gradients (Chapter 1, Protocol 10) or by column chromatography (Chapter 1, Protocol 9).
The plasmids pTet-tTAk and pTet-Splice are available from Life Technologies. Other vectors carrying selectable markers are commercially available (e.g., pCI-neo from Promega or pTK-HYG from CLONTECH). If another vector is substituted for pSV2-His, then use the appropriate selection media for the selectable marker carried by that plasmid.

pTet-Splice carrying the target gene ORF(s)
Optional, please see Step 2.

pTet-tTAk

Cells and Tissues

Cultured mammalian cells
This protocol uses NIH-3T3 cells, but other cells would certainly work. Grow the cells in the appropriate medium.

METHOD

1. Culture adherent cells in DMEM complete. The day before transfection, transfer the cells into DMEM complete containing 0.5 µg/ml tetracycline-HCl (tetracycline). Apply enough cells per 10-cm dish so that on the day of the transfection, the cells will be 33% confluent.
   IMPORTANT *From this point on, maintain cells in the presence of 0.5 µg/ml tetracycline-HCl at 37°C, in an atmosphere of 5% CO_2, unless otherwise stated.*
2. Linearize the plasmids at an appropriate restriction endonuclease site and adjust the DNA concentration of each plasmid to ≥ 0.5 mg/ml. Mix 10-20 µg of pTet-tTAk plasmid and 1-2 µg of pSV2-His (approx. 10:1 molar ratio of Tet plasmid to the selectable marker plasmid) with 500 µl of HEPES-buffered saline in a clear 4-ml polystyrene tube.
   Prepare a control for mock transfection containing the HEPES-buffered saline and no DNA. All of the mock-transfected cells should die in histidine-free DMEM containing L-histidinol.
3. Add 32.5 µl of 2 M $CaCl_2$ to the DNA mixture. Immediately mix the solution by gentle vortexing. Store the solution at room temperature, mixing it from time to time. A cloudy precipitate should form over the course of 15-30 minutes.
4. Aspirate all of the medium from the dishes of cells prepared in Step 1.
5. Mix the $CaCl_2$-DNA precipitate a few times by pipetting with a Pasteur pipette. Apply the mixture dropwise and distribute it evenly over the cell monolayers.
6. Incubate the cells in an atmosphere of 5% CO_2 for 30 minutes at 37°C, rocking the plate after 15 minutes to ensure even coverage of the DNA precipitate.
7. To each dish of cells, add 10 ml of DMEM complete containing tetracycline. Incubate the cells in an atmosphere of 5% CO_2 for 4-5 hours at 37°C.
8. Gently aspirate the medium from the cells. Avoid disruption of the precipitate that has settled onto the cells.
9. Subject the cells to a glycerol shock by adding 2.5 ml of 15% glycerol in HEPES-buffered saline warmed to 37°C. Store the cells for 2.5 minutes at room temperature. Add the glycerol dropwise to the culture.
10. Aspirate the glycerol solution after *exactly* 2.5 minutes. Work quickly, because glycerol can be very toxic to cells.
11. Immediately, gently, and quickly wash the cells by adding 10 ml of DMEM complete containing tetracycline. Immediately remove the medium by aspiration and repeat the wash.
    IMPORTANT *Because the cells tend to detach easily from the plate after glycerol shock, add all of the medium to a single spot on the plate.*
12. Add 10 ml of DMEM complete containing tetracycline to the cells, and incubate the cultures overnight at 37°C.

13. Approximately 16-24 hours after transfection, aspirate the medium and replace it with 10 ml of DMEM complete containing tetracycline. Incubate the cultures for a total of 48 hours at 37°C after the transfection (i.e., the sum of the incubation times in Steps 12 and 13).
14. Forty-eight hours after transfection, passage several dilutions of the cells into DMEM selection medium containing 125 µM L-histidinol and 0.5 µg/ml tetracycline-HCl. Cell densities should range from approx. 1 x 10^6 to 3 x 10^4 cells per 10-cm plate. Include several plates containing approx. 1 x 10^5 cells.
15. After incubating the cultures in DMEM selection medium for 4 days, feed them with a further 3-4 ml of DMEM selection medium containing 125 µM L-histidinol and 0.5 µg/ml tetracycline-HCl.
16. When colonies have formed (typically after approx. 10-12 days of selection), replace the medium with DMEM selection medium containing 250 µM L-histidinol and 0.5 µg/ml tetracycline-HCl.
17. When colonies are well-established (at day 12-15 of selection), delineate their borders by drawing a circle on the bottom of the culture dish around each colony. Aspirate the medium from the plate, and place a sterile plastic cloning ring on the plate to surround an individual clone. Repeat this process for each colony to be picked. Choose cells from plates on which individual colonies are well spaced and can be easily distinguished.
    IMPORTANT *After stable transfection with pTet-tTAk, it is imperative that the cells be maintained in medium containing 0.5 µg/ml tetracycline to prevent any toxic effects of tTA expression and subsequent selection against clones expressing high levels of tTA.*
18. Quickly wash the clones with approx. 100 µl of phosphate-buffered saline. To release the cells, add 2 drops of 1x trypsin-EDTA (approx. 100 µl) and incubate for 30-60 seconds. Loosen the cells by pipetting up and down with a Pasteur pipette. Transfer each colony to one well of a 24-well tissue culture plate that contains 1 ml of selection medium containing 250 µM L-histidinol and tetracycline.
19. When the cells in the wells have grown to 80% confluency, transfer them into 6-cm tissue culture dishes in DMEM selection medium containing 500 µM L-histidinol and tetracycline.
20. Expand the cells (typically use a 1:5 to 1:10 dilution of the cells) in DMEM selection medium containing 500 µM L-histidinol and tetracycline.
21. When the cell monolayers are again approx. 80% confluent, recover a portion of each clone of cells and store them in aliquots in liquid nitrogen. Passage the remainder of the cells until they have expanded sufficiently to allow testing for inducible expression of protein.
22. If cells were cotransfected with both tTA and the target plasmids, then directly analyze the products of the target genes, as described in Chapter 17, Protocol 10. If cells were transfected with only the pTet-tTAk plasmid, then prepare the cells to be tested for inducible expression as follows:
    a. The night before induction, plate the cells in DMEM selection medium containing 500 µM L-histidinol and 0.5 µg/ml tetracycline at an appropriate density so that they will be subconfluent at the time of harvest.
    b. The next day, wash the cells three times with PBS, swirling the plates gently each time.
    c. After the third wash, immediately add selection medium containing 500 µM L-histidinol, but lacking tetracycline. Culture the cells in the presence or absence of tetracycline for 6-48 hours.

    It is essential to include controls that are maintained in selection medium containing 500 µm L-histidinol and 0.5 µg/ml tetracycline.
23. *Test the cells for inducible expression of tTA, by northern analysis or immunoblotting. Cell lines expressing tTA may then be transfected with the target plasmid(s) as described in Chapter 17, Protocol 9.*

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Protocol 9

Tetracycline as Regulator of Inducible Gene Expression
Stage 2: Stable Transfection of Inducible tTA-expressing NIH-3T3 Cells with Tetracycline-regulated Target Genes

This stage of the procedure describes the transfection with target genes of cell lines already expressing inducible tTA. In this example, the target genes are transfected on a plasmid that carries puromycin resistance as a selectable marker.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Calf serum (10%)

- HEPES-buffered saline
- PBS

Enzymes and Buffers

Appropriate restriction endonuclease(s)

- 1x Trypsin-EDTA

Media

- DMEM selection medium containing 500 µM L-histidinol and 0.5 µg/ml tetracycline (with or without 3 µg/ml puromycin)

Additional Reagents

Step 3 of this stage requires the reagents listed in Chapter 17, Protocol 8.

Vectors and Bacterial Strains

pPGKPuro (or vector carrying another selectable marker)
Purify all plasmids by equilibrium centrifugation in CsCl-ethidium bromide gradients (Chapter 1, Protocol 10) or by column chromatography (Chapter 1, Protocol 9).The plasmids pTet-Splice and pUHC13-3 are available from Life Technologies. Other vectors carrying selectable markers are commercially available (e.g., pCI-neo from Promega and pTK-HYG or pPUR from CLONTECH). If another vector is substituted for pPGKPuro, then use the appropriate selection media for the selectable marker carried by that plasmid.

pTet-Splice carrying a reporter gene (optional, e.g., pUHC13-3)

pTet-Splice carrying the target gene ORF(s)

Cells and Tisssues

Stable cell lines that inducibly express autoregulatory tTA (Chapter 17, Protocol 8)

METHOD

1. Culture stable cell lines that inducibly express autoregulatory tTA (isolated in Chapter 17, Protocol 8) in complete selection medium containing 500 µm L-histidinol and 0.5 µg/ml tetracycline-HCl. The day before transfection, passage the cells into 10-cm tissue culture dishes containing complete selection medium. Transfer enough cells per dish so that on the day of the transfection, the cell monolayers will be 33% confluent.
   IMPORTANT *From this point on, maintain the cells in the presence of 0.5 µg/ml tetracycline-HCl at 37°C, in an atmosphere of 5% CO_2 unless otherwise stated.*
2. Linearize the plasmids to be used for transfection and adjust the DNA concentration of each to ≥0.5 mg/ml. Mix 10-20 µg of each target gene plasmid(s) and 1-2 µg of pPGKPuro (a 10:1 molar ratio of each tetracycline plasmid to selectable marker plasmid) with 500 µl of HEPES-buffered saline in a clear 4-ml polystyrene tube.
   Prepare a control for mock transfection containing HEPES-buffered saline and no DNA. All of the mock-transfected cells should die when incubated in a medium containing puromycin. Please see the note to Step 4.
3. Carry out Steps 3-13 from Chapter 17, Protocol 8.
   IMPORTANT *Be sure to substitute the selection medium containing 500 µM L-histidinol and 0.5 µg/ml tetracycline-HCl in this transfection, whenever Stage 1 calls for DMEM complete containing tetracycline.*
4. Forty-eight hours after transfection, passage the cells into DMEM selection medium containing 500 µM L-histidinol, 3 µg/ml puromycin, and 0.5 µg/ml tetracycline at several dilutions ranging from approx. 1 x 10^6 to 3 x 10^4 cells per 10-cm plate. Include several plates containing approx. 1 x 10^5 cells.
   The lowest concentration of puromycin that kills all untransfected cells within a few days should be determined empirically before transfection and varies with the cell type. A concentration of 3 µg/ml puromycin is sufficient for selection of transfected NIH-3T3 cells. Most types of cells are killed efficiently in concentrations of puromycin ranging from 0.1 µg/ml to 10 µg/ml.
5. After incubating cultures in DMEM selection medium for 4 days, feed them with a further 3-4 ml of DMEM selection medium containing 500 µM L-histidinol, 3 µg/ml puromycin, and 0.5 µg/ml tetracycline.
6. When colonies are well-established (at day 12-14 of selection), delineate their borders by drawing a circle on the bottom of the culture dish around each colony. Aspirate the medium from the plate, and place a sterile plastic cloning ring on the plate to surround an individual clone. Repeat the procedure with each colony that is to be picked. Choose cells from plates on which individual colonies are well spaced and can be easily distinguished.
7. Quickly wash the clones with approx. 100 µl of PBS. To release the cells, add 2 drops of 1x trypsin-EDTA (approx. 100 µl) and incubate for 30-60 seconds. Loosen the cells by pipetting up and down with a Pasteur pipette. Transfer each colony to one well of a 24-well tissue culture plate that contains 1 ml of DMEM selection medium containing 500 µm L-histidinol, 3 µg/ml puromycin, and 0.5 µg/ml tetracycline.
   Carry out all subsequent passaging of cells by a standard procedure, such as (i) a quick PBS wash, and (ii) a 1-3-minute incubation with trypsin-EDTA (2 ml per confluent 10-cm plate) using 10% calf serum (3 ml) to dilute/stop the activity of the trypsin.
8. When cells in the wells have grown to 80% confluency, transfer them into 6-cm dishes that contain DMEM selection medium containing 500 µM L-histidinol, 3 µg/ml puromycin, and 0.5 µg/ml tetracycline.
   NIH-3T3 cells become 80% confluent in approx. 4-7 days; however, the time required to reach 80% confluency varies from cell line to cell line and even from clone to clone.
9. Expand the cells (typically use a 1:5 to 1:10 dilution of the cells) in DMEM selection medium containing 500 µm L-histidinol, 3 µg/ml puromycin, and 0.5 µg/ml tetracycline.
10. When cell monolayers are again approx. 80% confluent, recover a portion of each clone of the cells and store them in aliquots in liquid nitrogen. Passage the remainder of the cells until they have expanded sufficiently to allow testing for inducible expression of the target gene product(s) as described in Chapter 17, Protocol 10.
    When the frozen cells are later used, they should be revived and grown in selection medium containing 500 µm L-histidinol, 3 µg/ml puromycin, and 0.5 µg/ml tetracycline-HCl.

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Protocol 10

Tetracycline as Regulator of Inducible Gene Expression
Stage 3: Analysis of Protein Expression in Transfected Cells

Stably transfected cells, generated in the first two stages of the procedure, are induced for expression of the target gene. After harvesting and lysis, the lysates are analyzed by SDS-PAGE and immunoblotting. For further details on expression using this system, please see http://www.clontech.com/tet/Refs/index.html.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Calf serum (10%)

PBS

1x Protein sample buffer

Antibodies

Antibodies appropriate for detecting the target proteins of interest by immunoblotting

Media

DMEM selection medium containing 500 µM L-histidinol and 3 µg/ml puromycin (with or without 0.5 µg/ml tetracycline)

Additional Reagents

Step 13 of this protocol requires reagents and equipment for immunoblotting.

Cells and Tissues

Stable cell lines that inducibly express autoregulatory tTA and contain the plasmids harboring the target genes of interest

METHOD

1. The night before induction, plate the cells in DMEM selection medium containing 500 µM L-histidinol, 3 µg/ml puromycin, and 0.5 µg/ml tetracycline at an appropriate density so that they will be subconfluent at the time of harvest.
2. The next day, wash the cells three times with PBS, swirling the plates gently each time.
3. After the third wash, immediately add DMEM selection medium containing 500 µM L-histidinol, 3 µg/ml puromycin, but lacking tetracycline. Culture the cells in the presence or absence of tetracycline for 6-48 hours.
   It is essential to include controls that are maintained in selection medium containing 500 µM L-histidinol, 3 µg/ml puromycin, and 0.5 µg/ml tetracycline.
4. After the cells have grown for the appropriate length of time, harvest them quickly and place them in a tube in an ice bucket.
5. For each clone and control, transfer 0.5 x 10^6 cells to a microfuge tube, and centrifuge all of the tubes at 3000 rpm (low to moderate speed) for 5 minutes at 4°C.
6. Wash the cell pellets by adding 1 ml of ice-cold PBS. Pellet the cells as in Step 5, and gently aspirate the supernatant without disturbing the cell pellet.
7. Keep the cell pellets on ice and loosen them by gently and quickly running the tubes over the open holes of a microfuge rack before freezing the cell pellets at -70°C.
8. Resuspend each cell pellet in 30 µl of protein sample buffer by gently pipetting the cells and then vortexing the tubes.
9. Boil the cells in protein sample buffer for 10 minutes.
10. Recover the cell debris by centrifugation at maximum speed for 2 minutes in a microfuge.
11. Load 10 µl of cell lysate per lane of a Tris-glycine SDS-polyacrylamide gel.
12. Run the gel for the appropriate length of time.
13. Electrotransfer the proteins from the SDS-polyacrylamide gel to a PVDF membrane, and probe the membrane with the appropriate antibodies.

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1. Gossen M. and Bujard H. 1995. Efficacy of tetracycline controlled gene expression in influenced of cell type: Commentary. *BioTechniques* 19:213-215.
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3. Yin D.X., Zhu L., and Schimke R.T. 1996. Tetracycline-controlled gene expression system achieves high level and quantitative control of gene expression. *Anal. Biochem.* 235:195-201.

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Protocol 11

Ecdysone as Regulator of Inducible Gene Expression in Mammalian Cells

This protocol is adapted from the information supplied by Invitrogen as part of their Edysone-inducible Mammalian Expression System.

MATERIALS

CAUTION: Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Neomycin

Ponasterone A
An alternative ecdysone analog is muristerone A (Sigma).

Zeocin

Media

Medium appropriate for growing the cells to be transfected
The medium must be variously supplemented with Zeocin, neomycin, and ponasterone A.

Additional Reagents

Steps 1, 2, and 6 of this protocol require the reagents listed in the appropriate transfection protocol in Chapter 16.

Step 4 of this protocol requires the reagents listed in Chapter 17, Protocol 6.

Step 8 of this protocol requires the reagents listed in Chapter 6, Protocol 8 or Chapter 7, Protocol 8.

Vectors and Bacterial Strains

An ecdysone-inducible expression plasmid (e.g., the pIND series; Invitrogen) carrying a luciferase reporter gene

An ecdysone-inducible plasmid harboring the gene of interest

pVgRXR (Invitrogen)

Cells and Tissues

Cultured mammalian cells

METHOD

1. Stably transfect cells with pVgRXR using the preferred method of transfection for the cells under study (for a selection of transfection protocols, please see Chapter 16).
   Perform a control mock transfection with no DNA added to the cells. All of the mock-transfected cells should die in the presence of Zeocin.
2. To choose clones capable of selectively inducing gene expression in the presence of ecdysone or one of its analogs, transiently transfect Zeocin-resistant colonies obtained in Step 1 with an ecdysone-inducible expression plasmid carrying a luciferase reporter gene (again, for a selection of transfection protocols, please see Chapter 16).
3. Twenty-four to ninety-six hours after transfection, induce the expression of luciferase by replacing the medium with fresh medium containing Zeocin, neomycin, and 5 µM ponasterone A. Incubate the cells for 20 hours at 37°C in a humidified incubator with an atmosphere of 5-7% CO_2.
4. Assay for luciferase activity according to Chapter 17, Protocol 6.
5. Use clones that exhibit the desired level of ecdysone-induced luciferase activity. Expand the cell culture in medium containing Zeocin and neomycin.
6. Stably transfect cells from Step 5 with an ecdysone-inducible plasmid harboring the gene of interest and a hygromycin resistance marker using the preferred method of transfection for the cells under study (for a selection of transfection protocols, please see Chapter 16).
   It is essential to include control cultures that are not exposed to ponasterone A. Incubate these control cultures in medium containing only Zeocin and neomycin.
7. Expand the chosen colonies of cells that are resistant to both antibiotics, hygromycin and neomycin.
8. Analyze expression of the target gene by immunoblotting, northern hybridization, or other appropriate assay.

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Chapter 18 Protein Interaction Technologies

Protocol 1: Two-hybrid Systems
Stage 1: Characterization of a Bait-LexA Fusion Protein

This protocol describes how to generate a plasmid construct (pBAIT) that expresses a target protein fused to the bacterial LexA protein. pBAIT is cotransformed into yeast with a *lexAop-lacZ* reporter plasmid carrying the bacterial *lacZ* gene under the control of the *lexA* operator. The recipient yeast strain contains a chromosomally integrated *leu2* reporter gene, also under the control of the *lexA* operator. pBAIT is analyzed in transformants to establish whether the bait protein is expressed as a stable nuclear protein of the correct size that does not independently activate transcription of either of the *lexA* operator-reporter genes to a significant extent. This protocol was provided by Erica A. Golemis and Ilya Serebriiskii (Fox Chase Cancer Center, Philadelphia, Pennsylvania).

Protocol 2: Two-hybrid Systems
Stage 2: Selecting an Interactor

In this stage of the protocol, a mammalian cDNA library constructed in a plasmid such as pJG4-5 is transformed into yeast strains containing pBAIT and the *lexAop-lacZ* reporter plasmid. pJG4-5 expresses the cloned cDNAs from a cassette containing a transcriptional activation domain and other moieties under the control of the yeast GAL1 promoter. The yeast transformants are plated under both noninducing and inducing conditions on appropriate dropout media. Plasmids encoding putative target proteins that (1) allow growth in the absence of leucine and (2) cause expression of *lacZ* are selected for further analysis. This protocol was provided by Erica A. Golemis and Ilya Serebriiskii (Fox Chase Cancer Center, Philadelphia, Pennsylvania).

Protocol 3: Two-hybrid Systems
Stage 3: Second Confirmation of Positive Interactions

Plasmids encoding putative target proteins that (1) allow growth of transformants in the absence of leucine and (2) cause expression of *lacZ* are recovered by passage through *E. coli*, characterized by restriction analysis, and retransformed into "virgin" strains of *lexAop-LEU2/lexAop-lacZ/pBAIT* yeast to confirm the specificity of the interaction of the putative target with pBAIT. This protocol was provided by Erica A. Golemis and Ilya Serebriiskii (Fox Chase Cancer Center, Philadelphia, Pennsylvania).

Protocol 4: Detection of Protein-Protein Interactions Using Far Western with GST Fusion Proteins

Far western analysis was developed to screen cDNA expression libraries for clones that can interact with a ^{32}P-labeled target protein fused to GST. The target-GST fusion protein is synthesized in bacteria, purified by affinity chromatography on glutathione agarose beads, and labeled in an in vitro reaction catalyzed by a commercially available protein kinase. The fusion protein is then digested with a protease to remove the GST moiety, and the labeled target is used to probe an expression library and/or membranes containing putative interacting proteins that have been separated by SDS-PAGE. This protocol was provided by Margret B. Einarson (Fox Chase Cancer Center, Philadelphia, Pennsylvania).

Protocol 5: Detection of Protein-Protein Interactions Using the GST Fusion Protein Pulldown Technique

GST pulldown experiments are used to identify novel interactions between a probe protein and unknown targets and to confirm suspected interactions between a probe and a known protein. In both cases, the probe protein is a GST fusion, which is expressed in bacteria and purified by affinity chromatography on glutathione beads. Target proteins are usually lysates of cells, which may be labeled with [^{35}S]methionine or unlabeled, depending on the method used to assay interaction between the target and the probe. The cell lysate and the GST fusion protein probe are incubated together with glutathione-agarose beads, which are then collected and washed. Complexes recovered from the beads are resolved by SDS-PAGE and processed for further analysis by western blotting, autoradiography, or staining. This protocol was provided by Margret B. Einarson (Fox Chase Cancer Center, Philadelphia, Pennsylvania).

Protocol 6: Identification of Associated Proteins by Coimmunoprecipitation

Coimmunoprecipitation is most commonly used to test whether two proteins of interest are associated in vivo, but it can also be used to identify novel interacting partners of a target protein. In both cases, the cells, which may have been labeled with [^{35}S]methionine, are harvested and lysed under conditions that preserve protein-protein interactions. The target protein is specifically immunoprecipitated from the cell extracts, and the immunoprecipitates are fractionated by SDS-PAGE. Coimmunoprecipitated proteins are detected by autoradiography and/or by western blotting with an antibody directed against that protein. The identity of interacting proteins may be established or confirmed by Edman degradation of tryptic peptides. This protocol was used to identify pVHL-associated proteins. Conditions should be optimized for the protein of interest. This protocol was provided by Peter D. Adams (Fox Chase Cancer Center, Philadelphia, Pennsylvania) and Michael Ohh (Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts).

Protocol 7: Probing Protein Interactions Using GFP and FRET
Stage 1: Labeling Proteins with Fluorescent Dyes

This stage describes how components (usually Fab fragments of antibodies) that are to be introduced into cells are labeled with fluorescent sulfoindocyanine dyes. For further details of this procedure, please see the introduction beginning on page 18.69 in the print version of the manual. This protocol was provided by Alisa G. Harpur and Philippe I.H. Bastiaens (Imperial Cancer Research Fund, London).

Protocol 8: Probing Protein Interactions Using GFP and FRET
Stage 2: Cell Preparation for FLIM-FRET Analysis

In preparation for FLIM-FRET analysis, the appropriate donor and acceptor components must be introduced into live or fixed cells. The method of introduction depends on the nature of the components and the state of the cells. For example, plasmid DNAs encoding a protein of interest fused to a variant of GFP may be introduced into live cells by transfection or microinjection, whereas labeled antibodies are delivered by microinjection. For studies on fixed cells, plasmid DNA is introduced by transfection or microinjection, and the cells are subsequently fixed in paraformaldehyde before staining with labeled protein or antibody. This stage describes the introduction of plasmid DNA into live cells by transfection. For alternative methods of introducing donor and acceptor molecules, please see pages 18.87-18.89 of the print version of the manual. This protocol was provided by Alisa G. Harpur and Philippe I.H. Bastiaens (Imperial Cancer Research Fund, London).

Protocol 9: Probing Protein Interactions Using GFP and FRET

Stage 3: FLIM-FRET Measurements

This stage presents a basic plan for capturing a series of images by fluorescent lifetime imaging microscopy. The protocol specifically describes data acquisition for a particular variant of GFP (EGFP) or Oregon Green as a donor fluorophore, but it can be adapted for image acquisition of other chromophore systems as described in the table below. This protocol was provided by Alisa G. Harpur and Philippe I.H. Bastiaens (Imperial Cancer Research Fund, London).

Protocol 10: Analysis of Interacting Proteins with SPR Spectroscopy Using BIAcore

Stage 1: Preparation of the Capture Surface and Test Binding

The introduction of commercial instruments, such as BIAcore, that are capable of measuring surface plasmon resonance (SPR), has simplified the study of macromolecular interactions by providing a format that may be used to measure molecular interactions in real time with small analytical amounts of material. This protocol describes the sequence of steps for analyzing a typical antibody-antigen system in a BIAcore experiment. The antibody (antithyroid stimulating hormone [anti-TSH]) and its ligand (TSH) are available commercially and can be used to optimize the parameters of the system. A complete description of the setup and use of the instrument may be found at www.biacore.com. For further theoretical and experimental details of the procedure, please see the introduction to this protocol on page 18.96 in the print version of the manual. This protocol was provided by Maxine V. Medaglia and Robert J. Fisher with the help of M. Fivash, C. Hixson, and L. Wilson (National Cancer Institute-FCRDC, Frederick, Maryland). Stage 1 describes how (1) to prepare a sensor chip containing immobilized rabbit anti-mouse C domain, (2) to capture mouse anti-TSH on the prepared surface, and (3) to test for the binding of TSH to its captured antibody.

Protocol 11: Analysis of Interacting Proteins with SPR Spectroscopy Using BIAcore

Stage 2: Kinetic Analysis of the Antibody-Antigen Interaction

Stage 2 presents a series of method blocks or analysis programs that allow the determination of the equilibrium constants for the interaction between ligand pairs. This protocol was provided by Maxine V. Medaglia and Robert J. Fisher with the help of M. Fivash, C. Hixson, and L. Wilson (National Cancer Institute-FCRDC, Frederick, Maryland).

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Protocol 1

Two-hybrid Systems
Stage 1: Characterization of a Bait-LexA Fusion Protein

This protocol describes how to generate a plasmid construct (pBAIT) that expresses a target protein fused to the bacterial LexA protein. pBAIT is cotransformed into yeast with a *lexAop-lacZ* reporter plasmid carrying the bacterial *lacZ* gene under the control of the *lexA* operator. The recipient yeast strain contains a chromosomally integrated *leu2* reporter gene, also under the control of the *lexA* operator. pBAIT is analyzed in transformants to establish whether the bait protein is expressed as a stable nuclear protein of the correct size that does not independently activate transcription of either of the *lexA* operator-reporter genes to a significant extent. This protocol was provided by Erica A. Golemis and Ilya Serebriiskii (Fox Chase Cancer Center, Philadelphia, Pennsylvania).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

2x SDS gel-loading buffer

Nucleic Acids and Oligonucleotides

Target DNA encoding the protein of interest (bait)

Antibodies

Monoclonal antibody to LexA (CLONTECH) or Polyclonal antibody to LexA (Invitrogen) or Specific antibody to the fusion domain of the target protein (if available)

Media

CM selective medium
Use the table below to prepare the necessary selective media.

Yeast selective X-gal medium

YPD medium

Yeast CM Selective Media for Two-hybrid Analysis

INGREDIENTS	CM(GLU) -U-H MEDIUM	CM(GLU) -U AGAR	CM(GLU) -U-H AGAR	CM(GLU) -U-H-L AGAR	CM(GLU, X-GAL)-U AGAR[a]	CM(GAL) -U-H-L AGAR	CM(GAL, X-GAL)-U-H-L AGAR[a]
YNB	6.7 g	6.7 g	6.7 g	6.7 g	6.7 g	6.7 g	6.7 g
Glucose	20 g	20 g	20 g	20 g	20 g		
Galactose						20 g	20 g
Dropout mix	2 g	2 g	2 g	2 g	2 g	2 g	2 g
Leucine	15 ml	15 ml	15 ml		15 ml		
Tryptophan	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml
Histidine		5 ml			5 ml		
Agar		20 g	20 g	20 g	20 g	20 g	20 g

To prepare liquid or agar medium, mix the ingredients in a final volume of 1 liter of H_2O. Autoclave for 20 minutes. Cool the agar medium to 55°C before pouring plates: Leucine stock = 4 mg/ml; Tryptophan stock = 4 mg/ml; Histidine stock = 4 mg/ml.
[a]For media containing X-gal, prepare the appropriate base medium in 900 ml of H_2O, and use it in the recipe for yeast selective X-gal medium.

Additional Reagents

Step 1 of this protocol requires the reagents for subcloning listed in Chapter 1, Protocol 17.

Step 2 of this protocol requires the reagents for transformation of yeast.

Step 17 of this protocol requires the reagents for immunoblotting.

Vectors and Yeast Strains

S. cerevisiae strains for selection and propagation of vectors

Vectors carrying LexA and activation domain fusion sequences and LacZ reporter plasmids (please see http://www.fccc.edu/research/labs/golemis/com_sources1.html)

METHOD

1. Clone the target DNA encoding the protein to be used as bait into the polylinker of a LexA fusion vector (e.g., pMW101 or pMW103) to synthesize an in-frame fusion to LexA. Ensure that a translational stop sequence is present at the carboxyl terminus of the desired bait sequence. The resulting plasmid is referred to as pBait.
2. Set up a series of transformations of the EGY48 *lexAop-LEU2* selection strain of yeast using the following combinations of LexA fusion and *lexAop-lacZ* reporter plasmids:
   - **a.** pBait + pMW112 (test for activation)
   - **b.** pSH17-4 + pMW112 (positive control for activation)
   - **c.** pRFHM1 + pMW112 (negative control for activation)
   - **d.** pBait + pJK101 (test for repression/DNA binding)
   - **e.** pRFHM1 + pJK101 (positive control for repression)
   - **f.** pJK101 alone (negative control for repression)
3. Plate each transformation mixture on selective dropout plates: CM(Glu)-Ura-His (for plasmid combinations **a-e**) or CM(Glu)-Ura (for plasmid combination **f**), as appropriate. Incubate the plates for 2-3 days at 30°C to select for transformed yeast colonies that contain the plasmids.
4. Make a master plate of transformants, from which specific colonies can be assayed for the phenotype of activation of *lacZ* and *LEU2* reporters as described in Steps 5-9.
   Steps 5-9 are used to test the bait-LexA fusion protein for transcriptional activity and to demonstrate that the fusion of the bait does not affect LexA DNA-binding activity. Several independent colonies are assayed for each combination of plasmids transformed in Step 2.
5. From each transformation **a-f** (from Step 2), use sterile, standard flat-edged toothpicks to pick approx. 8 colonies. Touch a clean toothpick to the colony to pick up cells, and restreak them as a 1-cm-long streak in a grid on a fresh CM(Glu)-Ura-His or CM(Glu)-Ura plate. As many as 60-80 streaks can generally be grown on a single plate. Incubate the plates overnight at 30°C.
6. On the second day, restreak from the two master plates to each of the following:
   *Transformations **a-f:*** Streak onto CM(Glu, X-gal)-Ura and onto CM(Gal, X-gal)-Ura

Transformations ***a-c:*** Streak onto CM(Glu)-Ura-His-Leu and onto CM(Gal)-Ura-His-Leu
Only uracil is dropped out (histidine is present) from the X-gal plates, to allow side-by-side comparison of the JK101 plasmid-only transformation **(f)** *with* **(d,e)**. *Lack of selection for the LexA-fused plasmid does not notably affect transcriptional activation over the period of this assay.*

7. Incubate the plates for up to 4 days at 30°C.
8. Assay for repression and activation activities:
   a. For repression, observe the X-gal phenotype at approx. 12-24 hours after streaking.
   b. For activation, observe the X-gal phenotype between 18 and 72 hours after streaking.
   c. Observe the Leu2 phenotype between 48 and 96 hours. The expected results for a well-behaved bait are summarized below.

   - Optimally, at 12-24 hours after streaking to CM(Gal, X-gal)-Ura, the **d** + **e** transformants should be discernibly lighter in color than **f**.
   - At 48 hours after streaking to CM(Glu, X-gal)-Ura, **d** transformants should be bright blue, **c** should be white, and **a** should be white or very light blue.
   - At 48 hours after streaking, **b** transformants should be as well grown on CM(Glu)-Ura-His-Leu or CM(Gal)-Ura-His-Leu as on a CM(Glu)-Ura-His master plate, whereas **a** and **c** should show no growth.
   - Ideally, **a** transformants will still display no apparent growth at 96 hours after streaking.

9. Select the appropriate candidate colonies, based on the results of the repression and activation assays.
10. On the master plate, mark the colonies that are to be assayed for protein expression. Use the colony that has been shown to express bait appropriately as the founder to grow a culture for transformation of a library (in Chapter 18, Protocol 2).
11. Analyze at least two primary transformants for each novel bait construct. Include two transformants as positive controls for protein expression (e.g., pRFHMI).
    a. Use a sterile toothpick to pick colonies from the CM(Glu)-Ura-His master plate into CM(Glu)-Ura-His liquid medium.
    b. Grow the cultures overnight on a roller drum or other shaker at 30°C.
    c. In the morning, dilute the saturated cultures into fresh tubes containing 3-5 ml of CM(Glu)-Ura-His, with a starting density of OD_{600} of approx. 0.15. Incubate the cultures for 4-6 hours at 30°C until the optical density has doubled approx. twice (OD_{600} approx. 0.45-0.7).
12. Transfer 1.5 ml of each culture to a microfuge tube, and centrifuge the cells at maximum speed for 3-5 minutes in a microfuge. The volume of the visible pellet should be 2-5 µl. Carefully decant or aspirate the supernatant.
13. Add 50 µl of 2x SDS gel-loading buffer to the tube, and vortex the tube rapidly to resuspend the pellet. Immediately place the tube either on dry ice or in a dry ice/ethanol bath.
14. Transfer the samples from the dry ice or -70°C directly to 100°C and boil them for 5 minutes.
15. Chill the samples on ice and centrifuge them at maximum speed for 5-30 seconds in a microfuge to pellet large cell debris. Load 20-50 µl into each lane of a SDS-polyacrylamide gel.
16. Run the gel and analyze the products to determine whether bait protein of the expected size is expressed at reasonable levels.
17. To anticipate and forestall potential problems, analyze the lysates of yeast containing LexA-fused baits by immunoblotting.

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Protocol 2

Two-hybrid Systems
Stage 2: Selecting an Interactor

In this stage of the protocol, a mammalian cDNA library constructed in a plasmid such as pJG4-5 is transformed into yeast strains containing pBAIT and the *lexAop-lacZ* reporter plasmid. pJG4-5 expresses the cloned cDNAs from a cassette containing a transcriptional activation domain and other moieties under the control of the yeast GAL1 promoter. The yeast transformants are plated under both noninducing and inducing conditions on appropriate dropout media. Plasmids encoding putative target proteins that (1) allow growth in the absence of leucine and (2) cause expression of *lacZ* are selected for further analysis. This protocol was provided by Erica A. Golemis and Ilya Serebriiskii (Fox Chase Cancer Center, Philadelphia, Pennsylvania).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ DMSO (Dimethylsulfoxide)

Ethanol
Optional, please see Step 9.

TE (pH 7.5) (sterile)

TE (pH 7.5) containing 0.1 M lithium acetate

TE (pH 7.5) containing 40% PEG 4000 and 0.1 M lithium acetate (sterile)

Yeast glycerol solution for freezing transformants

Nucleic Acids and Oligonucleotides

Carrier DNA
Sheared salmon sperm DNA is typically used as carrier.

Library to be screened for interaction
Interaction trap libraries are available commercially from, for example, CLONTECH (MatchMaker System), Invitrogen, OriGene, Display, MoBiTech (for a comprehensive listing of libraries from various species and tissue sources, please see www.fccc.edu/research/labs/golemis/lib sources.html).

Media

CM selective medium
Use the table in Chapter 18, Protocol 1 Stage 1 to prepare the necessary selective media.

Yeast selective X-gal medium

Vectors and Bacterial or Yeast strains

S. cerevisiae candidate strains carrying vectors expressing the bait and *lexAop-lacZ* reporter (from Chapter 18, Protocol 1 Stage 1)

METHOD

1. Select a yeast colony, expressing the bait and *lexAop-lacZ* reporter, that was shown in Chapter 18, Protocol 1 Stage 1 to be optimal in the initial control experiments. From this colony, grow a 20-ml culture in CM(Glu)-Ura-His liquid dropout medium overnight at 30°C on a roller drum.
   IMPORTANT *The bait and* lexAop-lacZ *reporter plasmids should have been transformed into the yeast <7-10 days before retransformation with the library. It is important to maintain sterile conditions throughout.*
2. Dilute the 20-ml overnight culture into 300 ml of CM(Glu)-Ura-His liquid dropout medium such that the diluted culture has an OD_{600} of approx. 0.10-0.15. Incubate the culture at 30°C on an orbital shaker until the culture has gone through 1.5 doublings, to reach an OD_{600} of approx. 0.50.
3. Transfer the culture to sterile 250-ml centrifuge bottles, and centrifuge them at 1000-1500*g* (2500-3000 rpm in a Sorvall GSA rotor) for 5 minutes at room temperature. Remove the supernatant and add 30 ml of sterile H_2O to the pellet. Resuspend the pellet by gently rapping the bottle against a countertop. Transfer the slurry to a sterile 50-ml Falcon tube.
4. Centrifuge the cells at 1000-1500*g* (2500-3000 rpm in a Sorvall GSA rotor) for 5 minutes. Pour off the H_2O and resuspend the yeast cells in 1.5 ml of TE (pH 7.5) containing 0.1 M lithium acetate.
5. Add 1 µg of library DNA and 50 µg of freshly denatured carrier DNA to each of 30 sterile 1.5-ml microfuge tubes. Immediately add 50 µl of the yeast suspension (from Step 4) to each of the 30 tubes.
6. To each tube of the cell suspensions, add 300 µl of sterile TE (pH 7.5) containing 40% PEG 4000 and 0.1 M lithium acetate. Mix by gently inverting the tubes a number of times (do not vortex). Incubate the tubes for 30-60 minutes at 30°C.
7. To each tube, add 40 µl of DMSO, and mix the suspensions by inversion. Place the tubes in a heating block for 10 minutes at 42°C.
8. Plate the transformation mixtures as follows:

   For 28 of the tubes to be used solely to generate transformants

   a. Pipette the contents of each tube onto 24 x 24-cm CM(Glu)-Ura-His-Trp dropout plates.
   b. Spread the cells evenly and incubate the plates at 30°C until colonies appear.

   For the 2 remaining tubes to be used to assess transformation efficiency

   a. Pipette 350 µl from each tube to 24 x 24-cm CM(Glu)-Ura-His-Trp dropout plates.
   b. Spread the cells evenly and incubate the plates at 30°C until colonies appear.
   c. Pipette the remaining 40 µl from each tube to make a series (at least 3) of 1:10 dilutions in sterile TE (pH 7.5) or H_2O.
   d. Plate 100 µl of each dilution on 100-mm CM(Glu)-Ura-His-Trp dropout plates, and incubate the plates at 30°C until colonies appear.
9. Harvest the library using one of the following methods.
   In Step 9, the first technique (by agitation) is faster to perform, and allows induction of the library and screening on selective plates to be carried out on the same day, and it also minimizes the time the plates are open. About one third of the yeast slurry will be left on the plates; however, normally no more than 2% of the collected slurry is used, so it is important to ensure that colonies are washed from the plates with approximately equal efficiency. The second technique (by scraping) in Step 9 is more economical of reagents and may be easier to use on plates from which molds and contaminants have been excised.

To harvest the library by agitation

a. Pour 10 ml of sterile H_2O and approximately 30 sterile glass beads on each of five 24 x 24-cm plates containing transformants.

b. Stack the five plates on top of one another, and, holding the plates tightly, shake the stack until all the colonies are resuspended (1-2 minutes).

c. Use a sterile pipette to collect 5 ml of yeast slurry from each plate (tilt the plate). Pool the slurry into sterile 50-ml conical tubes.

d. Proceed to the next five plates and repeat Steps a-c. Continue harvesting yeast cells from all 30 plates, resulting in a total volume of 150 ml of liquid contained in three 50-ml tubes.

To harvest the library by scraping

a. Wearing gloves, place the 30 24 x 24-cm plates containing transformants at 4°C to harden the agar (generally, 2 hours to overnight is acceptable).

b. Sterilize a glass microscope slide by immersing it in ethanol and flaming; use this slide to scrape yeast cells gently from the transformation plates into 50-ml conical tubes. Reflame or use a fresh slide at intervals (every 5-10 plates).

10. If necessary, fill each conical tube containing yeast to the 40-45-ml mark with sterile TE (pH 7.5) or H_2O, and vortex or invert the tubes to suspend cells.
11. Centrifuge the tubes in a benchtop centrifuge at 1000-1500*g* (2200-2700 rpm using a Sorvall H1000B rotor) for 5 minutes at room temperature, and discard the supernatant.
12. Repeat Steps 10 and 11.
13. Resuspend the packed cell pellet in 1 volume of sterile yeast glycerol solution. Combine the contents of the different Falcon tubes and mix thoroughly.
14. Transfer 1-ml aliquots into a series of sterile microfuge tubes, and freeze at -70°C (cells remain stable for at least 1 year).
    If proceeding directly to plating on selective medium (which requires 5 hours to complete), leave one aliquot unfrozen and carry out the next sequence of steps. Assume that the viability of the unfrozen culture is 100%.
15. Thaw one aliquot of library-transformed yeast (from Step 14), and dilute 1:10 with CM(Gal-Raff)-Ura-His-Trp dropout medium. Incubate the yeast with shaking for 4 hours at 30°C to induce transcription from the GAL1 promoter on the library.
    Aliquots of the transformed library are plated on -Leu selective media to test for the inability to promote LEU2 *transcription.*
16. Plate 10^6 cells (or 50 µl of a culture at OD_{600} = 1.0) on each of an appropriate number of 100-mm CM(Gal/Raff)-Ura-His-Trp-Leu dropout plates.
    IMPORTANT *The value of 10^6 cells per plate is the highest plating density that generally can be effectively used. Plating at higher densities (e.g., 3 x 10^6) can result in cross-feeding between yeast cells, resulting in high background growth.*
17. Incubate the plates for 5 days at 30°C.
18. Observe the plates for growth and mark colonies as they appear.
19. At day 5, create a master plate (CM[Glu]-Ura-His-Trp) on which colonies are grouped by day of appearance.
20. Incubate the plates at 30°C until patches/colonies form.
21. Assay for transcription activation.
    Steps 21 and 22 test for galactose-inducible transcriptional activation of both the lexAop-LEU2 *and* lexAop-lacZ *reporters. Simultaneous activation of both reporters in a galactose-specific manner generally indicates that the transcriptional phenotype is due to expression of library-encoded proteins, rather than mutation of the yeast host. A master plate containing glucose and leucine is used as a source for test colonies.*

To assay by direct streaking

a. On each of the four following plates, use a flat-edged toothpick to replicate the grid from the master plate. Use the same toothpick to streak an individual colony across the four plates. Try to get a thick streak of yeast on plates containing X-gal, and a thin streak of yeast on plates lacking leucine.

CM(Glu/Xgal)-Ura-His-Trp	1 plate
CM(Gal/Raff/Xgal)-Ura-His-Trp	1 plate
CM(Glu)-Ura-His-Trp-Leu	1 plate
CM(Gal/Raf)-Ura-His-Trp-Leu	1 plate

b. Incubate the plates for 3-4 days at 30°C.

To assay using a manifold/frogger

a. Deliver 25-30 µl of sterile H_2O into 48 wells of a 96-well microtiter plate.

b. Use a frogger to transfer patches simultaneously from a pregridded master plate to the wells of a microtiter plate. Agitate the plate gently.

c. Use the frogger to transfer yeast from the microtiter plate to each of the following plates. This approach allows approximately equal quantities of cells to be transferred to each plate.

CM(Glu)-Ura-His-Trp	2 plates
CM(Gal)-Ura-His-Trp	1 plate
CM(Glu)-Ura-His-Trp-Leu	1 plate
CM(Gal/Raff)-Ura-His-Trp-Leu	1 plate

d. Incubate the plates for 3-4 days at 30°C. After 1 day of incubation, use one CM(Glu)-Ura-His-Trp plate and one CM(Gal)-Ura-His-Trp plate to assay for activation of the *lacZ* reporter, using the chloroform overlay assay.

e. Continue to monitor growth: Differential growth on leucine will generally be apparent between 48 and 72 hours on the -Leu plates. The second CM(Glu)-Ura-His-Trp plate can be taken as a fresh master plate.

22. Interpret the results. Colonies and the library plasmids they contain are designated as first-round positives if:
    - X-Gal analysis indicates blue color following culture on CM(Gal)-Ura-His-Trp plates.
    - X-Gal analysis indicates white, or only faintly blue, following culture on CM(Glu)-Ura-His-Trp plates.
    - Colonies grow well on CM(Gal/Raff)-Ura-His-Trp-Leu plates.
    - Colonies grow poorly or not at all on CM(Glu)-Ura-His-Trp-Leu plates.

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Protocol 3

Two-hybrid Systems
Stage 3: Second Confirmation of Positive Interactions

Plasmids encoding putative target proteins that (1) allow growth of transformants in the absence of leucine and (2) cause expression of *lacZ* are recovered by passage through *E. coli*, characterized by restriction analysis, and retransformed into "virgin" strains of *lexAop-LEU2/lexAop-lacZ/pBAIT* yeast to confirm the specificity of the interaction of the putative target with pBAIT. This protocol was provided by Erica A. Golemis and Ilya Serebriiskii (Fox Chase Cancer Center, Philadelphia, Pennsylvania).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- ⚠ Ammonium acetate (7.5 M)
- ⚠ Chloroform
- Ethanol
- Isopropanol
- ⚠ Phenol (equilibrated to pH 8.0)
- ⚠ SDS (10% w/v)
- STES lysis solution
- TE (pH 8.0)
- Yeast lysis solution
- Yeast rescue buffer

Enzymes and Buffers

Restriction endonucleases *Eco*RI, *Xho*I, *Hae*III

Media

CM selective medium
Use the table in Chapter 18, Protocol 1 Stage 1 to prepare the necessary selective media.

- LB agar plates containing 50 µg/ml ampicillin

Minimal (*-trp*) medium for bacteria

- Yeast selective X-gal medium

Additional Reagents

Step 2 of this protocol requires the reagents for electroporation listed in Chapter 1, Protocol 26.

Steps 3 and 4 of this protocol require the reagents for the preparation of miniprep DNA from plasmids listed in Chapter 1, Protocol 1.

Step 10 of this protocol requires the reagents for sequencing of yeast DNA listed in Chapter 4, Protocol 13.

Vectors and Bacterial and Yeast Strains

E. coli DH5α or *E. coli* KC8 (*pyrF leuB600 trpC hisB463*; CLONTECH), competent for electroporation
Please see Chapter 1, Protocol 26.

Nonspecific bait plasmid
Please see the note to Step 6.

pMW112, pRFHM-1

pBait (from Chapter 18, Protocol 1 Stage 1)

Yeast colonies with the appropriate phenotype growing on a CM(Glu)-Ura-His-Trp master plate (identified in Chapter 18, Protocol 2 Stage 2, Step 21)

Yeast strain EGY48

METHOD

1. Prepare cell lysates from positive colonies.
   Two approaches are given for the isolation of discrete library plasmids. For the isolation of a small number of colonies (24-36 colonies), cells are lysed in SDS. For the isolation of a large number of colonies, cells are lysed in Zymolyase.

   For isolation of a small number of colonies

   a. Starting from the CM(Glu)-Ura-His-Trp master plate (Chapter 18, Protocol 2 Stage 2, Step 21), pick colonies that display the appropriate phenotype on selective plates into 5 ml of -Trp glucose medium. Grow the cultures overnight at 30°C.
   b. Centrifuge 1 ml of each culture at maximum speed for 1 minute at room temperature in a microfuge. Resuspend the pellets in 200 µl of STES lysis solution, and add 100 µl of 0.45-mm-diameter sterile glass beads. Vortex the tubes vigorously for 1 minute.
   c. Add 200 µl of equilibrated phenol to each tube, and vortex the tubes vigorously for another minute.
   d. Centrifuge the emulsions at maximum speed for 2 minutes at room temperature in a microfuge, and transfer each aqueous phase to a fresh microfuge tube.
   e. Add 200 µl of equilibrated phenol and 100 µl of chloroform to each aqueous phase, and vortex for 30 seconds. Centrifuge the emulsions at maximum speed for 2 minutes at room temperature in a microfuge, and transfer each aqueous phase to a fresh tube.
   f. Add two volumes (400 µl) of ethanol to each aqueous phase, mix by inversion, and chill the tubes for 20 minutes at -20°C. Recover the nucleic acid by centrifugation at maximum speed for 15 minutes at 4°C in a microfuge.
   g. Pour off the supernatants. Wash the pellets with ice-cold 70% ethanol, and dry the pellets briefly under vacuum. Resuspend each pellet in 5-10 µl of TE (pH 8.0). Proceed to Step 2.

   For isolation of a large number of colonies

   a. Transfer 2 ml of 2x CM(Glu)-Trp medium into each well of a 24-well microtiter plate. Use a toothpick to pick a putative positive colony from a master plate into each well (Chapter 18, Protocol 2 Stage 2, Step 21). Grow the cultures overnight at 30°C with shaking.

b. Centrifuge the plate(s) in a centrifuge with microplate holders at 1500*g* (3000 rpm in a Sorvall H1000B MPC rotor) for 5 minutes at 4°C. Shake off the supernatant with a snap of the wrist and return the plate to an upright position. Swirl or lightly vortex the plate to resuspend each cell pellet in the remaining liquid. Add 1 ml of H_2O to each well and swirl the plate gently.

c. Centrifuge as in Step b, shake off the supernatant, resuspend the cells in residual supernatant, and add 1 ml of rescue buffer.

d. Centrifuge as in Step b, shake off the supernatant, and resuspend the cells in the small volume of liquid remaining in the plate. To each well, add 25 µl of lysis solution. Swirl or vortex the plate. Incubate the plate (with the cover on) on a rotary shaker for approx. 1 hour at 37°C.

e. Add 25 µl of 10% SDS to each well. Disperse the precipitates completely by swirling the plates. Allow the plates to rest on the bench for 1 minute at room temperature. After 1 minute, the wells should contain a clear, somewhat viscous solution.

f. To each well, add 100 µl of 7.5 M ammonium acetate. Swirl the plates gently, and then store them for 15 minutes at -70°C or at -20°C until the lysates are frozen.

g. Remove the plates from the freezer. Once they begin to thaw, centrifuge the plates at 3000*g* (3800 rpm in a Sorvall H6000A MPC rotor) for 15 minutes at 4°C. Transfer 100-150 µl of the resulting clear supernatants to fresh 24-well plates.
IMPORTANT *In general, some contamination of the supernatants with pelleted material cannot be avoided. However, it is better to sacrifice yield in order to maintain purity.*

h. To each well, add approx. 0.7 volume of isopropanol. Mix the solutions by swirling and allow the nucleic acids to precipitate for 2 minutes at room temperature. Centrifuge as in Step g. Shake off the supernatants with a snap of the wrist.

i. To each well, add 1 ml of cold 70% ethanol. Swirl the plates, and then centrifuge them at 3000*g* (3800 rpm in a Sorvall H6000A MPC rotor) for 5 minutes at 4°C. Shake off the supernatant with a snap of the wrist, invert the plates, and blot them well onto paper towels. Allow the plates to dry in the air.

j. To each well, add 100 µl of TE (pH 8.0). Swirl the plates and allow them to rest on the bench for several minutes, until the pellets appear fully dissolved. Transfer the preparations to microfuge tubes or the wells of a 96-well plate for storage at -20°C. Proceed to Step 2.

1-5 µl of each of the resulting preparations can be used to transform competent E. coli *by electroporation. If insufficient numbers of colonies are obtained, reprecipitate the DNAs and dissolve them in 20 µl rather than 100 µl of TE, to concentrate the DNA stock.*

2. Introduce 1-5 µl of each preparation of plasmid DNA into competent *E. coli* DH5α or into strain KC8 (*pyrF leuB600 trpC hisB463*) by electroporation. Plate the bacteria on LB agar containing 50 µg/ml of ampicillin, and incubate the plates overnight at 37°C.
If the bait is cloned in one of the specialized LexA-fusion plasmids that carries an ampicillin resistance marker, a proportion of the E. coli *DH5α transformants will not contain the library plasmid. One option to resolve this problem is to analyze multiple transformants from each DNA preparation. Alternatively, it is possible to passage plasmids through a strain of* E. coli *possessing a* trpC *mutation, and select for a library plasmid by the ability of the yeast* TRP1 *gene to complement the* E. coli trpC *mutation.*
3. If plasmid DNA was transformed into DH5α, proceed to Step 4. If plasmid DNA was transformed into KC8:
   a. Use restreaking or replica plating to transfer colonies from LB/ampicillin plates to minimal (-*trp*) medium for bacteria. Incubate the bacteria overnight at 37°C.
   b. Prepare miniprep DNA from an isolated colony and use the DNA to transform DH5α cells as described in Step 2.
4. Prepare miniprep DNA from DH5α cells carrying the library plasmid.
IMPORTANT *In general, it is a good idea to prepare DNA from two or three separate bacterial colonies generated from each original positive interactor. By contrast to bacteria, yeast can tolerate multiple 2µ plasmids with identical selective markers. If a single yeast cell contains two or three distinct library plasmids, only one of which encodes an interacting protein, the relevant cDNA clone can be lost at the stage of plasmid isolation. Again, in general, this is not a major problem; on average, perhaps 10% of yeast cells will contain two or more library plasmids.*
5. Confirm by restriction endonuclease digestion that the duplicate samples prepared for each positive contain identical inserts, and/or determine whether a small number of cDNAs have been isolated repeatedly.
IMPORTANT *Some investigators sequence DNAs at this stage. A lot of money can be wasted on sequencing nonspecific interactors: It is strongly recommended that the transformation into yeast and specificity tests, as described below, be completed before sequencing.*
6. Transform yeast strain EGY48 with the following sets of plasmids, and select colonies on CM(Glu)-Ura-His plates.
The final test of the specificity of interacting proteins is the retransformation of library plasmids from E. coli *into "virgin"* lexAop-LEU2/lexAop-lacZ*/pBait-containing strains. The aim is to verify that interaction-dependent phenotypes are still observed and are specific to the starting pBait. This test will eliminate false positives, library-encoded cDNAs that interact with the LexA DNA-binding domain, and library-encoded proteins that are "sticky" and interact with multiple pBaits in a promiscuous manner.*
   a. pMW112 and pBait
   b. pMW112 and pRFHM-1
   c. pMW112 and a nonspecific bait

   If the pBait initially used in the screen was able to activate transcription, even slightly, we strongly recommend that a nonspecific control bait that can also weakly activate transcription be included as a control. In general, baits that activate transcription poorly are difficult to distinguish from the background of false positives. Some false positives interact generically with weakly activating LexA-fused proteins.
7. After 2-3 days, transformed yeast from Step 6 should be available. Use electroporation to introduce library plasmids prepared from *E. coli* KC8 or DH5α into individual transformants **a-c**. Plate each transformation mixture on CM(Glu)-Ura-His-Trp dropout plates and incubate the plates at 30°C until colonies grow (2-3 days).
IMPORTANT *As a negative control, also electroporate examples of transformants* ***a-c*** *with the pJG4-5 library vector.*
8. Create a CM(Glu)-Ura-His-Trp master dropout plate for each library plasmid being tested. It is generally helpful to streak tranformants **a-c** for each library plasmid in close proximity on the plate to facilitate detection of nonspecific interactions.
IMPORTANT *Each plate should also contain the* ***a-c*** *series transformed with the pJG4-5 negative control.*
9. Test for β-galactosidase activity and for leucine auxotrophy, exactly as described in Chapter 18, Protocol 2 Stage 2, Step 21.
10. Analyze the results of these specificity tests and sequence the positive isolates (please see Chapter 4, Protocol 13).

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Protocol 4

Detection of Protein-Protein Interactions Using Far Western with GST Fusion Proteins

Far western analysis was developed to screen cDNA expression libraries for clones that can interact with a ^{32}P-labeled target protein fused to GST. The target-GST fusion protein is synthesized in bacteria, purified by affinity chromatography on glutathione agarose beads, and labeled in an in vitro reaction catalyzed by a commercially available protein kinase. The fusion protein is then digested with a protease to remove the GST moiety, and the labeled target is used to probe an expression library and/or membranes containing putative interacting proteins that have been separated by SDS-PAGE. This protocol was provided by Margret B. Einarson (Fox Chase Cancer Center, Philadelphia, Pennsylvania).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- GST basic buffer
- GST blocking buffer
- GST Interaction buffer
- GST Wash buffer 1
  PBS containing 0.2% Triton X-100.
- GST Wash buffer 2
  PBS containing 0.2% Triton X-100 and 100 mM KCl.
- PBS
- 2x PK buffer
  Reduced glutathione (20 mM) in 50 mM Tris-Cl (pH 8.0)
  Optional, please see Step 3.

Enzymes and Buffers

Protease
Optional, please see Step 3.

Protein kinase A
Prepare fresh at each use according to the manufacturer's instructions.

Radioactive Compounds

⚠ [ɣ-^{32}P]ATP (6000 Ci/mmole)

Additional Reagents

Step 3 of this protocol requires reagents for cleavage of the fusion protein from its carrier or the fusion protein attached to glutathione-agarose beads in Chapter 15, Protocol 5.

Step 5 of this protocol requires either a polyacrylamide gel containing the proteins to be probed or plates containing a cDNA expression library (please see Chapter 14, Protocol 2), and reagents for immunoblotting.
A negative control of GST alone or a nonspecific protein should be loaded onto the SDS-polyacrylamide gel with the target proteins. If the protein of interest is a member of a conserved family, the interaction of the protein of interest can be compared to other proteins from the same family.

Vectors and Bacterial Strains

GST fusion protein bound to glutathione-agarose beads
This protocol is for proteins containing a protein kinase A phosphorylation site in the fusion protein. Also available is a vector that contains a cAMP-dependent protein kinase recognition sequence (Amersham Pharmacia Biotech).

METHOD

1. Prepare the following reaction mixture in a microfuge tube:

[ɣ-^{32}P]ATP (6000 Ci/mmole)	5 µl
protein kinase A	1 unit/µl
GST fusion protein on glutathione-agarose beads	1-3 µg
2x PK buffer	12.5 µl
H_2O	to 25 µl

   Incubate the reaction mixture for 30 minutes at 37°C.
   The fusion protein may be cleaved with a protease before the labeling reaction. In this case, substitute the cleaved protein for the protein bound to glutathione-agarose beads in the labeling reaction, incubate for 30 minutes at 37°C, and proceed to Step 4.
   If the GST moiety is retained on the fusion protein, the experiment must be replicated with GST alone (bound to glutathione-agarose beads) as a negative control. In the case of a library screen where this would be impractical, it is important to test positive plaques for their ability to bind GST alone. This control is usually carried out after quaternary purification, before clone characterization.
2. After the labeling reaction is complete, wash the beads by adding 200 µl of 1x PK buffer to the tube, and centrifuge the tube at maximum speed for 1 minute in a microfuge. Discard the supernatant containing the free radiolabeled nucleotide in an appropriate manner. Repeat the wash one more time.
3. Either cleave the labeled protein with a protease or elute the labeled GST fusion protein from the beads with 20 mM reduced glutathione in 50 mM Tris (pH 8.0) (please see Chapter 15, Protocol 5). Store the radiolabeled protein probe in an ice bucket and use it on the same day it is made.
4. If the labeled protein was cleaved from the GST moiety before the labeling reaction (please see the note to Step 1), then load the radiolabeled protein onto a Sephadex G-50 column equilibrated with 1x PK buffer to remove the free radiolabeled nucleotide. Once the probe protein is separated from the free nucleotide, it is ready for use. Store the radiolabeled protein probe in an ice bucket and use it on the same day it is made.
5. Prepare the membrane to be probed by transferring proteins to the membrane according to standard techniques.
6. Cover the membrane completely with GST basic buffer and wash it for 10 minutes at 4°C with gentle agitation.
7. Discard the GST basic buffer. Cover the membrane completely with GST blocking buffer and incubate it with gentle agitation for 4 hours to overnight at 4°C.
8. Prepare a solution of labeled protein by adding 1-3 µg of the stock probe (from either Step 3 or 4) to enough GST interaction buffer to make a final concentration of 1-5 nM. Transfer the membrane to a dish containing the diluted probe. Make sure that the probe solution contacts the entire surface of the membrane evenly. Incubate the membrane for 4-5 hours at 4°C with gentle agitation.
9. Discard the radioactive probe solution in an appropriate manner. Cover the membrane completely with GST Wash buffer 1, and incubate the membrane for 10 minutes at 4°C with gentle agitation. Repeat the wash three more times.
10. Cover the membrane completely with GST Wash buffer 2, and wash the membrane for 10 minutes at 4°C with gentle

agitation. Repeat this once.

11. Wrap the membrane carefully in plastic wrap and expose it to X-ray film.

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Protocol 5

Detection of Protein-Protein Interactions Using the GST Fusion Protein Pulldown Technique

GST pulldown experiments are used to identify novel interactions between a probe protein and unknown targets and to confirm suspected interactions between a probe and a known protein. In both cases, the probe protein is a GST fusion, which is expressed in bacteria and purified by affinity chromatography on glutathione beads. Target proteins are usually lysates of cells, which may be labeled with [^{35}S]methionine or unlabeled, depending on the method used to assay interaction between the target and the probe. The cell lysate and the GST fusion protein probe are incubated together with glutathione-agarose beads, which are then collected and washed. Complexes recovered from the beads are resolved by SDS-PAGE and processed for further analysis by western blotting, autoradiography, or staining. This protocol was provided by Margret B. Einarson (Fox Chase Cancer Center, Philadelphia, Pennsylvania).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

GST lysis buffer

Reduced glutathione (20 mM) in 50 mM Tris-Cl (pH 8.0)
Optional, please see Step 8.

2x SDS-PAGE gel-loading buffer

Probes

GST protein

GST fusion protein carrying the "bait" or probe sequence
Construct as described in Chapter 15, Protocol 1.

Cells and Tissues

Cell lysate in which the proteins are ^{35}S-labeled
It is possible to use unlabeled cell lysates depending on the goals of the experiment and the desired detection method.

Additional Reagents

Step 11 of this protocol requires reagents for immunoblotting and staining proteins separated by SDS-polyacrylamide gel electrophoresis.

METHOD

1. Incubate the cell lysate with 50 µl of a 50% slurry of glutathione agarose beads and 25 µg of GST for 2 hours at 4°C with end-over-end mixing. The amount of lysate needed to detect an interaction is highly variable. Start with a volume of lysate equivalent to 1 x 10^6 to 1 x 10^7 cells.
   Because the aim of the experiment is to compare GST with a GST fusion protein, it is necessary to prepare enough precleared lysate for each reaction. Efficient mixing of reagents is the key to success. This is best achieved if the reaction is carried out in a reasonable volume: 500-1000 µl is a good starting point.
2. Centrifuge the mixture at maximum speed for 2 minutes at 4°C in a microfuge.
3. Transfer the supernatant (i.e., the precleared cell lysate) to a fresh microfuge tube.
4. Set up two microfuge tubes containing equal amounts of precleared cell lysate and 50 µl of glutathione agarose beads. To one tube add approx. 10 µg of GST protein; to the other tube add approx. 10 µg of the GST fusion probe protein. The amount of probe and control protein added should be equimolar in the two reactions (i.e., the final molar concentration of GST should be the same as the GST fusion probe protein). Incubate the tubes for 2 hours at 4°C with end-over-end mixing.
   IMPORTANT *If the bound proteins will be removed from the beads by boiling (Step 10), it is important to include a control tube containing only glutathione agarose beads and cell lysate. This allows the detection of proteins that bind nonspecifically to the beads.*
5. Centrifuge the samples at maximum speed for 2 minutes in a microfuge.
6. Save the supernatants at 4°C in fresh microfuge tubes. These samples will be analyzed by SDS-polyacrylamide gel electrophoresis analysis in Step 10.
7. Wash the beads with 1 ml of ice-cold GST lysis buffer. Centrifuge the tubes at maximum speed for 1 minute in a microfuge. Discard the supernatants. Repeat the washes three times.
8. (*Optional*) Elute the GST fusion protein and any proteins bound to it by adding 50 µl of 20 mM reduced glutathione in 50 mM Tris-Cl (pH 8.0) to the beads. Centrifuge the tubes at maximum speed for 2 minutes in a microfuge.
9. Mix the beads (from Step 7) or the eluted proteins (from Step 8) with an equal volume of 2x SDS-PAGE gel-loading buffer.
10. Boil the samples for 4 minutes and analyze them by SDS-polyacrylamide gel electrophoresis.
11. The method of detecting proteins associated with the GST fusion protein will depend on whether or not the cell lysate was radiolabeled and on the goal of the experiment.
    - If the goal is to detect all of the ^{35}S-labeled proteins associated with the fusion protein, dry the gel on a gel dryer and expose it to X-ray film to produce an autoradiograph.
    - If the goal is to detect specific associated proteins, transfer the proteins from the SDS-polyacrylamide gel to a membrane and perform immunoblotting.
    - If the goal is to determine the sizes and abundance of proteins associated with the fusion protein from a nonradioactive lysate, stain the gel with Coomassie Blue or silver nitrate.

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Protocol 6

Identification of Associated Proteins by Coimmunoprecipitation

Coimmunoprecipitation is most commonly used to test whether two proteins of interest are associated in vivo, but it can also be used to identify novel interacting partners of a target protein. In both cases, the cells, which may have been labeled with [^{35}S]methionine, are harvested and lysed under conditions that preserve protein-protein interactions. The target protein is specifically immunoprecipitated from the cell extracts, and the immunoprecipitates are fractionated by SDS-PAGE. Coimmunoprecipitated proteins are detected by autoradiography and/or by western blotting with an antibody directed against that protein. The identity of interacting proteins may be established or confirmed by Edman degradation of tryptic peptides. This protocol was used to identify pVHL-associated proteins. Conditions should be optimized for the protein of interest. This protocol was provided by Peter D. Adams (Fox Chase Cancer Center, Philadelphia, Pennsylvania) and Michael Ohh (Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

- ⚠ Acetonitrile (50%)
- Coomassie Blue R-250
- EBC lysis buffer
- NETN
- NETN containing 900 mM NaCl
- PBS
- 1x SDS gel-loading buffer
- ⚠ TFA (Trifluoroacetic acid) (0.1% w/v, sequencing grade)

Enzymes and Buffers

- Trypsin
- Trypsin digestion buffer

Antibodies

Antibody or antibodies that will precipitate the target protein

Control antibody

Additional Reagents

Steps 10 and 11 of this protocol require the reagents and equipment for mapping, purification, and sequencing of peptides.

Cells and Tissues

Appropriate cell line(s) growing in culture

METHOD

1. Wash 30 10-cm plates of the appropriate cells (a total of approx. 6 x 10^7 cells) in PBS. Scrape each plate of cells into 1 ml of ice-cold EBC lysis buffer.
2. Transfer each milliliter of cell suspension into a microfuge tube, and centrifuge the tubes at maximum speed for 15 minutes at 4°C in a microfuge.
3. Pool the supernatants (approx. 30 ml) and add 30 µg of the appropriate antibody. Rock the immunoprecipitate for 1 hour at 4°C.
4. Add 0.9 ml of the protein A-Sepharose slurry. Rock the immunoprecipitate for another 30 minutes at 4°C.
5. Wash the protein A-Sepharose mixture in NETN containing 900 mM NaCl. Repeat this wash five more times. Finally, wash the mixture once in NETN.
6. Remove the liquid portion of the mixture by aspiration. Add 800 µl of 1x SDS gel-loading buffer to the beads, and boil them for 4 minutes.
7. Load the sample into the large well of the discontinuous SDS-PAGE gradient gel, and run the gel at 10 mA constant current overnight.
8. Visualize the protein bands by staining with Coomassie Blue.
9. Excise the band of interest from the gel, place it in a microfuge tube, and wash it twice for 3 minutes each in 1 ml of 50% acetonitrile.
10. Digest the protein with trypsin while it is still in the gel, and electroelute the peptides.
    a. Remove the gel slice to a clean surface and allow it to partially dry.
    b. Add 5 µl of trypsin digestion buffer and 2 µl of trypsin solution.
    c. After the gel absorbs the trypsin solution, add 5-µl aliquots of trypsin buffer until the gel slice regains its original size.
    d. Place the gel slice in a microfuge tube, immerse it in trypsin digestion buffer, and incubate it for 4 hours at 30°C. Stop the reaction by addition of 1.5 µl of 0.1% trifluoroacetic acid.
11. Fractionate the peptides by narrow-bore high-performance liquid chromatography. Subject the collected peptides to automated Edman degradation sequencing on an ABI 477A or 494A machine.

REFERENCES

1. Harlow E. and Lane D. 1988. Antibodies: A laboratory manual *Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.* null:null.
2. Harlow E. and Lane D. 1999. Using antibodies: A laboratory manual. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.* null:null.

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Protocol 7

Probing Protein Interactions Using GFP and FRET
Stage 1: Labeling Proteins with Fluorescent Dyes

This stage describes how components (usually Fab fragments of antibodies) that are to be introduced into cells are labeled with fluorescent sulfoindocyanine dyes. For further details of this procedure, please see the introduction beginning on page 18.69 in the print version of the manual. This protocol was provided by Alisa G. Harpur and Philippe I.H. Bastiaens (Imperial Cancer Research Fund, London).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ Ab digestion buffer
Bicine (0.1 M, pH 7.5) adjusted with NaOH
Bicine (1 M, pH 9.0-9.5) adjusted with NaOH

⚠ DMF (*N,N*-dimethylformamide)

Sodium phosphate (20 mM) with 10 mM EDTA (pH 7.0)

PBS

TE (pH 7.0)

Enzymes and Buffers

Papain immobilized to agarose beads (50% slurry in digestion buffer; Sigma)

Labeled Compounds

Cy3 and Cy5 OSu monofunctional sulfoindocyanine succinimide esters (Amersham)
Succinimide esters of sulfoindocyanine (Cy) dyes covalently bind to free amino groups (the α-amino terminal or ε-amino groups on lysine side chains). Cy dyes are provided as lyophilized samples that must be maintained in a desiccated environment at all times to prevent reaction with H_2O. The amount of dye in each vial must be individually quantitated as there can be variations between batches. In the example given here, the succinimide ester of Oregon Green (Molecular Probes) is used to label the MC5 PKCα antibody.

Antibodies

Purified antibody such as MC5, monoclonal or T250, polyclonal or protein to be labeled
The labeling reaction must be performed in a buffer free of amine groups. Many commercial antibody preparations are provided with stabilizing agents that contain free amino groups (such as bovine serum albumin or gelatin) which will compete for the Cy dye. A number of suppliers provide the antibody preparations free of these compounds upon request; in this case, request that they are provided at 1 mg/ml in PBS.

Additional Reagents

Step 7 of this protocol requires the reagents for determining protein concentration.

METHOD

1. Concentrate the antibodies to 15-20 mg/ml in 20 mM Na-phosphate, 10 mM EDTA (pH 7.0), by centrifugation using a disposable concentrator such as a Centricon YM100.
2. To 250 µl of the concentrated antibody preparation (approx. 4-5 mg), add 500 µl of Ab digestion buffer and 500 µl of a 50% slurry (in digestion buffer) of papain immobilized on agarose beads. Allow the digestion to proceed for 6-10 hours at 37°C with shaking (300-400 rpm in a shaking incubator).
   IMPORTANT *Excessive digestion (i.e., in excess of 16 hours) will result in the cleavage of the Fab fragments into smaller polypeptides. It is therefore advisable to perform a pilot experiment where small aliquots of the digestion (approx. 5 µl) are withdrawn at 1-hour intervals for analysis by reducing SDS-polyacrylamide gel electrophoresis. Under reducing conditions, the heavy and light chains of IgG migrate as 50-kD and 25-kD polypeptides. The papain-digested antibody migrates at approx. 25 kD.*
3. Purify the Fab fragments from the Fc portion and nondigested antibodies by passage through a protein A-Sepharose column. Apply the digestion mixture to the equilibrated column and immediately begin collecting 1-ml fractions of the flowthrough.
4. To identify which fraction contains the purified Fab fragments, measure the OD_{280} of each fraction. Collect and pool three or four fractions containing the highest concentration of Fab.
5. Concentrate the pooled fractions by centrifugation through a Centricon YM10 (10-kD cut-off; Amicon) to a volume of <0.5 ml.
6. Apply the sample to a low-molecular-mass (6-kD exclusion) gel filtration chromatography column equilibrated in PBS. Elute the Fab fragments with PBS (according to manufacturers instructions).
7. Following elution, concentrate the Fab fragments through a Centricon concentrator and determine the protein concentration. Add 1/10 volume of 1 M bicine (pH 9.0).
8. Resuspend the contents of a single vial of lyophilized dye in 20 µl of dry *N,N*-dimethylformamide, to give a Cy solution with a concentration of approx. 10 mM.
9. To prepare the dye, dilute 1 µl of the dye mixture 1:10,000 in PBS and calculate the dye concentration from the visible peak absorption.
   The extinction coefficients for Cy3 and Cy5 are 150,000 mM^{-1} and 250,000 mM^{-1} at 554 nm and 650 nm, respectively.
10. To label the proteins, suspend them in a buffer that does not contain free amino groups. If the protein solution is not in an appropriate buffer, perform Steps 6-7.
11. React the protein sample (antibody, Fab fragment, or protein) with a 10-40-fold molar excess of Cy3 for 30 minutes at room temperature (prepared as described in Steps 8-9). Add the dye very slowly while stirring the solution with a pipette tip (carefully avoiding direct dye contact with the microfuge tube).
    To avoid protein denaturation by DMF, the volume of Cy3/DMF added must not exceed 10% of the total volume.
12. Terminate the labeling reaction by the addition of a free-amine-containing buffer such as Tris, to a final concentration of 10 mM.
13. Remove the excess unreacted dye by exchanging the buffer using gel filtration column chromatography (10DG, Bio-Rad), and eluting the protein into PBS.
    a. Equilibrate the column with three bed volumes of PBS or the buffer of choice (approx. 30 ml).
    b. To maximize separation, load the labeling reaction mixture directly onto the resin, taking care to apply it in as small a volume as possible.
    c. Wash the column with 2.5 ml of buffer (approx. 3.3 ml is void) and discard.
    d. Add a further 2 ml of buffer and collect the visible protein fraction, discarding the remainder. The labeled product will run at the leading front and should be visible. Free dye migrates more slowly through the column.
14. Concentrate the labeled protein once again using a Centricon concentrator (of the appropriate molecular-mass cut-off).

15. Analyze the labeled product by electrophoresis through an SDS-acrylamide gel to verify successful covalent labeling of the protein. Following electrophoresis, examine the gel directly on a UV transilluminator (302 nm) to visualize the labeled product.
16. Following the labeling reaction, calculate the labeling ratio using the following formula:
$A\lambda$ x M/[(A_{280} - f x $A\lambda$) x $\varepsilon\lambda$]
Where $A\lambda$ is the absorption of the dye at its absorption maximum at wavelength λ, A_{280} is the absorption of the protein at 280 nm, M is the molecular mass of the protein in kD, $\varepsilon\lambda$ is the molar extinction coefficient of the dye at wavelength λ in $mM^{-1}cm^{-1}$. The equation also corrects for absorption of the dye at 280 nm. This factor f is the ratio between absorption of the dye at 280 nm and its maximal visible absorption at wavelength λ. For example, the labeling ratio formula for Cy3-labeled antibody becomes:
A_{554} x 170/[(A_{280} - 0.05 x A_{554}) x 150]
To verify that dye coupling has not damaged the biological function of the Fab, whole antibody, or protein, compare the specific activity of the labeled product with its unlabeled counterpart.

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Protocol 8

Probing Protein Interactions Using GFP and FRET
Stage 2: Cell Preparation for FLIM-FRET Analysis

In preparation for FLIM-FRET analysis, the appropriate donor and acceptor components must be introduced into live or fixed cells. The method of introduction depends on the nature of the components and the state of the cells. For example, plasmid DNAs encoding a protein of interest fused to a variant of GFP may be introduced into live cells by transfection or microinjection, whereas labeled antibodies are delivered by microinjection. For studies on fixed cells, plasmid DNA is introduced by transfection or microinjection, and the cells are subsequently fixed in paraformaldehyde before staining with labeled protein or antibody. This stage describes the introduction of plasmid DNA into live cells by transfection. For alternative methods of introducing donor and acceptor molecules, please see pages 18.87-18.89 of the print version of the manual. This protocol was provided by Alisa G. Harpur and Philippe I.H. Bastiaens (Imperial Cancer Research Fund, London).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.
RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

Gelatin solution (0.1%) in PBS
Optional, please see Step 1.

PBS

Poly-L-lysine
Optional, please see Step 1.
Available commercially from Sigma as a 0.1% (w/v) solution in H_2O; dilute 1:10 in H_2O before use.

Nucleic Acids and Oligonucleotides

Plasmid DNA carrying the probe
GFP fusion vector (CLONTECH) encoding the protein of interest.

Media

CO_2-independent imaging medium
This low-background fluorescence medium is commercially available from Life Technologies or adjust the standard formulation of Dulbecco's modified Eagle's medium by omitting each of the following: pH indicator phenol red, penicillin, streptomycin, folic acid, and riboflavin. Just before use, supplement the medium with sterile HEPES (final concentration 50 mM) adjusted with NaOH to pH 7.4.The imaging medium must not contain components that are autofluorescent.

Additional Reagents

Step 2 of this protocol requires the reagents for a transfection method as described in Chapter 16.

Cells and Tissues

Cell line (adherent or suspension culture) to be transfected
The only consideration for the choice of cell type, other than their suitability for the system to be investigated, is that a facility be available to immobilize them in some manner (see note to Step 1 for suggestions).

METHOD

1. Seed the cells onto an appropriate surface for microscopy:
   For live cell preparations: Seed cells onto glass-bottomed dishes or coverglass chamber slides.
   For cell preparations to be fixed after transfection: Seed the cells onto glass coverslips placed in 6- or 12-well tissue culture dishes.
   For suspension cells, immobilization can be facilitated with the use of media such as gelatin (0.1% w/v in PBS; autoclaved) or poly-L-lysine (0.01% w/v in H_2O). In either case, coat the surface by covering either the wells, coverslips, or coverglass slides for approx. 30 minutes with the chosen medium and then aspirate the excess. Cells can then be directly seeded onto the coated surface.
2. Transfect the cells with the plasmid(s) encoding the GFP-tagged protein(s) of interest, using any one of the transfection methods described in Chapter 16.
3. Incubate the transfected cells under the appropriate conditions for 16-24 hours to allow the cells to express the protein of interest.
4. Identify cells that are expressing the protein of interest.
5. (*Optional*) If appropriate for the experimental design, introduce another probe (e.g., labeled protein) using one of the alternative protocols following Step 6: microinjection (for live cells) or fixation and staining (for fixed cells).
6. Immediately before performing an experiment at the microscope, replace the culture medium with CO_2-independent imaging medium (commercial medium or Dulbecco's modified Eagle's medium adjusted as described in the Materials list).

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Protocol 9

Probing Protein Interactions Using GFP and FRET
Stage 3: FLIM-FRET Measurements

This stage presents a basic plan for capturing a series of images by fluorescent lifetime imaging microscopy. The protocol specifically describes data acquisition for a particular variant of GFP (EGFP) or Oregon Green as a donor fluorophore, but it can be adapted for image acquisition of other chromophore systems as described in the table below. This protocol was provided by Alisa G. Harpur and Philippe I.H. Bastiaens (Imperial Cancer Research Fund, London).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Cells and Tissues

Cells (live or fixed) expressing the probe proteins, prepared as described in Chapter 18, Protocol 8 Stage 2

Filter Specifications for FLIM-FRET Using Selected Donor and Acceptor Pairs

FRET Pair Donor and Acceptor	Donor Filter Set			Acceptor Filter Set		
	Excitation	Dichroic	Emission	Excitation	Dichroic	Emission
ECFP and EYFP	laser line 457.9 nm	455DRLP (Omega)	480DF30 (Omega)	HQ500/40 (Chroma)	Q525LP (Chroma)	HQ555/50 (Chroma)
EGFP and DsRed	laser line 488 nm	Q495LP (Chroma)	HQ510/20 (Chroma)	HQ545/30 (Chroma)	HQ565LP (Chroma)	HQ610/75 (Chroma)
Cy3 and Cy5	laser line 514.5 nm	HQ565LP (Chroma)	HQ610/75 (Chroma)	HQ620/60 (Chroma)	HQ660LP (Chroma)	HQ700/75 (Chroma)
EGFP and Cy3	laser line 488 nm	Q495LP (Chroma)	HQ510/20 (Chroma)	HQ545/30 (Chroma)	HQ565LP (Chroma)	HQ610/75 (Chroma)

METHOD

1. Select the 488-nm excitation wavelength on the argon laser by adjusting the wavelength selector prism. Optimize the output power by fine tuning the position of the high-reflector mirror using the control knobs at the back of the laser.
2. Set the frequency synthesizer to drive the AOM to approx. 40 MHz at a resonance frequency (for the experiments described herein, a driving frequency of 40.112 MHz is used). This gives rise to intensity oscillations in the laser light beam at twice the driving frequency (80.224 MHz).
3. Optimize the diffraction in the AOM and thereby the modulation depth by adjusting the angle of incidence and monitoring the intensity of the undiffracted zero order beam with a power meter. The optimal angle of diffraction (corresponding to maximal diffraction) gives rise to a minimum in the output power of the zero-order beam.
4. Turn on the MCP and the CCD. Set the bias of the photocathode voltage to -2 V and adjust the gain to match the full dynamic range on the CCD. This is dependent on the fluorescence intensity of the sample and must be determined empirically. Ideally, keep the gain as low as possible to reduce noise. Typically, the gain is set at 1 for the Hamamatsu C5825, with the gain of the Photometrics Quantix CCD set at 3. Set the readout of the CCD to 2 x 2 binning.
5. Set the master frequency synthesizer driving the MCP to a value exactly double (for the above example: 80.224 MHz) that driving the AOM.
6. Choose the most suitable objective for the experiment. For this example, a Zeiss Fluar 100x/1.4 NA oil objective is used.
7. To obtain a zero lifetime reference image, record a cycle of 16 phase-dependent images, each separated by 22.5° from a strong scatterer (e.g., a small piece of aluminum foil placed on the imaging surface of a coverslip or glass bottom dish).
   a. Exchange the fluorescence filter set for a half-silvered mirror and reduce the intensity of the incident beam to a minimum with a variable density filter wheel. Adjust the focus on the foil surface.
   IMPORTANT *When setting up the foil image, take extreme care not to look directly into the microscope occular until the incident laser source has been reduced to a minimum.*
   b. Take a single image of the aluminum foil with an exposure time of approx. 100 msec. This image is taken to select an ROI and to gauge the likely exposure time required. Because the phase of the master frequency synthesizer may not be at a maximum, and to avoid saturation of the detector, select an exposure time that generates approx. 1000 counts.
   c. Record a cycle of 16 phase images, each separated by 22.5°. Determine the phase setting at which maximum intensity is reached in the image series. Reset this phase to zero degrees on the master frequency synthesizer.
   d. Record another cycle of 16 phase images from the foil to save as a zero lifetime reference.
   While performing lifetime imaging, we recommend that phase stability be monitored over time. In our setup, the phase is stable within 0.3° during a period of 1 hour. For our setup, a reference foil sequence is recorded and saved each hour.
8. Restore the incident excitation source to a maximum (using the variable density filter wheel); the system is now ready for the acquisition of cell imaging data.
9. Acquire a donor image using the GFP filter set (Dichroic: Q495LP, emission: HQ510/20). Select an exposure time such that 75% of the dynamic range of the CCD (3000 counts for a 12-bit CCD) is filled. Select a region of interest in the image on which to measure fluorescence lifetimes.
10. Create a donor fluorescence lifetime map:
    a. Take two contiguous series of 16 phase-dependent images (45° phase-stepped), one forward and one reverse cycle to correct for photobleaching. Optimize the set exposure time for each phase image as in Step 9.
    b. Take an additional image in the absence of sample illumination. This background offset image is subtracted from all phase images in the series. Each of the pairs of equivalent phase images of the forward and reverse cycles are then summed to first-order to correct for bleaching of the donor.
    c. From these data and the zero lifetime reference (foil), calculate a donor fluorescence lifetime map as described in the discussion on Image Processing in the protocol introduction, pages 18.75-18.78 in the print version of the manual.
11. Record an image of the acceptor, and then photobleach the acceptor by changing to the 100-W mercury arc lamp (Zeiss Attoarc) as a source of illumination. Move the Cy3 filter set (excitation: HQ545/30, dichroic: Q565LP, emission: HQ610/75 chroma) into the detection path. Take an image of the acceptor by optimizing the exposure time to occupy the full dynamic range of the CCD. Close the detector port and illuminate the acceptor until there is no discernible Cy3 fluorescence.
    When performing acceptor photobleaching experiments, it is crucial to establish that there is no fluorescence from the photoproduct of the photobleached acceptor in the donor channel.

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Protocol 10

Analysis of Interacting Proteins with SPR Spectroscopy Using BIAcore
Stage 1: Preparation of the Capture Surface and Test Binding

The introduction of commercial instruments, such as BIAcore, that are capable of measuring surface plasmon resonance (SPR), has simplified the study of macromolecular interactions by providing a format that may be used to measure molecular interactions in real time with small analytical amounts of material. This protocol describes the sequence of steps for analyzing a typical antibody-antigen system in a BIAcore experiment. The antibody (antithyroid stimulating hormone [anti-TSH]) and its ligand (TSH) are available commercially and can be used to optimize the parameters of the system. A complete description of the setup and use of the instrument may be found at www.biacore.com. For further theoretical and experimental details of the procedure, please see the introduction to this protocol on page 18.96 in the print version of the manual. This protocol was provided by Maxine V. Medaglia and Robert J. Fisher with the help of M. Fivash, C. Hixson, and L. Wilson (National Cancer Institute-FCRDC, Frederick, Maryland). Stage 1 describes how (1) to prepare a sensor chip containing immobilized rabbit anti-mouse C domain, (2) to capture mouse anti-TSH on the prepared surface, and (3) to test for the binding of TSH to its captured antibody.

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

⚠ EDC (0.2 M *N*-ethyl-*N*′-(dimethylaminopropyl)-carbodiimide in H_2O)
Commercially available as part of the Amine-Coupling Kit from BIAcore.

⚠ Ethanolamine (1 M ethanolamine hydrochloride, adjusted to pH 8.5 with NaOH)
Commercially available as part of the Amine-Coupling Kit from BIAcore.

Extraclean
Extraclean is a solution for surface regeneration and is commercially available from BIAcore.

HEPES-buffered saline buffer

⚠ HCl (20 mM)

NHS (0.05 M *N*-hydroxysuccinimide in H_2O)
Commercially available as part of the Amine-Coupling Kit from BIAcore.

Antibodies

Mouse anti-TSH monoclonal antibody (1 mg/ml in HEPES-buffered saline)
This antibody is commercially available from Alexon Trend, Inc.

Rabbit anti-mouse Fc domain (RAMc) (30 µg/ml in 10 mM Na-acetate pH 5.0)
This antibody is commercially available from BIAcore.

TSH (20 µM in HEPES-buffered saline)
TSH is commercially available from Alexon Trend, Inc.
The Molecular Interactions Research Group (MIRG) within the Association of Biomolecular Resource Facilities (ABRF) (www.abrf.org) recommends that protein samples be prepared for BIAcore analysis by buffer exchange dialysis (3 times in 500 volumes) against HEPES-buffered saline, and then centrifuged at maximum speed in a refrigerated microfuge and finally passed through a 0.22-µm filter. The resulting protein concentration is determined by absorbance at 280 nm using the calculated extinction coefficient for the particular protein or peptide.

METHOD

1. Dock Sensor Chip CM-5 in the BIAcore instrument.
   The procedure for primary amine coupling of RAMc to the sensor chip CM-5 surface (Steps 1-14) takes approx. 45 minutes. The commands are accessed through the pulldown menus or by the icons of the toolbar of the BIAcore control software.
2. Prime using filtered and degassed HEPES-buffered saline.
3. Place tubes containing 100 µl each of NHS, EDC, ethanolamine, and RAM Fc and 20 mM HCl in appropriate positions in the autosampler rack in the BIAcore.
4. Place an empty tube in the autosampler rack in the BIAcore.
5. Start the instrument at a flow of 5 µl/minute over one flow cell.
6. Transfer 75 µl of NHS to the empty tube.
7. Transfer 75 µl of EDC to the same tube.
8. Mix the contents of the tube containing the NHS and EDC.
9. Inject 35 µl of the NHS/EDC mixture to activate the surface.
10. Inject 35 µl of the RAM Fc to couple the antibody to the activated surface.
11. Inject 35 µl of ethanolamine to deactivate excess reactive groups.
12. Quickinject 10 µl of 20 mM HCl followed by Extraclean to remove noncovalently bound material.
13. Determine the level of RAMc bound by placing a baseline report point before the start of the RAMc injection and a second report point 2 minutes after the end of the 20 mM HCl injection.
14. Stop the flow, close the command queue window, and save the report file.
15. Start the instrument at a flow of 10 µl/minute over the flow cell with the coupled RAMc.
    The procedure for testing the binding of the anti-TSH to the RAMc surface (Steps 15-20) takes approx. 20 minutes. The commands are accessed through the pulldown menus or by the icons of the toolbar of the BIAcore control software.
16. Inject 10 µl of 2 µg/ml anti-TSH (this requires a total of 40 µl of anti-TSH solution).
    10 µl of the anti-TSH should result in an increase of approx. 250 RUs. If this level of binding to the RAMc surface is not obtained, regenerate (see Step 17) and repeat the injection of anti-TSH using different volumes until the necessary injection volume is determined.
17. Quickinject 10 µl of 20 mM HCl followed by Extraclean to regenerate the RAMc surface.
18. To test the reproducibility of the binding to the RAMc surface, repeat the injection using the volume of anti-TSH required to give 250 RUs bound to anti-TSH.
19. Quickinject 10 µl of 20 mM HCl followed by Extraclean to regenerate the RAMc surface.
20. Stop the flow, close the command queue window, and save the report file.
21. Start the instrument at a flow of 10 µl/minute over the flow cell with the coupled RAMc.
    The procedure for testing the binding of TSH to the captured anti-TSH surface (Steps 21-25) takes approx. 20 minutes. The commands are accessed through the pulldown menus or by the icons of the toolbar of the BIAcore control software.
22. Inject 10 µl of 2 µg/ml anti-TSH (or the volume determined to give 250 RUs bound anti-TSH as determined in Steps 15-20).
23. Inject 25 µl of 200 nM TSH, specifying a 120-second dissociation time (this requires a total of 65 µl of TSH solution). The 120-second dissociation time allows the rate at which the antibody-antigen complex dissociates to be gauged.
24. Quickinject 10 µl of 20 mM HCl followed by Extraclean to regenerate the RAMc surface.
25. Stop the flow, close the command queue window, and save the report file.

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Protocol 11

Analysis of Interacting Proteins with SPR Spectroscopy Using BIAcore
Stage 2: Kinetic Analysis of the Antibody-Antigen Interaction

Stage 2 presents a series of method blocks or analysis programs that allow the determination of the equilibrium constants for the interaction between ligand pairs. This protocol was provided by Maxine V. Medaglia and Robert J. Fisher with the help of M. Fivash, C. Hixson, and L. Wilson (National Cancer Institute-FCRDC, Frederick, Maryland).

MATERIALS

⚠ **CAUTION:** Please click for information about appropriate handling of materials.

RECIPE: Please click for components of stock solutions, buffers, and reagents.

Buffers and Solutions

HEPES-buffered saline buffer or appropriate running buffer
This buffer is commercially available from BIAcore.

Antibodies

Mouse anti-TSH monoclonal antibody (1 mg/ml in HEPES-buffered saline)
This antibody is commercially available from Alexon Trend, Inc.Dilute the antibody to 2 µg/ml and prepare 1000 µl (or the volume required) to achieve the target resonance units as determined in Steps 15-20 of Chapter 18, Protocol 10.

TSH (20 µM in HEPES-buffered saline)
TSH is commercially available from Alexon Trend, Inc.

METHOD

1. For an example of a sequence of commands used to determine the equilibrium constants of an interaction by the analysis program, please see the description beginning on page 18.108 of the print version of the manual.
2. The sequence of commands consists of three method blocks or sections: the MAIN, the DEFINE APROG, and the DEFINE SAMPLE. There are four DEFINE APROG blocks, one for each of the four APROGs referred to in the MAIN (bind1, bind2, bind3, and bind4). When the BIAcore software edits a method, the method commands will be written in capitals and the user-defined parameters will be in lowercase letters (further details may be found at www.biacore.com).

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