EXERCISE

5

Southern Blot Detection of DNA

Background

Southern blots are used to identify a specific restriction fragment (containing a DNA sequence of interest) among a large heterogeneous population of DNA fragments. First, agarose gel electrophoresis is used to separate restriction fragments according to size. Next the DNA is denatured in situ (in the gel) and transferred to a membrane. The DNA is bound to the membrane by UV cross-linking. Finally, the DNA sequence of interest is detected using a hybridization probe, which must be complementary (or nearly complementary) to its target. After a prehybridization treatment to block sites on the membrane where the hybridization probe may bind nonspecifically, the membrane is incubated with the probe. Posthybridization washes remove all probe except that annealed to target DNA bound to the filter. The probe–DNA fragment complex is then detected by autoradiography or a colorimetric method.

To demonstrate the Southern (DNA) blot technique, we will isolate genomic DNA from several different strains of *Agrobacterium tumefaciens* and examine restriction fragment length polymorphisms (RFLPs) in their *virD2* genes. RFLPs are used to estimate relatedness. Using RFLPs from *virD2,* we will group the different *A. tumefaciens* strains by RFLP phenotype.

Steps of the experiment are

A. Prepare genomic DNA

B. Perform restriction digest and agarose gel electrophoresis of products

C. Denature and blot DNA

D. Prepare probe by nick translation

E. Hybridize and wash Southern blot

A. PREPARATION OF GENOMIC DNA FROM *AGROBACTERIUM TUMEFACIENS*

Introduction

Methods for preparing genomic DNA from prokaryotes and eukaryotes (plants, animals, and fungi) share common features. Cells are harvested and then dispersed into a homogeneous suspension; tissues of multicellular organisms must be disrupted, often by grinding. Next, cells are lysed, usually in a detergent–EDTA extraction buffer. Nucleic acids are separated from other cell components, typically by phenol extraction and ethanol precipitation.

Although *virD2* lies on the tumor-inducing (Ti) plasmid in *A. tumefaciens*, isolation of this plasmid is tedious because of its large size (200 kb) and low copy number (1 per chromosome). Therefore, you will isolate genomic (both chromosomal and Ti plasmid) DNA for analysis. You will isolate DNA from the following *A. tumefaciens* strains: A208, A277, A856, ACH5, C58, EHA101, GV3111, K599, A348, A348 with pVK225, R1000, 1855, and 15955.

Technical Tips

Chromosomal DNA is more fragile than the smaller closed circular plasmid DNAs; take care handling and mixing.

Phenol is equilibrated with buffer before use and stored under buffer. Therefore, 2 phases are apparent in the phenol bottle; **do not** mix these! A layer of aqueous buffer is on top. To obtain phenol, insert a pipet into the phenol layer beneath the buffer. Afterward, dispose of phenol properly.

When performing an organic extraction, leave the entire interphase with the phenol phase; you will have to leave behind some of the aqueous phase to avoid debris at the interphase.

Protocol

1. TAs will provide *A. tumefaciens* cultures grown in YEP broth at 28°C with aeration overnight. Each team will receive 4 strains.
2. Centrifuge 1.5 mL of culture for 1 minute at full speed in microfuge.
3. Discard supernatant; resuspend pellet in 480 μL of TE (25/10); add 20 μL of 25% SDS.
4. Incubate 15 minutes at 37°C.
5. Add 57 μL of 5 *M* NaCl; vortex at full power 1 minute.
6. Incubate at 68°C for 10 minutes, then vortex as above.
7. Add 0.5 mL phenol (equilibrated with 1 *M* Tris, pH 8.0); mix well, then add 0.3 mL $CHCl_3$ and mix again. Wear eye protection, gloves, and lab coat.
8. Mix well, then centrifuge 10 minutes at full speed in microfuge.
9. Save aqueous (top) phase and extract with 0.5 mL $CHCl_3$:isoamyl alcohol (24:1).
10. Centrifuge 1 minute in microfuge; remove aqueous (top) phase to a clean 1.5-mL tube.
11. Add 1 mL of 95% ethanol; mix thoroughly and hold on ice for 5 minutes.
12. Centrifuge 3 minutes in microfuge.
13. Discard supernatant; dissolve pellet in 200 μL TE (10/0.1). Add 100 μL 7.5 *M* ammonium acetate.
14. Add 0.6 mL ethanol; mix and hold on ice for 5 minutes.
15. Centrifuge 3 minutes in microfuge.

16. Discard supernatant. Dissolve pellet in 50 μL TE (10/0.1); store frozen.

17. Quantify concentration of nucleic acids (DNA and RNA) by measuring absorption at 260 nm. Dilute samples 1:100 (1 μL DNA in 99 μL TE). $A_{260\ nm} \times$ dilution factor $\times$ 50 = μg/mL.

Strain	Concentration (μg/μL)	Total yield (μg)
1.		
2.		
3.		
4.		

Solutions for Preparation of Genomic DNA

TE (25/10): 25 mM Tris, pH 8.0; 10 mM EDTA (autoclave)

TE (10/0.1): 10 mM Tris, pH 7.5; 0.1 mM EDTA (autoclave)

7.5 *M* Ammonium acetate: 57.8 g/100 mL (autoclave)

5 *M* NaCl: 29.2 g/100 mL (autoclave)

25% SDS: 25 g/100 mL

Phenol: Melt phenol in distilled H_2O, then add 1 *M* Tris base (12.1 g/100 mL; pH not adjusted) until pH of phenol phase reaches 8.0 (check with pH paper, not a meter); store frozen. Phenol is colorless; pink color indicates oxidized phenol. Quality is important: buy nucleic acids grade, or distill reagent grade yourself.

$CHCl_3$ and $CHCl_3$:isoamyl alcohol (24:1)

YEP broth: 10 g/L peptone (Difco) + 5 g/L NaCl + 10 g/L yeast extract (Difco) (no need to adjust pH). Autoclave 22 minutes

Notes

B. RESTRICTION DIGESTION OF GENOMIC DNA

Technical Tips

Store restriction endonucleases at −20°C. Keep restriction enzymes on ice for the short period that you have them out of the freezer. Add them last to a restriction digest mixture, and begin the incubation immediately.

Protocol

1. Obtain genomic DNAs from different strains from other lab groups. Calculate the volume of each genomic DNA preparation to add to the restriction digestion (add 10 μg total nucleic acids, including RNA).
2. Combine:
   __ μL of genomic DNA preparation
   1.5 μL of 10× reaction buffer (NEBuffer *Eco*RI)
   1.5 μL of *Eco*RI restriction endonuclease (10 units/μL)
   __ μL water to bring to 15 μL
3. Incubate at 37°C for 2 hours to ensure complete digestion.

Solution for Restriction Digest

NEBuffer *Eco*RI: 50 mM NaCl, 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.025% Triton X-100

Notes

C. AGAROSE GEL ELECTROPHORESIS OF RESTRICTION FRAGMENTS

Protocol

1. Mix agarose with 1× TAE buffer (0.8% = 0.24 g/30 mL for minigel); melt in microwave until thoroughly dissolved; add 1.25 μL of 10 mg/mL ethidium bromide.
2. Pour gel into bed with comb (well former) in place; allow to solidify; place in gel apparatus, cover with 1× TAE running buffer, and remove comb.
3. Add 2 μL of 6× load buffer to 10 μL of each sample. Load into individual wells (initially full of 1× TAE). As a molecular weight standard, use *Hin*dIII-cut lambda DNA; **do not use the 1-kb DNA ladder.** Mix 5 μL of a 100-ng/μL solution of the *Hin*dIII-cut lambda DNA ladder with 1 μL 6× load buffer and load at one side of the gel; leave an empty lane between the molecular weight standard and the genomic DNAs.
4. Apply 100 V. DNA will migrate to the positive electrode.
5. When bromphenol blue nears bottom of gel, observe DNA under UV light and photograph. Use the fluorescent ruler in your photograph. Protect gel from contact with the UV light source and other surfaces with plastic wrap.

Solutions for Agarose Gel Electrophoresis

TAE: 0.04 *M* Tris–acetate, 0.002 *M* EDTA
1 L 50×: 242 g Tris base
57.1 mL glacial acetic acid
100 mL 0.5 *M* EDTA, pH 8.0

Ethidium bromide: 10 mg/mL stock

6× load buffer
0.05% bromphenol blue
40% (w/v) glycerol in H_2O

*Hin*dIII-cut lambda DNA (100 ng/μL)

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D. SOUTHERN BLOT: DENATURATION AND BLOTTING OF DNA

Introduction

During the Southern blot procedure, we denature DNA in the agarose gel, transfer this single-stranded DNA to a nylon membrane, and hybridize it to a labeled DNA probe (*virD2*). The agarose gel contains both large and small DNA fragments. Because large fragments transfer less efficiently than small ones, we use limited chemical hydrolysis to reduce fragment length. Acid hydrolysis partially depurinates DNA (embedded in the agarose gel), and subsequent incubation with NaOH denatures the DNA and breaks the phosphodiester backbone at apurinic sites. Capillary action moves liquid and DNA from the agarose gel upward; the liquid continues through the nylon membrane and into dry paper towels, but the membrane traps DNA. Application of a vacuum or electric current speeds DNA transfer, but requires special apparatus. DNA can be cross-linked to nylon or nitrocellulose membranes by UV irradiation or baking at 80°C in a vacuum for 2 hours. Positively charged nylon membranes do not require cross-linking, but UV irradiation may improve signal and is recommended when blots will be stripped and reprobed.

Protocol

1. Wash the gel for 15 minutes in 0.25 *M* HCl (100 to 200 mL/wash); rinse the gel with distilled water.
2. Wash the gel twice for 15 minutes each in NaOH–NaCl solution; rinse the gel with distilled water after the 2nd wash.

3. Wash the gel twice for 15 minutes each in Tris–NaCl neutralization buffer.

4. Cut Nytran membrane to exact size of gel. Wear gloves and use the liner sheet to keep the nylon membrane clean. Mark one corner of your membrane with a soft pencil.

5. Float the membrane on distilled water in a tray to wet it by capillary action.

6. Soak the membrane in 10× SSC for 15 minutes.

7. Cut 8 sheets of Whatman 3 MM filter paper to the exact size of the gel; saturate the filters with 10× SSC and set 7 sheets on a large piece of plastic wrap.

8. Place the agarose gel on the SSC-saturated Whatman 3 MM paper. Invert the gel so the bottom face will contact the nylon membrane. Use finger pressure (wear gloves) to remove air bubbles trapped between the gel and filters.

9. Lay the nylon membrane on top of the gel, with the pencil mark down. Once the membrane contacts the gel, do not move it, even if the gel and filter are not properly aligned. Use finger pressure to remove air bubbles.

10. Place 1 sheet of SSC-saturated Whatman 3 MM paper on top of the nylon membrane and remove air bubbles. Cover this with a 3-inch stack of dry paper towels (also cut to the same size as the gel). Wrap the entire stack in the plastic film, and set a modest weight on top of the paper towels.

11. Allow DNA transfer to continue for 2 to 16 hours. Transfer is complete when the gel becomes 1 mm thick.

12. Wash nylon filter for 20 minutes at room temperature with 0.2 *M* Tris, pH 7.5, + 2× SSC. Place filter (pen-

cil mark [DNA] side up) on dry Whatman 3 MM paper. Just as the filter begins to dry, irradiate it with 1200 μJ of UV light (using the Stratagene 1800 Stratalinker). This procedure links the DNA permanently to the membrane.

Solutions for Denaturation and Blotting of DNA

0.25 *M* HCl: 12.5 mL of concentrated HCl + 487.5 mL distilled water

NaOH–NaCl solution (0.5 *M* NaOH + 1.5 *M* NaCl): 10 g NaOH + 43.9 g NaCl + 485 mL distilled water

Tris–NaCl neutralization buffer (1 *M* Tris, pH 7.5, + 1.5 *M* NaCl): 157.6 g Tris base + 87.7 g NaCl + 67.7 mL concentrated HCl + 810 mL water

20× SSC (3 *M* NaCl + 0.3 *M* sodium citrate, pH 7.0): 350.4 g NaCl + 176.5 g sodium citrate·2 H_2O + 7.2 mL concentrated HCl + distilled water to final volume of 2 L

0.2 *M* Tris, pH 7.5, + 2× SSC: 24.2 g Tris base + 14 mL concentrated HCl + 880 mL distilled water + 100 mL 20× SSC

Notes

E. PREPARATION OF PROBE BY NICK TRANSLATION

Introduction

Probes may be labeled in vitro by several procedures. The nick translation method uses DNAse I, an endonuclease, to nick double-stranded DNA, and DNA polymerase I to synthesize labeled DNA fragments from these nicks. The random prime technique uses random-sequence 8-base oligonucleotides to prime synthesis of labeled DNA from single-stranded DNA. Many cloning vectors have a phage-specific promoter positioned to transcribe cloned DNA. In vitro transcription from such a promoter, using the corresponding phage RNA polymerase, produces a strand-specific RNA probe.

The most common labels are radioactive (^{32}P) or biotinylated deoxynucleoside triphosphates (dNTPs), or ribonucleoside triphosphates for labeled RNA. Each method of labeling has advantages, disadvantages, and commercial kits to ensure success.

You will use a nick translation kit from GIBCO/BRL to ^{32}P-label pWR160 plasmid DNA, which contains *virD2*. To separate unincorporated label from labeled probe DNA, you will use a Sephadex spin column.

Safety Precautions

Wear film badges when using radioactive isotopes. Wear finger badges **under** your gloves, with the film side turned toward your palm. Work behind plexiglass shields. Use a radiation meter (Geiger counter) to scan your fingers, Pipetman, and work area.

Protocol

1. In a 1.5-mL microfuge tube (on ice) mix:

   1 μg of plasmid DNA (pWR160)
   25 ng of λ DNA
   5 μL of dNTP solution A2 (contains dATP, dGTP, and dTTP; from BRL nick translation kit)
   water to 40 μL total.

2. Add 5 μL of ^{32}P-labeled dCTP.

3. Add 5 μL of DNase I–DNA polymerase I mixture (solution C, supplied with the kit).

4. Incubate at 15°C for 1 hour.

5. Remove the plunger from a 1-mL syringe and plug with siliconized glass wool. Fill with Sephadex G-50 (medium). Or use a commercial spin column, follow the manufacturer's directions, and skip steps 5, 6, and 7.

6. Remove the top of a 1.5-mL microfuge tube and place the tube in the bottom of a 15-mL Falcon tube; place the syringe barrel inside the Falcon tube so that the tip drains into the microfuge tube.

7. Centrifuge 2.5 minutes at 2900 rpm in a Beckman GP (clinical bench-top) centrifuge. Use the GH3.7 swinging bucket rotor. This will pack the column. Fill the void with more Sephadex and repeat the centrifugation.

8. Layer the nick translation reaction on top of the column and centrifuge as in step 7; room temperature is fine. The probe DNA will move rapidly through the Sephadex column and into the microfuge tube; smaller molecules, such as unincorporated label, will be retarded by the pores in the Sephadex beads and remain in the column during the centrifugation.

9. Measure the incorporation with a Geiger counter. To obtain a more accurate assessment, place 2 μL of probe in 4 mL of scintillation fluor and determine the number of counts per minute (cpm) in a scintillation counter. A good probe should have 10 to 100 million cpm/μg of input DNA.
10. Boil the probe for 3 minutes.
11. Chill on ice. Use immediately or store frozen at −20°C.

Solutions for Nick Translation

dNTP mixes in the BRL nick translation kit:
0.2 mM each of the 3 included dNTPs (in 500 mM Tris, pH 7.8, + 50 mM $MgCl_2$ + 100 mM 2-mercaptoethanol + 100 μg/mL bovine serum albumin)

solution A1: no dATP; contains dCTP, dGTP, dTTP
solution A2: no dCTP; contains dATP, dGTP, dTTP
solution A3: no dGTP; contains dATP, dCTP, dTTP
solution A4: no dTTP; contains dATP, dCTP, dGTP
solution A5: no dCTP, no dGTP; contains dATP, dTTP

DNA polymerase I–DNase I (solution C from kit): 0.4 Units/μL DNA polymerase I, 40 pg/μL DNase I, 50 mM Tris, pH 7.5, 5 mM Mg-acetate, 1 mM 2-mercaptoethanol, 100 μM phenylmethylsulfunyl fluoride (proteinase inhibitor), 50% (v/v) glycerol,100 μg/ml bovine serum albumin

^{32}P-labeled dATP: [α-^{32}P]-deoxyadenosine 5'-triphosphate; 3000 Ci/mmol, 10 mCi/mL

Nick spin columns: Pharmacia

Sephadex G-50 (medium): Hydrate the Sephadex by steaming for 1 hour in 5 to 10 volumes of 10 mM Tris–1 mM EDTA, pH 8.0.

λ (phage lambda) DNA

Siliconized glass wool: Untreated glass will bind nucleic acids (consider the Geneclean procedure, which depends on this binding). Immerse in 5% (v/v) dichlorodimethylsilane for 5 minutes, rinse thoroughly with distilled water, and dry. Rinse again with distilled water, then bake at 230°C.

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F. HYBRIDIZATION AND WASHING OF SOUTHERN BLOTS

Introduction

Detection of a specific DNA with a labeled probe involves 4 steps: blocking, hybridization, washes, and detection. Blocking prevents labeled probe, when it is added later, from binding nonspecifically to the membrane. The extent of hybridization between probe and target DNA depends on the "Cot" value; "Co" is the concentration of the free probe at time zero, and "t" is time. To keep Co high, keep the volume as small as practical. Washes remove probe not bound specifically to target sequences. By altering the temperature and salt concentrations of the final washes, we can control the amount of mismatch permitted between probe and target. High-stringency washes (low salt, high temperature) permit fewer mismatches than low-stringency washes.

Technical Tips

Handle blots at an edge with forceps. Bring wash solutions to temperature before use; correct wash temperatures are important.

Protocol

1. Blocking: treat the membrane at 42°C with 10 mL of hybridization solution. Use a sealed plastic tray or a hybridization tube. Agitate gently for 1 hour.
2. Add 100 to 200 ng of denatured probe DNA (10^6 to 10^7 cpm); mix thoroughly. Incubate with gentle agitation

overnight at 42°C. (To denature probe DNA, boil for 3 minutes and then chill in ice water.)

3. Remove hybridization solution and wash the membrane (with constant agitation) twice for 5 minutes with 25 mL of 2× SSC at 42°C.

4. Wash membrane 3 times for 20 minutes (each) with 25 mL of 2× SSC + 1% SDS at 65°C.

5. Wash the membrane 3 times for 20 minutes (each) with 25 mL of 0.1× SSC + 1% SDS at 42°C.

6. Seal the membrane in plastic wrap while it is still damp. This will allow you to later strip the probe from the membrane and rehybridize with a different probe. (If Gene Screen dries completely, the probe may bind irreversibly and delay rehybridization studies.)

7. Under a safelight, load the filter into a film cassette, set a sheet of Kodak XAR X-ray film on top, and place an intensifying screen (Dupont Cronex) over both. Expose the film for 1 to 7 days at −80°C. Exposure time will vary with the specific activity of the probe and amount of probe bound to the target.

   Alternatively, place the plastic-wrapped blot on a Phosphor Imager screen.

Solutions for Hybridization and Washing of Southern Blots

Hybridization solution: 50% formamide, 1% SDS, 1 *M* NaCl, 10% dextran sulfate, 0.01% calf thymus DNA (to make 500 mL: 250 mL formamide + 5 g SDS + 29.2 g NaCl + 50 g dextran sulfate + 5 mL of 10 mg/mL denatured, sonicated calf thymus DNA + 175 mL H_2O to bring to 500 mL)

Calf thymus DNA: dissolve 100 mg DNA in 10 mL distilled water, sonicate for 2 minutes to shear, boil for 3 minutes, then chill on ice.

20× SSC (3 *M* NaCl + 0.3 *M* sodium citrate, pH 7.0): 350.4 g NaCl + 176.5 g sodium citrate·2 H_2O + 7.2 mL concentrated HCl + distilled water to final volume of 2 L

Notes

EXERCISE 5. STUDY QUESTIONS

1. In pictures of the agarose gels showing *A. tumefaciens* genomic DNA digested with *Eco*RI, there are 2 bright bands in most lanes at the lower section of the gel. What do these bands represent?

2. Why does the DNA appear to migrate as a smear rather than as distinct restriction fragments?

3. Before blotting, you soaked this gel in acid and then in base. Why?

4. How could you check whether the DNA transferred to the membrane during the blotting procedure?

5. Which wash is the stringent wash? Why?

6. Will the length of time you perform your stringent wash affect the results? Why or why not?

7. Group the *A. tumefaciens* strains according to their *virD2* RFLPs.