EXERCISE

6

Northern Blot Detection of mRNA

Background

We use Northern blot analysis to estimate the amount and molecular weight of a specific messenger RNA (mRNA) within a preparation of total RNA. The Northern blot procedure measures mRNA accumulation, which reflects both the rate of transcription and the stability of the RNA in question. A hybridization probe can detect a particular mRNA among a population of RNAs separated by electrophoresis and transferred to a membrane.

To demonstrate the Northern blot technique, you will examine expression of the *RbsS* gene in tobacco leaves. This light-regulated nuclear gene encodes the small subunit of the photosynthetic enzyme, RuBisCO. The large subunit of the enzyme is encoded by chloroplast DNA. The RuBisCO holoenzyme catalyzes the addition of CO_2 to ribulose 1,5-bisphosphate, the first step in the Calvin cycle. *RbsS* mRNA is the most abundant message in tobacco leaves, making it easily detectable by Northern blot analysis.

The most difficult part of a Northern blot is preparing undegraded RNA. RNases are ubiquitous and highly stable; they are found in tissue from which RNA is extracted, as well as on human skin. RNases are not destroyed by autoclaving, and are resistant to metal chelating agents. To prevent RNase contamination from fingers, handle all equipment with gloves. During RNA extraction, grind leaves in liquid nitrogen and keep them frozen until they are in phenol or another denaturant.

To eliminate RNases from glassware and solutions, treat with diethylpyrocarbonate (DEPC). Use DEPC at 0.1% (v/v) for at least 10 hours; DEPC and reaction products (CO_2 and H_2O) can then be removed from reagents by autoclaving or heating to 60 to 80°C. DEPC is incompatible with Tris buffer; use MOPS buffer or make Tris buffer from an unopened bottle of Tris and DEPC-treated water. Baking at 230°C eliminates RNases from glassware. DEPC

will destroy polycarbonate and polystryrene (e.g., electrophoresis tanks). To decontaminate them, soak in 3% hydrogen peroxide for 10 minutes; remove peroxide by rinsing in DEPC-treated water.

Steps of the experiment are

A. Prepare RNA from tobacco

B. Run agarose–formaldehyde gel electrophoresis

C. Denature and blot RNA

D. Prepare probe by nick translation

E. Hybridize and wash Northern blot

Safety Precautions

DEPC may be carcinogenic; use gloves. DEPC solution will build up pressure in its storage bottle if it contacts water. Always use a clean dry pipet tip when removing DEPC from the stock bottle, and open DEPC stock bottles in a fume hood.

Notes

A. PREPARATION OF RNA FROM TOBACCO LEAVES

Introduction

Methods of isolating RNA must inhibit endogenous RNases and deproteinize the RNA. The most common procedures include either phenol extraction followed by ethanol precipitation, or the use of some other strong denaturant such as guanidine hydrochloride or guanidinium isothiocyanate. When separated by gel electrophoresis, the ribosomal RNA (rRNA) bands provide a visual measure of the yield and purity of the RNA preparation. mRNAs, which have 3' polyA tails, can be separated from ribosomal and transfer RNAs (tRNAs) by oligo(dT) cellulose chromatography.

To prepare total RNA (mRNA + rRNA + tRNA) from tobacco leaves, freeze tobacco leaves in liquid nitrogen and grind with a mortar and pestle. Extract with phenol, and precipitate RNA with lithium chloride.

Freeze and grind 4 leaf samples; each of these will be divided into 3 portions.

Safety Precaution

Use eye and skin protection when using liquid nitrogen and phenol.

Technical Tip

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; insert a pipet into the phenol layer beneath the buffer.

Procedure:

1. Chill a mortar and pestle in the freezer.
2. Weigh fresh leaf tissue approximately the size of a quarter and grind to a **fine** powder in liquid nitrogen.
3. Pour into a 15-mL disposable centrifuge tube; solution may spatter, so wear eye protection.
4. Allow liquid nitrogen to boil off completely, then immediately add 2.5 mL RNA extraction buffer.
5. Immediately add 2.5 mL phenol:chloroform 1:1. Vortex for 1 minute.
6. While solution is still homogeneous, divide into three 1.5-mL microfuge tubes; discard remainder.
7. Centrifuge at top speed for 10 minutes.
8. Remove 500 μL of the aqueous (upper) phase from each of the 3 tubes and combine; be careful to leave the interphase behind. Divide into two 1.5-mL microfuge tubes and add an equal volume of 4 *M* LiCl to each.
9. Precipitate RNA overnight at −20°C.
10. Centrifuge tubes at top speed for 10 minutes.
11. Remove supernatant and dissolve pellets in 100 to 200 μL DEPC-treated ddH_2O. Combine.
12. Add 0.4 (sample) volumes of 5 *M* ammonium acetate and 2.5 (final) volumes cold 95% ethanol.
13. Precipitate RNA at −20°C for 2 hours.
14. Centrifuge at top speed for 10 minutes.
15. Decant supernatant. Wash pellet with cold 70% ethanol. Air dry pellet. Dissolve in 20 μL DEPC-treated water.

16. Quantify RNA by measuring absorbance at 260 nm ($A_{260\ nm}$ × dilution factor × 40 = μg/mL). Measure a 1:100 dilution in 100 μL.

Solutions for Preparation of RNA from Tobacco Leaves

Extraction buffer: 100 mM LiCl, 1% SDS, 100 mM Tris, pH 9.0, 10 mM EDTA (do not treat with DEPC; the tissue contains RNase, so the extraction buffer need not be RNase free)

Phenol: (equilibrate with Tris base, pH 7.5; purchase high-quality phenol or redistill it; store frozen at −20°C; should be colorless)

Chloroform

4 *M* LiCl: treat with DEPC, then autoclave

5 *M* Ammonium acetate

70% Ethanol

Distilled water: treat with DEPC, then autoclave

Liquid nitrogen

Notes

B. AGAROSE–FORMALDEHYDE GEL ELECTROPHORESIS

Introduction

Single-stranded nucleic acids such as mRNAs fold into secondary structures (under nondenaturing conditions) that affect their electrophoretic mobilities. Thus we cannot determine the molecular weights of RNA molecules by agarose gel electrophoresis unless the RNA is denatured. Formamide, formaldehyde, and high temperatures are used in sample preparation, electrophoresis buffers, and gels to keep RNAs denatured. Otherwise, agarose gel electrophoresis and blotting techniques are similar to those used for DNA.

Technical Tips

With gloved hands, wipe gel apparatus, combs, gel casting trays, and plastic boxes with RNase Away solution before use. Prepare a supply of baked or DEPC-treated glassware and equipment. To avoid contaminating your RNAs and the RNA size standard, use aerosol-resistant (plugged) tips for your Pipetman.

Procedure

1. Heat a 1% agarose–water suspension (in microwave) until thoroughly dissolved. Hold at 60°C. 1% agarose = 0.5 g/50 mL final volume for minigel. Weigh agarose, using a baked spatula, into DEPC-treated beaker; use a 25-mL disposable pipet to add 43.5 mL of DEPC-treated distilled water; other solutions will be added in the next step.

2. Add 5.0 mL of 10× MOPS buffer and 1.5 mL of 37% formaldehyde, then pour the gel (50 mL total).

3. Combine: 1 to 10 μg of RNA sample (in 5.6 μL)
   2.5 μL of 10× MOPS
   4.4 μL of 37% formaldehyde
   12.5 μL of formamide

4. Incubate at 55°C for 15 minutes.

5. Microfuge sample to bottom of tube, add 5 μL of RNA loading buffer, mix, and load gel. Use 2 size standards: 1) 5 μg of BRL low-range RNA standards, mixed with MOPS, formaldehyde, and formamide and heated as above. **Load in outside lane and leave an empty lane between this marker and RNA**—you will cut this off your gel after it has been photographed; 2) mix 5 μL of a 100 ng/μL solution of the *Hind*III-cut lambda DNA with 1 μL 6× load buffer and load in an outside lane.

6. Apply 50 V (5 V/cm) until bromphenol blue dye migrates halfway down the gel; RNA will migrate toward the positive electrode. Electrophoresis buffer is 1× MOPS.

7. Rinse gel 3× with 200 mL water to remove formaldehyde; 6 minutes total.

Solutions for Agarose–Formaldehyde Gel Electrophoresis

10× MOPS buffer: Add 41.8 g MOPS [3-(*N*-morpholino)-propanesulfonic acid] to 800 mL DEPC-treated water; adjust to pH 7.0 (with NaOH or acetic acid as needed). Add 16.6 mL of 3 *M* DEPC-treated sodium acetate, pH 5.2 + 20 mL of 0.5 *M* DEPC-treated EDTA, pH 8.0. Bring to 1 L final volume with DEPC-treated water.

6× RNA loading buffer: 1 mM EDTA, pH 8.0 + 0.25% bromphenol blue + 50% glycerol

37% Formaldehyde

Formamide: To deionize, mix 100 mL formamide with 5 g of AG 501-X8(D) mixed bed resin (Bio-Rad); stir 30 minutes at room temperature, then filter. Freeze at −20°C or prepare fresh daily.

BRL RNA molecular weight markers

*Hind*III-cut lambda DNA

RNase Away solution

Notes

C. NORTHERN BLOT: DENATURATION AND BLOTTING OF RNA

Procedure

1. Soak the gel with agitation in 250 mL of 50 mM NaOH for 30 minutes.
2. Rinse the gel with distilled water.
3. Neutralize for 30 minutes in Tris-NaCl neutralization buffer; place the gel in 10× SSC.
4. Cut Gene Screen Plus to exact size of gel. Wear gloves and use the liner sheet to keep the nylon membrane clean. Mark one corner of 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 contacts 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 bub-

bles. 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 RNA transfer to continue for 2 to 16 hours. Transfer is complete when the gel becomes 1 mm thick.

12. Rinse membrane in 2× SSC for 5 minutes at room temperature with agitation. Place filter (pencil mark [RNA] side up) on dry Whatman 3 MM paper. Just as the filter begins to dry, irradiate it with 1200 μJ of UV light (use the Stratagene 1800 Stratalinker). This links the RNA permanently to the membrane.

13. Soak membrane in 5% acetic acid for 15 minutes.

14. Soak the membrane in 0.5 *M* sodium acetate + 0.04% methylene blue for 5 to 10 minutes. Rinse with DEPC-treated water until bands appear.

Solutions for Denaturation and Blotting of RNA

50 mM NaOH

Tris-NaCl neutralization buffer (0.5 *M* Tris, pH 7.5 + 1.5 *M* NaCl): 121.1 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

5% Acetic acid

0.5 *M* Sodium acetate + 0.04% methylene blue

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D. PROBE PREPARATION

Introduction

You will use a nick translation kit from GIBCO/BRL to ^{32}P-label the pUC18-based plasmid, pRbcS, which contains gene for the small subunit of RuBisCO. To separate unincorporated label from labeled probe DNA, 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 (pRbcS)
   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 phenylmethylsulfonyl 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.

Siliconized glass wool: Untreated glass will bind nucleic acids (consider the Gene Clean 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.

λ (phage lambda) DNA

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E. HYBRIDIZATION AND WASHING OF NORTHERN BLOTS

Introduction

Detection of a specific DNA with a radiolabeled probe involves 4 steps: blocking, hybridization, washes, and autoradiography.

Safety Precautions

Wear gloves and lab coat. Limit exposure to radioactivity. Dispose of radioactive washes properly.

Protocol

1. Prehybridization: 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); 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 Northern 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 (or salmon sperm) 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

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EXERCISE 6. STUDY QUESTIONS

1. Identify bands from the total RNA extract on the photograph of the agarose–formaldehyde gel.

2. Estimate the molecular weight of the RuBisCO mRNA. Measure the distance each molecular weight marker migrated, and plot on semilog paper. Measure the distance RuBisCO mRNA migrated, and use the semilog plot to estimate its molecular weight.

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