Analysis of RNA by Northern and Slot Blot Hybridization

Specific sequences in RNA preparations can be detected by blotting and hybridization analysis using techniques very similar to those originally developed for DNA (*UNITS 2.9A, 2.9B, & 2.10*). Fractionated RNA is transferred from an agarose gel to a membrane support (northern blotting); unfractionated RNA is immobilized by slot or dot blotting. The resulting blots are studied by hybridization analysis with labeled DNA or RNA probes. Northern blotting differs from Southern blotting largely in the initial gel fractionation step. Because they are single-stranded, most RNAs are able to form secondary structures by intramolecular base pairing and must therefore be electrophoresed under denaturing conditions if good separations are to be obtained. Denaturation is achieved either by adding formaldehyde to the gel and loading buffers or by treating the RNA with glyoxal and dimethyl sulfoxide (DMSO) prior to loading. Basic Protocol 1 describes blotting and hybridization of RNA fractionated in an agarose-formaldehyde gel. This is arguably the quickest and most reliable method for northern analysis of specific sequences in RNA extracted from eukaryotic cells. Alternate Protocol 1 gives details of the glyoxal/DMSO method for denaturing gel electrophoresis, which may provide better resolution of some RNA molecules. Alternate Protocol 2 describes slot-blot hybridization of RNA samples, a rapid method for assessing the relative abundance of an RNA species in extracts from different tissues. Stripping hybridization probes from blots can be done under three different sets of conditions; these methods are outlined in the Support Protocol.

Analysis of small noncoding RNA (microRNA, or miRNA) has received much attention as a new tool for analyzing gene expression. Because these miRNAs range from 20 to 30 nucleotides, traditional agarose gels will not separate the products adequately. Basic Protocol 2 describes a hybridization procedure using a polyacrylamide gel, adapted for these small RNAs. Alternate Protocol 3 describes a hybridization procedure for miRNAs that uses a non-formamide-containing hybridization solution.

NOTE: The ubiquity of contaminating RNases in solutions and glassware and the concomitant difficulties in ensuring that an RNA preparation remains reasonably undegraded throughout the electrophoresis, blotting, and hybridization manipulations can make it difficult to obtain good hybridization signals with RNA. To inhibit RNase activity, all solutions for northern blotting should be prepared using sterile deionized water that has been treated with diethylpyrocarbonate (DEPC) as described in *UNIT 4.1*. The precautions described in the introduction to Section I of this chapter (e.g., baking of glassware) should be followed religiously. In addition, RNA should not be electrophoresed in gel tanks previously used for DNA separations—a new tank plus accessories should be obtained and saved exclusively for RNA work. For full details on the establishment of an RNase-free environment, see Wilkinson (1991).

CAUTION: DEPC is a suspected carcinogen and should be handled carefully. Because DEPC reacts with ammonium ions to produce ethyl carbamate, a potent carcinogen, special care should be exercised when treating ammonium acetate solution with DEPC.

CAUTION: Investigators should wear gloves for all procedures involving radioactivity and should be careful not to contaminate themselves or their clothing. When working with ^{32}P, investigators should frequently check themselves and the working area for radioactivity using a hand-held radiation monitor. Any radioactive contamination should be cleaned up using appropriate procedures. Radioactive waste should be placed in appropriately designated areas for disposal. Follow the guidelines provided by the local radiation safety adviser; also see *APPENDIX 1F*.

Contributed by Terry Brown, Karol Mackey, and Tingting Du

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BASIC PROTOCOL 1

NORTHERN HYBRIDIZATION OF RNA FRACTIONATED BY AGAROSE-FORMALDEHYDE GEL ELECTROPHORESIS

The protocol is divided into three sections: electrophoresis of an RNA preparation under denaturing conditions in an agarose-formaldehyde gel, transfer of the RNA from the gel to a nylon or nitrocellulose membrane by upward capillary transfer, and hybridization analysis of the RNA sequences of interest using a labeled DNA or RNA probe. Hybridization is carried out in formamide solution, which permits incubation at a relatively low temperature, reducing degradation of the membrane-bound RNA. Nitrocellulose and nylon membranes are equally effective for northern hybridization analysis, although high backgrounds are likely with nylon membranes if the protocol is not followed carefully.

This protocol should be read in conjunction with *UNITS 2.9A & 2.10*, which describe the equivalent Southern procedures for DNA blotting and hybridization. Details of alternative transfer systems (upward capillary blots, electroblotting, and vacuum blotting) can be found in *UNIT 2.9A*. Modifications to the Southern hybridization procedure described in the Commentary to *UNIT 2.10* can also be used with northern blots, and the troubleshooting guide for DNA blotting and hybridization is also applicable to northern analysis. Other relevant units are located elsewhere in the manual: *UNIT 2.5A* covers the general features of agarose gel electrophoresis; *UNITS 3.18 & 3.19* describe the preparation of alternate nonradioactive probes and their use in hybridization analysis; and *UNIT 6.4* explains how to use labeled oligonucleotides as hybridization probes.

Materials

10× and 1× MOPS running buffer (see recipe for 10× buffer)
12.3 M (37%) formaldehyde, pH >4.0
RNA sample: total cellular RNA (*UNITS 4.1-4.4*) or poly(A)$^+$ RNA (*UNIT 4.5*)
Formamide
Formaldehyde loading buffer (see recipe)
0.5 µg/ml ethidium bromide in 0.5 M ammonium acetate *or* 10 mM sodium phosphate (pH 7.0; see recipe)/1.1 M formaldehyde with and without 10 µg/ml acridine orange
0.05 M NaOH/1.5 M NaCl (optional)
0.5 M Tris·Cl (pH 7.4; *APPENDIX 2*)/1.5 M NaCl (optional)
20×, 2×, and 6× SSC (*APPENDIX 2*)
0.03% (w/v) methylene blue in 0.3 M sodium acetate, pH 5.2 (optional)
DNA suitable for use as probe *or* for in vitro transcription to make RNA probe (Table 2.10.1)
Formamide prehybridization/hybridization solution (*UNIT 2.10*)
2× SSC/0.1% (w/v) SDS
0.2× SSC/0.1% (w/v) SDS, room temperature and 42°C
0.1× SSC/0.1% (w/v) SDS, 68°C

55°, 60°, and 100°C water baths
Oblong sponge slightly larger than the gel being blotted
RNase-free glass dishes (*UNIT 4.1*)
Whatman 3MM filter paper sheets
UV-transparent plastic wrap (e.g., Saran Wrap or other polyvinylidene wrap)
Nitrocellulose or nylon membrane (see Table 2.9.1 for list of suppliers)
Glass plate of appropriate size (Fig. 2.9.1)
Vacuum oven
UV transilluminator, calibrated (*UNIT 2.9A*)
Hybridization oven (e.g., Hybridiser HB-1, Techne) and tubes

Additional reagents and equipment for agarose gel electrophoresis (*UNIT 2.5A*), radiolabeling of DNA by nick translation or random oligonucleotide priming (*UNIT 3.5*), RNA labeling by in vitro synthesis (*UNIT 2.10*), measuring specific activity of labeled nucleic acids and separating unincorporated nucleotides from labeled nucleic acids (*UNIT 3.4*), and autoradiography (*APPENDIX 3A*)

NOTE: All solutions should be prepared with sterile deionized water that has been treated with DEPC as described in *UNIT 4.1*; see unit introduction for further instructions and precautions regarding establishment of an RNase-free environment.

Prepare gel

1. Dissolve 1.0 g agarose in 72 ml water and cool to 60°C in a water bath (see *UNIT 2.5A*).

   UNIT 2.5A provides details on preparing, pouring, and running the agarose gel; vary as described here.

   This step will make a 1.0% gel, which is suitable for RNA molecules 500 bp to 10 kb in size. A higher-percentage gel (1.0 to 2.0%) should be used to resolve smaller molecules or a lower-percentage gel (0.7 to 1.0%) for longer molecules. The recipe may be scaled up or down depending on the size of gel desired; the gel should be 2 to 6 mm thick after it is poured and the wells large enough to hold 60 µl of sample.

2. When the flask has cooled to 60°C, place in a fume hood and add 10 ml of 10× MOPS running buffer and 18 ml of 12.3 M formaldehyde.

   CAUTION: *Formaldehyde is toxic through skin contact and inhalation of vapors. All operations involving formaldehyde should be carried out in a fume hood.*

   The formaldehyde concentration in the gel is 2.2 M. Lower concentrations (down to 0.4 M) may be used; these result in less brittle gels but may not provide adequate denaturation for runs longer than 2 to 3 hr.

3. Pour the gel and allow it to set. Remove the comb, place the gel in the gel tank, and add sufficient 1× MOPS running buffer to cover to a depth of ~1 mm.

Prepare sample and run gel

4. Adjust the volume of each RNA sample to 11 µl with water, then add:

   5 µl 10× MOPS running buffer
   9 µl 12.3 M formaldehyde
   25 µl formamide.

   Mix by vortexing, microcentrifuge briefly (5 to 10 sec) to collect the liquid, and incubate 15 min at 55°C.

   CAUTION: *Formamide is a teratogen and should be handled with care.*

5. Add 10 µl formaldehyde loading buffer, vortex, microcentrifuge to collect liquid, and load onto gel.

   0.5 to 10 µg of RNA should be loaded per lane (see Commentary). Duplicate samples should be loaded on one side of the gel for ethidium bromide or acridine orange staining.

6. Run the gel at 5 V/cm until the bromphenol blue dye has migrated one-half to two-thirds the length of the gel.

   This usually takes ~3 hr. Lengthy electrophoresis (>5 hr) is not recommended for northern transfers as this necessitates more formaldehyde in the gel (e.g., the recipe for a gel run overnight would be 1.0 g agarose, 60 ml water, 10 ml of 10× MOPS, and 30 ml of 12.3 M formaldehyde). Increasing the amount of formaldehyde causes the gel to become more brittle and prone to breakage during transfer and also increases the health hazard from volatilization of formaldehyde during electrophoresis.

Stain and photograph gel

7a. Remove the gel and cut off the lanes that are to be stained. Place this portion of the gel in an RNase-free glass dish, add sufficient 0.5 M ammonium acetate to cover, and soak for 20 min. Change solution and soak for an additional 20 min (to remove the formaldehyde). Pour off solution, replace with 0.5 µg/ml ethidium bromide in 0.5 M ammonium acetate, and allow to stain for 40 min.

If necessary (i.e., if background fluorescence makes it difficult to visualize RNA fragments), destain in 0.5 M ammonium acetate for up to 1 hr.

7b. Alternatively, remove gel, cut off lanes, and stain 2 min in 1.1 M formaldehyde/10 mM sodium phosphate containing 10 µg/ml acridine orange.

If necessary, destain 20 min in the same buffer without acridine orange.

8. Examine gel on a UV transilluminator to visualize the RNA and photograph with a ruler laid alongside the gel so that band positions can later be identified on the membrane.

Molecular weight markers are not usually run on RNA gels as the staining causes rRNA molecules present in cellular RNA to appear as sharp bands that can be used as internal markers. In mammalian cells, these molecules are 28S and 18S (corresponding to 4718 and 1874 nucleotides respectively; Fig. 4.9.1). Mitochondrial rRNAs (16S and 12S in mammalian cells) may also be visible in some extracts, and plant extracts usually contain chloroplast rRNAs (23S and 16S). Bacterial rRNAs are smaller than the eukaryotic nuclear counterparts (E. coli: 23S and 16S, 2904 and 1541 nucleotides) and in some species one or both of the molecules may be cleaved into two or more fragments. If poly(A)$^+$ RNA is being fractionated, commercial RNAs (e.g., 0.24- to 9.5-kb RNA ladder from Life Technologies) can be used as molecular weight markers.

Prepare gel for transfer

9. Place unstained portion of gel in an RNase-free glass dish and rinse with several changes of sufficient deionized water to cover the gel.

The rinses remove formaldehyde, which would reduce retention of RNA by nitrocellulose membranes and hinder transfer onto nylon. The portion of the gel that will be blotted is not stained with ethidium bromide as this can also reduce transfer efficiency.

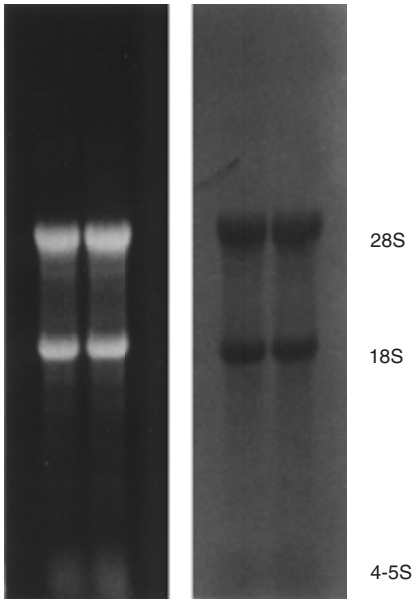

Figure 4.9.1 Rat liver RNA (5 µg) was electrophoresed on a formaldehyde 1% agarose gel containing ethidium bromide (left), transferred to a hybridization membrane and stained with methylene blue stain (Molecular Research Center; Herrin and Schmidt, 1988, right). Shown are 28S (4.7 kb) and 18S (1.9 kb) ribosomal RNAs, as well as 4S to 5S (0.10 to 0.15 kb) RNA containing mix of tRNA and 5S ribosomal RNA.

10. Add ~10 gel volumes of 0.05 M NaOH/1.5 M NaCl to dish and soak for 30 min. Decant and add 10 gel volumes of 0.5 M Tris·Cl (pH 7.4)/1.5 M NaCl. Soak for 20 min to neutralize.

    This step is optional. It results in partial hydrolysis of the RNA which in turn leads to strand cleavage; the length reduction improves transfer of longer molecules. However, RNA is extremely sensitive to alkaline hydrolysis and smaller molecules may be fragmented into lengths too short for efficient retention by the membrane. Neutralization should be carried out only if efficient transfer of molecules >5 kb is required from a gel that has an agarose concentration of >1.0% and is >5 mm thick.

11. Replace solution with 10 gel volumes of 20× SSC and soak for 45 min.

    This step is also optional but improves transfer efficiency with some brands of membrane.

Transfer RNA from gel to membrane

12. Place an oblong sponge slightly larger than the gel in a glass or plastic dish (if necessary, use two or more sponges placed side by side). Fill the dish with enough 20× SSC to leave the soaked sponge about half-submerged in buffer.

    Refer to Figure 2.9.1A for a diagram of the transfer setup. The sponge forms the support for the gel. Any commercial sponge will do, but before a sponge is used for the first time, it should be washed thoroughly with distilled water to remove any detergents that may be present. Two or more sponges can be placed side by side if necessary. As an alternative, a solid support with wicks made out of Whatman 3MM paper (Fig. 2.9.1B) may be substituted. Do not use an electrophoresis tank, as the high-salt transfer buffer will corrode the electrodes.

    If using a nylon membrane, a lower concentration of SSC (e.g., 10×) may improve transfer of molecules >4 kb; reduction of the SSC concentration is not recommended for a nitrocellulose membrane as the high salt is needed for retention of RNA.

13. Cut three pieces of Whatman 3MM paper to the same size as the sponge. Place them on the sponge and wet them with 20× SSC.

14. Place the gel on the filter paper and squeeze out air bubbles by rolling a glass pipet over the surface.

15. Cut four strips of plastic wrap and place over the edges of the gel.

    This is to prevent buffer from "short-circuiting" around the gel rather than passing through it.

16. Cut a piece of nylon or nitrocellulose membrane just large enough to cover the exposed surface of the gel. Pour distilled water ~0.5 cm deep in an RNase-free glass dish and wet the membrane by placing it on the surface of the water. Allow the membrane to submerge. For nylon membrane, leave for 5 min; for nitrocellulose membrane, replace the water with 20× SSC and leave for 10 min.

    Avoid handling nitrocellulose and nylon membranes even with gloved hands—use clean blunt-ended forceps instead.

17. Place the wetted membrane on the surface of the gel. Try to avoid getting air bubbles under the membrane; remove any that appear by carefully rolling a glass pipet over the surface.

18. Flood the surface of the membrane with 20× SSC. Cut five sheets of Whatman 3MM paper to the same size as the membrane and place on top of the membrane.

19. Cut paper towels to the same size as the membrane and stack on top of the Whatman 3MM paper to a height of ~4 cm.

20. Lay a glass plate on top of the structure and add a weight to hold everything in place. Leave overnight.

The weight should be sufficient to compress the paper towels to ensure good contact throughout the stack. Excessive weight, however, will crush the gel and retard transfer.

An overnight transfer is sufficient for most purposes. Make sure the reservoir of 20× SSC does not run dry during the transfer.

Prepare membrane for hybridization

21. Remove paper towels and filter papers and recover the membrane and flattened gel. Mark in pencil the position of the wells on the membrane and ensure that the up-down and back-front orientations are recognizable.

Pencil is preferable to pen, as ink marks may wash off the membrane during hybridization. With a nylon membrane only, the positions of the wells can be marked by slits cut with a razor blade (do not do this before transfer or the buffer will short-circuit). The best way to record the orientation of the membrane is by making an asymmetric cut at one corner.

22. Rinse the membrane in 2× SSC, then place it on a sheet of Whatman 3MM paper and allow to dry.

The rinse has two purposes: to remove agarose fragments that may adhere to the membrane and to leach out excess salt.

Immobilize the RNA and assess transfer efficiency

23a. *For nitrocellulose membranes:* Place between two sheets of Whatman 3MM filter paper and bake in a vacuum oven for 2 hr at 80°C.

Baking results in noncovalent attachment of RNA to the membrane; the vacuum is needed to prevent the nitrocellulose from igniting.

23b. *For nylon membranes:* Bake as described above *or* wrap the dry membrane in UV-transparent plastic wrap, place RNA-side-down on a UV transilluminator (254-nm wavelength), and irradiate for the appropriate length of time (determined as described in *UNIT 2.9A*, Support Protocol).

CAUTION: *Exposure to UV irradiation is harmful to the eyes and skin. Wear suitable eye protection and avoid exposure of bare skin.*

UV cross-linking is recommended for a nylon membrane as it leads to covalent attachment and enables the membrane to be reprobed several times. The membrane must be completely dry before UV cross-linking; check the manufacturer's recommendations, which may suggest baking for 30 min at 80°C prior to irradiation. The plastic wrap used during irradiation must be UV transparent—e.g., polyvinylidene (Saran Wrap). A UV light box (e.g., Stratagene Stratalinker) can be used instead of a transilluminator (follow manufacturer's instructions).

24. If desired, check transfer efficiency by either staining the gel in ethidium bromide or acridine orange as in steps 7 and 8 or (if using nylon membrane) staining the membrane in 0.03% (w/v) methylene blue in 0.3 M sodium acetate, pH 5.2, for 45 sec and destaining in water for 2 min.

If significant fluorescence is observed in the gel, not all the RNA has transferred. RNA bands on a nylon membrane will be stained by the methylene blue (Herrin and Schmidt, 1988).

Membranes can be stored dry between sheets of Whatman 3MM filter paper for several months at room temperature. For long-term storage they should be placed in a desiccator at room temperature or 4°C.

25. Prepare DNA or RNA probe labeled to a specific activity of $>10^8$ dpm/μg and with unincorporated nucleotides removed.

    Probes are ideally 100 to 1000 bp in length. DNA for a double-stranded probe is obtained as a cloned fragment (Chapter 1) and purified from the vector by restriction digestion (UNIT 3.1) followed by recovery from an agarose gel (UNIT 2.6). The DNA is labeled by nick translation or random oligonucleotide priming (UNIT 3.5) to create the radioactive probe. A single-stranded DNA probe is created in the same fashion but using a single-stranded vector; the probe should be antisense so it will hybridize to the sense RNA strands that are bound to the membrane. An RNA probe, which should also be antisense, is created by in vitro synthesis from a single-stranded sense DNA fragment (UNIT 2.10).

26. Wet the membrane carrying the immobilized RNA (from step 23) in 6× SSC.

27. Place the membrane RNA-side-up in a hybridization tube and add ~1 ml formamide prehybridization/hybridization solution per 10 cm^2 of membrane.

    Prehybridization and hybridization are usually carried out in glass tubes in a commercial hybridization oven. Alternatively, a heat-sealable polyethylene bag and heat-sealing apparatus can be used. The membrane should be placed in the bag, all edges sealed, and a corner cut off. Hybridization solution can then be pipetted into the bag through the cut corner and the bag resealed.

28. Place the tube in the hybridization oven and incubate with rotation 3 hr at 42°C (for DNA probe) or 60°C (for RNA probe).

    If using a bag, it can be shaken slowly in a suitable incubator or water bath. If using a nylon membrane, the prehybridization period can be reduced to 15 min.

29. If the probe is double-stranded, denature by heating in a water bath or incubator for 10 min at 100°C. Transfer to ice.

30. Pipet the desired volume of probe into the hybridization tube and continue to incubate with rotation overnight at 42°C (for DNA probe) or 60°C (for RNA probe).

    The probe concentration in the hybridization solution should be 10 ng/ml if the specific activity is 10^8 dpm/μg or 2 ng/ml if the specific activity is 10^9 dpm/μg.

    For denatured probe, add to hybridization tube as soon after denaturation as possible.

    If using a bag, a corner should be cut, the probe added, and the bag resealed. It is very difficult to do this without contaminating the bag sealer with radioactivity. Furthermore, the sealing element (the part that gets contaminated) is often difficult to clean. Hybridization in bags is therefore not recommended.

Wash membrane and perform autoradiography

31. Pour off hybridization solution and add an equal volume of 2× SSC/0.1% SDS. Incubate with rotation 5 min at room temperature, change wash solution, and repeat.

    CAUTION: *Hybridization solution and all wash solutions must be treated as radioactive waste and disposed of appropriately.*

    To reduce background, it may be beneficial to double the volume of the wash solutions. If using a bag, transfer the membrane to a plastic box for the washes.

32. Replace wash solution with an equal volume of 0.2× SSC/0.1% SDS and incubate 5 min with rotation at room temperature. Change wash solution and repeat (this is a low-stringency wash; see *UNIT 2.10* Commentary).

33. If desired, carry out two further washes using prewarmed (42°C) 0.2× SSC/0.1% SDS for 15 min each at 42°C (moderate-stringency wash).

34. If desired, carry out two further washes using prewarmed (68°C) 0.1× SSC/0.1% SDS for 15 min each at 68°C (high-stringency wash).

35. Remove final wash solution and rinse membrane in 2× SSC at room temperature. Blot excess liquid and cover in UV-transparent plastic wrap.

    Do not allow membrane to dry out if it is to be reprobed.

36. Perform autoradiography.

    If the membrane is to be reprobed, the probe can be stripped from the hybridized membrane without removing the bound RNA (see Support Protocol). Do not add NaOH. The membrane must not be allowed to dry out between hybridization and stripping, as this may cause the probe to bind to the matrix.

ALTERNATE PROTOCOL 1

NORTHERN HYBRIDIZATION OF RNA DENATURED BY GLYOXAL/DMSO TREATMENT

In this procedure denaturation of the RNA is achieved by treating samples with a combination of glyoxal and DMSO prior to running in an agarose gel made with phosphate buffer. The glyoxal/DMSO method produces sharper bands after northern hybridization than do formaldehyde gels, but is more difficult to carry out as the running buffer must be recirculated during electrophoresis.

Additional Materials *(also see Basic Protocol 1)*

10 mM and 100 mM sodium phosphate, pH 7.0 (see recipe)
Dimethyl sulfoxide (DMSO)
6 M (40%) glyoxal, deionized immediately before use (see recipe)
Glyoxal loading buffer (see recipe)
20 mM Tris·Cl, pH 8.0 (*APPENDIX 2)*

Apparatus for recirculating running buffer during electrophoresis
50° and 65°C water baths

NOTE: All solutions should be prepared with sterile deionized water that has been treated with DEPC as described in *UNIT 4.1*; see unit introduction for further instructions and precautions regarding establishment of an RNase-free environment.

Denature and carry out agarose gel electrophoresis

1. Prepare a 1.0% agarose gel by dissolving 1.0 g agarose in 100 ml of 10 mM sodium phosphate, pH 7.0. Cool to 60°C in a water bath, pour gel, and allow to set. Remove comb, place gel in gel tank, and add 10 mM sodium phosphate (pH 7.0) until gel is submerged to a depth of ~1 mm (see *UNIT 2.5A*).

   UNIT 2.5A provides details on preparing, pouring, and running the agarose gel; vary as described here.

   A 1.0% gel is suitable for RNA molecules 500 bp to 10 kb in size. A higher-percentage gel (1.0 to 2.0%) should be used to resolve smaller molecules or a lower percentage (0.7 to 1.0%) for longer molecules. The recipe may be scaled up or down depending on the size of gel desired; the gel should be 2 to 6 mm thick after it is poured and the wells large enough to hold 60 µl of sample.

2. Adjust volume of each RNA sample to 11 µl with water, then add:

   4.5 µl 100 mM sodium phosphate, pH 7.0
   22.5 µl DMSO
   6.6 µl 6 M glyoxal.

   Mix samples by vortexing, spin briefly (5 to 10 sec) in a microcentrifuge to collect the liquid, and incubate 1 hr at 50°C.

3. Cool samples on ice and add 12 µl glyoxal loading buffer to each sample. Load samples onto gel.

   0.5 to 10 µg of RNA should be loaded per lane (see Commentary). Duplicate samples should be loaded at one side of gel for ethidium bromide staining.

4. Run the gel at 4 V/cm with constant recirculation of running buffer for ~3 hr or until bromphenol blue dye has migrated one-half to two-thirds the length of the gel.

   Recirculation is needed to prevent an H^+ gradient forming in the buffer. If a gradient forms, the pH in parts of the gel may rise to >8.0, resulting in dissociation of the glyoxal from the RNA followed by renaturation. If no recirculation apparatus is available, electrophoresis should be paused every 30 min and the tank shaken to remix the buffer.

5. Remove the gel, cut off lanes, and stain with ethidium bromide (see Basic Protocol 1, steps 7a and 8).

   The RNA transfer (using the remaining portion of the gel) should be set up as soon as the gel is cut, before starting the staining.

Carry out northern transfer and hybridization analysis

6. Transfer RNA (see Basic Protocol 1, steps 9 to 24).
7. Immediately before hybridization, soak the membrane in 20 mM Tris·Cl (pH 8.0) for 5 min at 65°C to remove glyoxal.
8. Continue with hybridization analysis (see Basic Protocol 1, steps 25 to 36).

NORTHERN HYBRIDIZATION OF UNFRACTIONATED RNA IMMOBILIZED BY SLOT BLOTTING

ALTERNATE PROTOCOL 2

RNA slot blotting is a simple technique that allows immobilization of unfractionated RNA on a nylon or nitrocellulose membrane. Hybridization analysis is then carried out to determine the relative abundance of target mRNA sequences in the blotted samples. The technique is based on the DNA dot- and slot-blotting procedure (*UNIT 2.9B*), the main difference being the way in which the samples are denatured prior to immobilization.

RNA dot blots can be prepared by hand but slot blots constructed using a manifold apparatus are preferable because the slots make it easier to compare hybridization signals by densitometry scanning.

Additional Materials *(also see Basic Protocol 1)*

0.1 M NaOH
10× SSC (*APPENDIX 2*)
20× SSC (*APPENDIX 2*), room temperature and ice-cold
Denaturing solution (see recipe)
100 mM sodium phosphate, pH 7.0 (see recipe)
Dimethyl sulfoxide (DMSO)
6 M (40%) glyoxal, deionized immediately before use (see recipe)
Manifold apparatus with a filtration template for slot blots (e.g., Bio-Rad Bio-Dot SF, Schleicher and Schuell Minifold II)
50° and 60°C water baths

NOTE: All solutions should be prepared with sterile deionized water that has been treated with DEPC as described in *UNIT 4.1*; see unit introduction for further instructions and precautions regarding establishment of an RNase-free environment.

Set up membrane for transfer

1. Clean the manifold with 0.1 M NaOH and rinse with distilled water.
2. Cut a piece of nylon or nitrocellulose membrane to the size of the manifold. Pour 10× SSC (for nylon membrane) or 20× SSC (for nitrocellulose membrane) into a glass dish; place membrane on top of liquid and allow to submerge. Leave for 10 min.

   Avoid handling nitrocellulose and nylon membranes even with gloved hands—use clean blunt-ended forceps instead.

3. Place the membrane in the manifold. Assemble the manifold according to manufacturer's instructions and fill each slot with 10× SSC. Ensure there are no air leaks in the assembly.

Denature RNA samples

4a. Add 3 vol denaturing solution to RNA sample. Incubate 15 min at 65°C, then place on ice.

   Up to 20 µg of RNA can be applied per slot. Total cellular RNA (UNITS 4.1-4.4) or poly(A)$^+$ RNA (UNIT 4.5) can be used, although the latter is preferable (see Commentary).

4b. Alternatively, mix:

   11 µl RNA sample
   4.5 µl 100 mM sodium phosphate, pH 7.0
   22.5 µl DMSO
   6.6 µl 6 M glyoxal.

   Mix by vortexing, spin briefly in a microcentrifuge to collect liquid, and incubate 1 hr at 50°C.

5. Add 2 vol ice-cold 20× SSC to each sample.

Pass samples through manifold

6. Switch on the suction to the manifold device and allow the 10× SSC added in step 3 to filter through. Leave the suction on.

   The suction should be adjusted so that 500 µl buffer takes ~5 min to pass through the membrane. Higher suction may damage the membrane. Slots that are not being used can be blocked off by placing masking tape over them or by applying 500 µl of 3% (w/v) gelatin to each one. The former method is preferable as use of gelatin may lead to a background signal after hybridization. Alternatively, keep all slots open and apply 10× SSC instead of sample to the slots not being used.

7. Load each sample to the slots and allow to filter through, being careful not to touch the membrane with the pipet tip.
8. Add 1 ml of 10× SSC to each slot and allow to filter through. Repeat.
9. Dismantle the apparatus, place the membrane on a sheet of Whatman 3MM paper, and allow to dry.

Immobilize RNA and carry out hybridization

10. Immobilize the RNA (see Basic Protocol 1, step 23).

    If glyoxal/DMSO denaturation has been used, immediately before hybridization soak the membrane in 20 mM Tris·Cl (pH 8.0) for 5 min at 65°C to remove glyoxal.

11. Carry out hybridization analysis as described in steps 25 to 36 of Basic Protocol 1.

REMOVAL OF PROBES FROM NORTHERN BLOTS

SUPPORT PROTOCOL

Hybridization probes can be removed from northern blots on nylon membranes without damage to the membrane or loss of the transferred RNA. Some probes (particularly RNA probes) are more resistant to stripping. In these cases, higher temperatures, longer incubation periods, or the inclusion of formamide may be necessary for complete probe removal. The following stripping procedures are appropriate for both radioactive and chemiluminescent probes. Begin with the mildest conditions (step 1a) and monitor results to determine the extent of stripping. If the hybridization signal is still evident, proceed with the more stringent treatments (steps 1b and 1c) until stripping is complete.

Materials

Northern hybridization membrane containing probe (see Basic Protocol 1, Alternate Protocol 1, or Alternate Protocol 2)
Stripping solution (see recipe)

Hybridization bags
65°, 80°, or 100° (boiling) water bath
UV-transparent plastic wrap (e.g., Saran Wrap or other polyvinylidene wrap)

Additional reagents and equipment for autoradiography (*APPENDIX 3A*)

CAUTION: If hybridization probes include a radioactive label, dispose of stripping solutions as radioactive waste. Observe appropriate caution when working with the toxic compound formamide.

1a. *To remove probes at 80°C:* Place membrane in a hybridization bag containing stripping solution without formamide. Place bag in water preheated to 80°C for 5 min. Pour out solution, then repeat this washing process three to four times.

Add sufficient solution to cover the membrane completely when using a bag; alternatively, stripping can be done in an open container, again with sufficient solution to cover the membrane.

1b. *To remove probes at 100°C:* Place membrane in a hybridization bag containing stripping solution without formamide. Place bag in boiling water for 5 min. Pour out solution, then repeat this washing process three to four times.

1c. *To remove probes with formamide:* Place membrane in a hybridization bag containing stripping solution with formamide. Place bag in water preheated to 65°C for 5 min. Pour out solution, then repeat this washing process three times using stripping solution with formamide and once using stripping solution without formamide.

2. Place membrane on filter paper to remove excess solution. Wrap membrane in plastic wrap and perform autoradiography to verify probe removal.

If a chemiluminscent probe was used, verify probe removal by chemiluminscent detection (UNIT 3.19). The membrane may be immediately rehybridized or air-dried and stored for future use.

NORTHERN HYBRIDIZATION OF SMALL RNA FRACTIONATED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

BASIC PROTOCOL 2

This protocol is adapted for analysis of small RNAs. The major differences between this procedure and the traditional northern hybridization procedure are the fractionation system and the transfer system applied. Fractionation using denaturing polyacrylamide gel electrophoresis (PAGE) allows better separation of small RNAs. The introduction of a semidry transfer system reduces the time of the experimental procedure to 2 days.

Tissue or cell samples
TRIzol reagent (Invitrogen)
RNase-free H_2O (*UNIT 4.1*)
15% denaturing polyacrylamide sequencing (urea/TBE) gel (*UNIT 7.6*)
0.5× TBE electrophoresis buffer (*APPENDIX 2*)
Formamide loading dye (see recipe)
2× SSC (*APPENDIX 2*)
50 µM probe oligonucleotide (DNA or RNA; *UNIT 2.11*) in RNase-free H_2O
≥10 mCi/ml [γ-^{32}P]ATP (6000 Ci/mmol; ICN Biomedicals)
10× T4 polynucleotide kinase buffer (New England Biolabs)
200 U/µl T4 polynucleotide kinase (New England Biolabs)
Prehybridization/hybridization solution (see recipe), prewarmed to 37°C
2× SSC (*APPENDIX 2*) containing 0.1× (w/v) SDS, prewarmed to 37°C

95°C heating block or water bath
Hybond N+ Nylon Transfer Membrane (Amersham Biosciences)
Extra-thick blotting paper (Bio-Rad), slightly larger than the gel being blotted
Semi-dry transfer apparatus (e.g., Bio-Rad Trans-Blot SD cell)
Sephadex G-25 spin column
Hybridization oven with rotating glass hybridization bottles, 37°C
Image-analysis software (also see *UNIT 10.5*): e.g., QuantityOne (Bio-Rad) or ImageGauge (Fuji)

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (*UNIT 7.6*), phosphor imaging (*APPENDIX 3A*), and digital electrophoresis analysis (*UNIT 10.5*)

Prepare RNA sample and run the gel

1. Isolate total RNA from tissue or cell samples with TRIzol reagent according to the manufacturer's instructions. At the end of the procedure, dissolve the RNA to a final concentration of 10 µg/µl in RNase-free water.
2. Prerun 15% denaturing polyacrylamide sequencing (urea/TBE) gel for 15 min at 25 W in 0.5× TBE electrophoresis buffer. Mix 5 µl RNA sample (10 µg/µl) with equal volume of formamide loading dye. Heat 2 min at 95°C, and load onto gel (*UNIT 7.6*).
3. Run the gel at 25 W until the bromophenol blue dye has migrated to the bottom of the gel.

Transfer RNA from gel to membrane

4. Cut a piece of Hybond N+ nylon membrane slightly larger than the gel. Soak the membrane and four pieces of blotting paper of appropriate size in 0.5× TBE buffer for 10 min.
5. Stack two pieces of blotting paper on the anode platform of the transfer cell. Avoid getting air bubbles under or between the papers; remove any that appear by carefully rolling a glass pipet over the surface.
6. Place the membrane on top of the blotting paper and squeeze out air bubbles by rolling a glass pipet over the surface.
7. Carefully transfer the gel from glass plate to the top of the membrane and squeeze out air bubbles.
8. Stack another two pieces of blotting paper on the gel and squeeze out air bubbles.
9. Set the cathode assembly and the safety lid on the sandwich. Transfer for 1 hr at 300 mA.

Prepare membrane for hybridization

10. Disassemble the transfer cell. Remove the paper and the gel. Rinse the membrane in 2× SSC, then place it on a sheet of filter paper and allow it to air dry.

11. Place membrane RNA-side-down on a UV transilluminator (254-nm wavelength) or in a UV light box for the appropriate length of time to covalently attach the RNA to the membrane.

Prepare probe

The authors typically use a chemically synthesized 21-22 nt DNA or RNA oligonucleotide (see *UNIT 2.11* for oligonucleotide synthesis) perfectly complementary to the small RNA to be detected.

12. Set up the 5′-end-labeling reaction by combining the following reagents:

    1 µl of 50 µM probe oligonucleotide (DNA or RNA)
    1 µl of [γ- ^{32}P]ATP (6000 Ci/mmol, ≥10 mCi/ml)
    4 µl of 10× T4 polynucleotide kinase buffer
    H_2O to a final volume of 40 µl
    1 µl of 200 U/µl T4 polynucleotide kinase.

    Incubate reaction 1 hr at 37°C.

13. Pass reaction mixture through Sephadex G-25 spin column (centrifuging per manufacturer's instructions) to remove unincorporated [γ- ^{32}P]ATP.

Perform prehybridization and hybridization

14. Place membrane (from step 11) RNA-side-up in a hybridization bottle and add ~1 ml prewarmed (37°C) prehybridization/hybridization solution per 10 cm^2 of membrane. Place the bottle in a hybridization oven and incubate with rotation for 30 min at 37°C.

15. Pipet the entire reaction mix (from step 13) into the hybridization bottle and continue to incubate with rotation overnight at 37°C.

Wash membrane

16. Pour off hybridization solution and wash the membrane briefly with 2× SSC for 5 min. Add prewarmed (37°C) 2× SSC containing 0.1% SDS to the bottle. Incubate with rotation for 15 min at 37°C. Replace solution with fresh solution and repeat. Remove final wash solution, blot excess liquid, and wrap with plastic wrap.

    Do not allow membrane to dry out if it is to be reprobed.

Perform phosphor imaging and analyze the hybridization signals

17. Visualize the hybridization signals by phosphor imaging (*APPENDIX 3A*). Analyze hybridization result using appropriate software (*UNIT 10.5*). Subtract the background from the original signal to obtain the specific hybridization.

18. To compare the amount of small RNA in different samples, normalize the amount of small RNA detected to the nonspecific hybridization of the probe to 5S rRNA.

    Alternatively, the blot can be reprobed with a probe specific for 5S rRNA (for Drosophila 5′-CAA CAC GCG GTG TTC CCA AGC CG-3′) or, for Drosophila, the 2S RNA (5′-TAC AAC CCT CAA CCA TAT GTA GTC CAA GCA-3′).

    The precise amount (pmol or molecules) of small RNA can be determined if concentration standards of synthetic RNA are included on the blot. The assay is typically linear with respect to concentration over a 10,000-fold range.

ALTERNATE PROTOCOL 3

NORTHERN HYBRIDIZATION OF RNA USING CHURCH'S HYBRIDIZATION BUFFER

Church's hybridization buffer can be used as an alternative for the standard prehybrization/hybridization solution in this assay. It provides similar sensitivity.

Additional Materials *(also see Basic Protocol 2)*

Church's hybridization buffer without BSA (see recipe)

1. Perform northern blotting and prepare probe (see Basic Protocol 2, steps 1 through 13).
2. Perform prehybridization and hybridization (see Basic Protocol 2, steps 14 to 15) using Church's hybridization buffer (without BSA) in place of the prehybridization/hybridization solution.
3. Wash membrane and proceed with development and analysis (see Basic Protocol 2, steps 16 to 18).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Church's hybridization buffer without BSA

0.5 M sodium phosphate buffer, pH 7.2 (*APPENDIX 2*)
1 mM EDTA, pH 8.0 (*APPENDIX 2*)
7% (w/v) SDS
Store up to 1 year at room temperature

Denaturing solution

500 µl formamide
162 µl 12.3 M (37%) formaldehyde
100 µl MOPS buffer (see recipe)
Make fresh from stock solutions immediately before use

If formamide has a yellow color, deionize as follows: add 5 g of mixed-bed ion-exchange resin (e.g., Bio-Rad AG 501-X8 or X8(D) resins) per 100 ml formamide, stir 1 hr at room temperature, and filter through Whatman #1 filter paper.

CAUTION: *Formamide is a teratogen. Handle with care.*

Formaldehyde loading buffer

1 mM EDTA, pH 8.0 (*APPENDIX 2*)
0.25% (w/v) bromphenol blue
0.25% (w/v) xylene cyanol
50% (v/v) glycerol
Store up to 3 months at room temperature

Formamide loading dye

98% (v/v) deionized formamide
10 mM EDTA pH 8.0 (*APPENDIX 2*)
0.025% (w/v) xylene cyanol
0.025% (w/v) bromphenol blue
Store indefinitely at –20°C

Glyoxal, 6 M, deionized

Immediately before use, deionize glyoxal by passing through a small column of mixed-bed ion-exchange resin (e.g., Bio-Rad AG 501-X8 or X8(D) resins) until the pH is >5.0.

Glyoxal loading buffer

10 mM sodium phosphate, pH 7.0 (see recipe)
0.25% (w/v) bromphenol blue
0.25% (w/v) xylene cyanol
50% (v/v) glycerol
Store up to 3 months at room temperature

MOPS buffer

0.2 M MOPS [3-(*N*-morpholino)-propanesulfonic acid], pH 7.0
0.5 M sodium acetate
0.01 M EDTA
Store up to 3 months at 4°C

Store in the dark and discard if it turns yellow.

MOPS running buffer, 10×

0.4 M MOPS, pH 7.0
0.1 M sodium acetate
0.01 M EDTA
Store up to 3 months at 4°C

Prehybridization/hybridization solution

5× SSPE (see recipe)
5× Denhardt solution (*APPENDIX 2*)
50% (v/v) formamide
0.5% (w/v) SDS
72 µg/ml denatured herring sperm DNA (Promega)
Make fresh from stock solutions immediately before use

The herring sperm DNA is denatured by heating 10 min at 75°C just before it is added.

Sodium phosphate, pH 7.0, 100 mM and 10 mM

100 mM stock solution:
5.77 ml 1 M Na_2HPO_4
4.23 ml 1 M NaH_2PO_4
H_2O to 100 ml
Store up to 3 months at room temperature

10 mM solution:
Dilute 100 mM stock 1/10 with H_2O
Store up to 3 months at room temperature

SSPE, 10×

1.5 M NaCl
50 mM $NaH_2PO_4 \cdot H_2O$
5 mM EDTA
Store indefinitely at room temperature

Stripping solution

1% (w/v) SDS
0.1× SSC (*APPENDIX 2*)
40 mM Tris·Cl, pH 7.5 to 7.8 (*APPENDIX 2*)
Store up to 1 year at room temperature

Where formamide stripping is desired, prepare the above solution and add an equal volume of formamide just before use.

COMMENTARY

Background Information

The development of Southern blotting (*UNIT 2.9A*; Southern, 1975) was quickly followed by an equivalent procedure for the immobilization of gel-fractionated RNA (Alwine et al., 1977). The term northern blotting, initially used in a humorous fashion, has become enshrined in molecular biology jargon. Northern hybridization is a standard procedure for identification and size analysis of RNA transcripts and RNA slot blotting is frequently used to assess the expression profiles of tissue-specific genes (Kafatos et al., 1979).

Procedures for the removal of hybridization probes from northern blots are similar to those for Southern blots, except that NaOH is omitted to prevent hydrolysis of the RNA, and formamide may be included.

The recent discovery of microRNAs (miRNAs) revealed an entire new class of molecules that regulate gene expression. miRNAs are small noncoding RNAs that range from 20 to 30 nucleotides, making traditional formaldehyde-agarose gel electrophoresis unsuitable for their size fractionation. The modified northern protocol here (Basic Protocol 2) combines denaturing polyacrylamide gel electrophoresis (PAGE), which is ideal for the separation of small RNAs, with standard blotting and hybridization procedures.

Critical Parameters

Gel electrophoresis and northern blotting

The main distinction between northern and Southern blotting lies with the initial gel fractionation step. Because single-stranded RNA can form secondary structures, samples must be electrophoresed under denaturing conditions to ensure good separation.

A variety of denaturants for RNA gels have been used, including formaldehyde (Basic Protocol 1; Lehrach et al., 1977), glyoxal/DMSO (Alternate Protocol 1; Thomas, 1980), and the highly toxic methylmercuric chloride (Bailey and Davidson, 1976). Because of the substantial health risks, use of methylmercuric chloride is not advised. Formaldehyde gels are recommended, as they are easy to run and reasonably reliable. The formaldehyde must be rinsed from the gel before the transfer is set up, but this is a minor inconvenience compared to assembling the buffer recirculation system required for electrophoresis of glyoxal-denatured RNA.

Total cellular RNA (*UNITS 4.1-4.4*) or poly(A)$^+$ RNA (*UNIT 4.5*) can be used for northern transfers and slot blots. Total RNA is less satisfactory because nonspecific hybridization, however slight, to one or both of the highly abundant rRNA molecules will lead to a substantial hybridization signal. Any hybridizing band that appears in the vicinity of an rRNA should be treated with suspicion and its identity confirmed by blotting with poly(A)$^+$ RNA.

Under ideal conditions, a band that contains as little as 1 pg of RNA can be detected by northern hybridization with a probe labeled to a specific activity of 10^9 dpm/μg. In practice, the effective detection limit with an overnight exposure is ~5 pg RNA. An mRNA is usually considered to be abundant if it constitutes >1% of the mRNA fraction. In a typical mammalian cell, the mRNA fraction makes up about 0.5% of total RNA, so >5 pg of an abundant mRNA should be present in just 100 ng of total RNA. If 10 μg of total RNA is transferred, abundant mRNAs should give strong hybridization signals and less abundant ones (down to 0.01% of the mRNA population) should be detectable with an overnight exposure. For rarer molecules, the poly(A)$^+$ fraction must be prepared. In this sample 3 μg is sufficient for detecting an mRNA that makes up 0.0002% of the polyadenylated population.

Unlike probing for mRNA, which often requires enrichment by poly(A) selection prior to analysis, total cellular RNA can always be used for the detection of miRNA, because individual miRNA species can be present in thousands to tens of thousands of copies per cell. The highly abundant rRNA and microRNAs are well separated in a 15% denaturing polyacrylamide gel; thus, the nonspecific hybridization to rRNA will not affect the interpretation of the desired hybridizing signal.

RNA slot blots

Although easy to perform, RNA slot-blot hybridization is one of the most problematic techniques in molecular biology. A number of criteria must be satisfied if slot blotting is to be used to make meaningful comparisons of mRNA abundance in different extracts. The first requirement is that equal amounts of RNA must be loaded in each slot. In practice this is difficult to achieve, especially if RNA concentrations are estimated by absorbance spectroscopy (*APPENDIX 3D*), which is subject to errors

due to the small quantities being measured and the presence of contaminants such as protein and DNA.

Even if equal amounts of RNA are loaded, a difference in hybridization signal does not necessarily mean that the gene whose transcript is being studied is more active in a particular tissue. The analysis provides information on the abundance of an mRNA (i.e., the fraction of total RNA that it constitutes), not its absolute amount. To illustrate this point, consider a tissue in which a highly active gene is switched on at time *t*, where the transcripts of this gene constitute 0% of the mRNA at $t - 1$ but 20% of the mRNA at $t + 1$. If the slot blots of RNA from $t - 1$ and $t + 1$ are probed with the highly active gene, there will be a clear increase in hybridization signal after time *t*. In contrast, hybridization of the same slot blots with a second gene whose transcription rate is unchanged will show a decreased hybridization signal at $t + 1$. Transcripts of this gene are present in the same absolute amounts at $t - 1$ and $t + 1$, but their abundance decreases as the total mRNA population becomes larger due to activation of the highly expressed gene. To the unwary, the result of the hybridization analysis could appear to indicate down-regulation of a gene whose expression rate in fact remains constant.

Choice of membrane and transfer system

General information relating to the choice of membrane for a nucleic acid transfer is given in the Commentary to *UNIT 2.9A*. Because of the greater tensile strength of nylon, together with the fact that the RNA can be bound covalently by UV cross-linking, most transfers are now carried out using nylon rather than nitrocellulose. Nylon has the added advantage of being able to withstand the highly stringent conditions (50% formamide at 60°C) that may be required during hybridization with an RNA probe; nitrocellulose tends to disintegrate under these conditions.

For DNA transfer, a major advantage of positively charged nylon is that nucleic acids become covalently bound to the membrane if the transfer is carried out with an alkaline buffer. RNA can also be immobilized on positively charged nylon by alkaline transfer, but the procedure is not recommended as the alkaline conditions result in partial hydrolytic degradation of the RNA. This hydrolysis is difficult to control and smaller molecules are easily broken down into fragments too short for efficient retention by the membrane (see Table 2.9.1). This results in a loss of signal after hybridization, a problem that is exacerbated by the increased background caused by lengthy exposure of the membrane to the alkaline solution. Only if the signals are expected to be strong should an alkaline transfer be considered. In this case, Basic Protocol 1 should be modified as follows: omit the pre-transfer alkaline hydrolysis (step 10), use 8 mM NaOH rather than 20× SSC as the transfer buffer, do not transfer for more than 6 hr, and rinse the membrane in 2× SSC/0.1% (w/v) SDS rather than plain SSC immediately after transfer (step 22). If using Alternate Protocol 1, omit step 7 as well, as the alkaline transfer buffer removes glyoxal from the RNA.

The standard northern transfer system can be modified as described for Southern blotting (*UNIT 2.9A*). Aqueous transfers onto nylon can be performed using a variety of buffers, although SSC is still most frequently used. Changes can be made to the transfer time and architecture of the blot (e.g., downward transfer; Chomczynski, 1992), and alternative methods such as electroblotting (Smith et al., 1984) and vacuum transfer (Peferoen et al., 1982) can be used.

Hybridization procedures

Hybridization analysis of an RNA blot is subject to the same considerations as DNA hybridization (see *UNIT 2.10* Commentary). The factors that influence sensitivity and specificity are the same, and incubation times, hybridization solutions, probe length, and mechanics of hybridization all have similar effects. There are just two additional points that need to be made with respect to RNA blots.

The first point is that formamide is almost always used in RNA hybridization solutions. The primary reason for this is to permit a lower hybridization temperature to be used, minimizing RNA degradation during the incubations.

The second point concerns the stability of the hybrids formed between the immobilized RNA and the probe molecules. For a DNA probe the relevant equation is (Casey and Davidson, 1977):

$$T_m = 79.8°C + 18.5(\log M) + 0.58(\%GC) + 11.8(\%GC)^2 - 0.50(\%form) - {}^{820}/_{L}$$

and for an RNA probe (Bodkin and Knudson, 1985):

$$T_m = 79.8°C + 18.5(\log M) + 0.58(\%GC) + 11.8(\%GC)^2 - 0.35(\%form) - {}^{820}/_{L}$$

where T_m is the melting temperature, *M* is the molarity of monovalent cations, %GC is the

percentage of guanosine and cytosine nucleotides in the DNA, %form is the percentage of formamide in the hybridization solution, and *L* is the length of the hybrid in base pairs. What these equations indicate is that an RNA-RNA hybrid is more stable than a DNA-RNA hybrid: if %form is 50%, the T_m for an RNA-RNA hybrid is 7.5°C higher than that for an equivalent RNA-DNA hybrid. The greater stability of the RNA-RNA hybrid means that an RNA probe requires a more stringent hybridization and washing regime than a DNA probe (e.g., hybridization at 60°C in 50% formamide and final wash at 68°C in 0.1× SSC/0.1% SDS).

Reprobing conditions

Nitrocellulose presents problems regarding both membrane integrity and RNA retention (see *UNIT 2.10*). UV cross-linking of RNA to a neutral nylon membrane presents optimal conditions for northern blot reprobing.

Because of the sensitivity of RNA to alkaline hydrolysis, NaOH, which is included in protocols for probe removal from Southern blots, should not be used when removing probes from northern blots. It is recommended that probes be removed prior to membrane storage, because unstripped probes remain permanently attached if the blot dries.

Troubleshooting

The appearance of the agarose gel after staining gives a first indication of how successful a northern experiment is likely to be. If total RNA has been used, the rRNA bands should be clear and sharp (Fig. 4.9.1) with no "smearing" toward the positive electrode. The only exception is when the RNA has been prepared by the guanidinium isothiocyanate procedure (*UNIT 4.2*), in which case some smearing is normal. If the rRNA bands are not sharp, the RNA preparation may be of poor quality (usually because insufficient care has been taken in establishing an RNase-free environment) or the denaturing gel electrophoresis system may not have worked adequately. If the latter problem is suspected, make sure that the formaldehyde concentration in the gel is 2.2 M or, if glyoxal denaturation has been used, that the buffer recircularization is sufficient to maintain the gel pH at 7.0. Whatever the problem, if the rRNAs are not distinct, there is no point in proceeding with the transfer as the bands obtained after hybridization will also be fuzzy. In fact, even if the rRNA bands are clear there is no guarantee that the mRNAs are intact.

An indication of the efficiency of transfer onto nylon can be obtained by staining the membrane with methylene blue (see Basic Protocol 1, step 24), but often a problem with transfer is not recognized until after hybridization. If poor signals are obtained, the troubleshooting section of *UNIT 2.10* (including Table 2.10.4) should be consulted to identify the likely cause. Note that it is relatively easy to detach RNA from a membrane before immobilization, so some loss may occur when the membrane is rinsed in 2× SSC to wash off agarose fragments and leach out salt (see Basic Protocol 1, step 22). If necessary, this rinse can be postponed until immediately before hybridization, after the RNA has been immobilized.

Other problems, such as high backgrounds, extra bands, and difficulties with probe stripping, should be dealt with by referring to Table 2.10.4.

To increase the sensitivity of the miRNA assay, RNA probes can be used instead of DNA probes. In some cases, RNA probes work better to detect miRNA precursors (~60 to 70 nt long) which contain the immature miRNAs in a stem-loop whose structure can prevent the hybridization of DNA probes.

Anticipated Results

Using either a nylon or nitrocellulose membrane and a probe labeled to ≥5× 10^8 dpm/μg, it should be possible to detect transcripts that represent 0.01% of the mRNA population with a blot of 10 μg total mammalian RNA or 0.0002% of the population with a blot of 3 μg poly(A)$^+$ RNA.

It should be possible to detect small RNAs at levels as low as 0.3 fmol miRNA by following the Basic Protocol 2. Small RNAs that differ by as little as 1 nt (or even by a single phosphate group) can be separated on 15% denaturing polyacrylamide gel (50 to 100 cm), particularly when a long gel is used. For long gels, only the lower portion of the gel is used for transfer to the membrane.

Time Considerations

Traditional blots

A northern experiment can be completed in 3 days. The agarose gel is prepared and electrophoresed during the first day and the transfer carried out overnight. On the second day the blot is prehybridized and then hybridized overnight. Washes are completed early on the third day. A slot-blot experiment takes only two days, as the blot can be prepared and prehybridized

on the first day, hybridized overnight, and washed on the second day.

The length of time needed for the autoradiography depends on the abundance of the target sequences in the blotted RNA. Adequate exposure can take anything from overnight to several days.

With blots that are intended for reprobing, stripping procedures can be completed in ~1 hr, not including the verification steps.

miRNA blots

Using a semidry transfer system, the northern experiment can be completed in 2 days.

The length of time needed for the autoradiography depends on the abundance of the small RNA sequence and the detection system used. Optimal exposure can range from overnight to several days.

Under ideal conditions, a band that contains as little as 0.3 fmol of small RNA can be detected by northern hybridization with a 1-day exposure to a phosphor imager plate when scanned at 25 μm resolution. The amount of RNA loaded and the exposure time may vary with the abundance of the individual miRNA species. For abundant miRNAs, loading of 5 μg total RNA and overnight exposure will give strong hybridization signals.

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Contributed by Terry Brown
University of Manchester Institute of Science and Technology
Manchester, United Kingdom

Karol Mackey (probe removal)
Molecular Research
Cincinnati, Ohio

Tingting Du (miRNA blots)
University of Massachusetts Medical School
Worcester, Massachusetts