

Protocol

Northern Blots for Small RNAs and MicroRNAs

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This protocol describes the detection of small RNAs (~10–200 nucleotides) by blot hybridization. The RNA samples, denatured in formamide, are separated by denaturing polyacrylamide gel electrophoresis. Because high-percentage polyacrylamide gels are required to separate RNAs in this size range, it is necessary to perform electrophoretic transfer to positively charged nylon membranes. After transfer, the immobilized RNAs are subjected to hybridization with a ^{32}P -radiolabeled DNA or RNA probe and detected by phosphorimaging or autoradiography. This procedure is commonly used to detect small, U-rich spliceosomal small nuclear RNAs (snRNAs) and miRNAs. It should be possible also to detect most miRNAs using high-percentage (e.g., 15%) urea–polyacrylamide gel electrophoresis.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Electrophoretic transfer buffer (20×) <R>

Dilute the 20× stock to 1× in H₂O and precool to 4°C before use. Alternatively, use TBE electrophoresis buffer (10×) <R>, diluted to 0.5×. See Steps 4 and 5.

Formamide gel-loading buffer <R>

Polyacrylamide-urea gel (10%–15%) and electrophoresis reagents

Prehybridization/hybridization mix for northern blots <R>

Probe DNA or RNA, labeled at 2×10^8 to 5×10^8 cpm/μg

The probe is typically a DNA oligonucleotide, end-labeled by kinasing, or an antisense in vitro RNA transcript, labeled internally.

RNA marker ladder (10–150 nucleotides; e.g., U.S. Biochemicals Low Molecular Weight Marker or Ambion Decade Marker System)

The marker can be radiolabeled by kinasing and run in parallel with the unlabeled RNA samples in Step 2. It also serves as a control for electrophoretic transfer from the gel.

RNA samples (each ~10–15 μg of total RNA)

SSC for northern blots (20×) <R>

SDS (10%)

Adapted from *RNA: A Laboratory Manual* by Donald C. Rio, Manuel Ares Jr, Gregory J. Hannon, and Timothy W. Nilsen. CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot080838

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Equipment

Autoradiography or phosphorimaging equipment

Ballpoint ink pen for marking the filter

Containers for hybridization (polyethylene heat-seal bags or cylindrical glass hybridization bottles)

If heat-seal bags are used, a heat sealer (e.g., National Instruments, Model 310) will be required.

Electroblot transfer tank and power supply (e.g., Bio-Rad Trans-Blot cell with wire electrodes and PowerPac HC Power Supply)

Nylon transfer membrane (positively charged; e.g., GE Healthcare, Hybond-N+)

The membrane should be cut to the same size as the gel.

Oven with shaker or hybridization oven with a rotating rack for glass hybridization bottles, set at 42°C

Plastic wrap

Polyacrylamide gel electrophoresis system and power supply

The gel is typically ~18 × 18-cm, 1.5 mm thick, set with a 20-well comb. For details, see [Polyacrylamide Gel Electrophoresis of RNA](#) (Rio et al. 2010).

Stratalinker (Stratagene) with 254-nm bulbs

Alternatively, use a 254-nm UV transilluminator or vacuum oven set at 80°C (see Step 7).

Water bath or temperature block (90°C)

Whatman 3MM chromatography paper (four sheets must be the same size as the gel; see Steps 3 and 4)

METHOD

Denaturing Polyacrylamide-Urea Gel and Electrophoretic Transfer

1. Resuspend each RNA sample (~10–15 µg of total RNA) in 8–12 µL of formamide gel-loading buffer. Heat the samples for 2 min at 90°C.
2. Load the samples and the RNA marker ladder onto a high percentage (10%–15%) denaturing polyacrylamide-urea gel. Run the gel between 15 and 20 W on constant power, usually for 3–4 h, or until the bromophenol blue (in the formamide gel-loading buffer) is near the bottom of the gel. For details, see [Polyacrylamide Gel Electrophoresis of RNA](#) (Rio et al. 2010).
3. Remove the gel from the tank and separate the plates. Wet two sheets of Whatman 3MM paper with 20× SSC and place them on top of the gel.
4. Set up the electroblot transfer:
 - i. Soak a sponge from the electroblot apparatus in electrophoretic transfer buffer until wet.
 - ii. Transfer the gel with the paper onto the sponge, such that the paper is on top of the sponge.
 - iii. Float the nylon transfer membrane in H₂O until wet, then immerse it in 20× SSC.
 - iv. Place the nylon membrane on top of the gel and remove any bubbles.
 - v. Wet two more sheets of Whatman 3MM paper with 20× SSC, and place them on top of the membrane.
 - vi. Add the second sponge on top of the Whatman paper sheets and clamp the cassette closed.

The layers of the sandwich are as follows, starting with the negative (–) pole: sponge, two sheets of Whatman 3MM paper, gel, nylon membrane, two sheets of Whatman 3MM paper, sponge. The RNA will migrate to the positive (+) pole.
5. Place the assembled gel cassette into the electroblot chamber and fill the chamber with 1× electrophoretic transfer buffer, precooled to 4°C. Carry out the transfer at 250 mA for 2 h at 4°C and then at 350 mA for a further 2 h at 4°C.

Cross-Linking, Prehybridization, and Hybridization

6. Prepare the membrane for cross-linking:
 - i. Disassemble the apparatus. Remove and save the nylon membrane.
 - ii. Carefully remove any gel fragments from the membrane and mark the orientation of the membrane with a ballpoint ink pen.
 - iii. Wrap the membrane in plastic wrap and place it on a sheet of Whatman 3MM paper.
7. To cross-link the RNA to the nylon transfer membrane, place the membrane in a Stratalinker with the RNA surface facing up. Expose it to short-wave UV light (254 nm; the 1200 mJ auto-cross-linking setting in a Stratalinker) for 1 min.

Alternatively, use a 254-nm UV transilluminator, exposing the membrane with the RNA surface facing down against the glass surface; or bake the membrane in a vacuum oven for 1 h at 80°C.

For microRNAs (miRNAs; RNAs <50 nucleotides), covalent chemical cross-linking (Pall and Hamilton 2008) may be used to fix the small RNAs to the nylon membrane.
8. Place the membrane in a heat-seal bag or cylindrical glass hybridization bottle and cover with prehybridization/hybridization mix, using 10 mL for a heat-seal bag or ~3–5 mL for a small hybridization tube. Prehybridize the membrane with shaking or rotation for at least 2 h at 42°C.
9. Replace the buffer with an appropriate volume of fresh prehybridization/hybridization mix containing radiolabeled probe at 10⁶ cpm/mL, and hybridize overnight at 42°C.

Washing and Detection

10. Remove and safely dispose of the radioactive hybridization buffer.
11. Prepare three wash solutions: 2× SSC/1% SDS, 1× SSC/1% SDS, and 0.1× SSC.
12. Perform the following series of 15-min washes on the membrane, each at 42°C with agitation:
 - i. 2× SSC/0.1% SDS
 - ii. 1× SSC/0.1% SDS
 - iii. 0.1× SSC
13. Air-dry the membrane, cover it with plastic wrap and expose it to a phosphor screen for phosphorimaging or to X-ray film for autoradiography.

Exposure for several hours to overnight may be necessary.
14. Image on a phosphorimager or develop the X-ray film.

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 14): A high background is observed on the membrane.

Solutions: High background may be reduced by one of the following approaches.

1. Try prehybridizing for a longer period of time (Step 8).
2. When preparing the prehybridization/hybridization mix, include tRNA and/or poly(A) at a concentration of 200 µg/mL.
3. Polyacrylamide sticking to the membrane can cause high background by retaining the excess probe or unincorporated label. After transfer and while the membrane is still wet (Step 6.ii), “wash” the side of the membrane in contact with the gel in 1× electrophoretic transfer buffer using gloved fingers.

Problem (Step 14): The bands are faint or absent.

Solution: For the detection of miRNAs and other 20- to 30-nucleotide RNAs, it may be useful to try using low-stringency hybridization conditions (e.g., a low-stringency Church and Gilbert hybridization buffer containing 10 mg/mL BSA, 0.5 M sodium phosphate [pH 7.2], 1 mM EDTA, and 7% SDS) and to omit the high-stringency wash in Step 12.iii.

RELATED INFORMATION

One disadvantage of northern blotting is that it is relatively insensitive. For greater detection sensitivity, the splinted RNA ligation method is preferred (see **Splinted Ligation Method to Detect Small RNAs** [Nilsen 2013]).

RECIPES

Denhardt's Solution for Northern (100×)

Reagent	Quantity (for 1 L)	Final concentration (100×)
Polyvinylpyrrolidone	20 g	2%
Bovine serum albumin (Type V)	20 g	2%
Ficoll 400	20 g	2%
EDTA (250 mM, pH 8.0)	10 mL	2.5 mM
H ₂ O	to 1 L	

Store a working stock at 4°C. For long-term storage (>1 yr), store at -20°C.

Electrophoretic Transfer Buffer (20×)

Reagent	Quantity (for 1 L)	Final concentration (20×)
Trisodium citrate	35.29 g	120 mM
Sodium phosphate, dibasic (Na ₂ HPO ₄)	22.71 g	160 mM
H ₂ O	to 1 L	

Adjust the pH to 8.3, autoclave, and store for up to 1–2 yr at room temperature.

Formamide Gel-Loading Buffer

Reagent	Quantity (for 10 mL)	Final concentration
Deionized formamide	9.5 mL	95%
Bromophenol blue	2.5 mg	0.025% (w/v)
Xylene cyanol FF	2.5 mg	0.025% (w/v)
EDTA (0.5 M, pH 8.0)	100 μL	5 mM
H ₂ O	400 μL	

Store in aliquots at -20°C.

Herring Testis Carrier DNA

To make a 5 mg/mL solution, dissolve 10 g of herring testis carrier DNA (Type XIV) in H₂O to a final volume of 2 L. Stir until the DNA is no longer opaque, up to overnight. Autoclave the solution, aliquot in 50-mL tubes and store for >1 yr at -20°C. Alternatively, instead of autoclaving, the carrier DNA can be sonicated and denatured.

Prehybridization/Hybridization Mix for Northern Blots

Reagent	Quantity (for 50 mL)	Final concentration
Formamide	25 mL	50%
SSC for northern blots (20×)	12.5 mL	5×
Denhardt's solution for northern blots (100×)	1.5 mL	3×
Herring testis carrier DNA	2 mL	200 µg/mL
SDS (10%)	0.5 mL	0.1%
H ₂ O	8.5 mL	

Prepare fresh before use.

SSC for Northern Blots (20×)

Reagent (20×)	Quantity (for 1 L)	Quantity (for 2 L)	Final concentration
NaCl	175.3 g	350.6 g	3 M
Trisodium citrate	88.3 g	176.5 g	0.3 M
H ₂ O	to 1 L	to 2 L	

Adjust the pH to 7.0 with NaOH or HCl. Autoclave the solution and store it at room temperature. It will last for 1 yr or longer.

TBE Electrophoresis Buffer (10X)

Reagent	Quantity (for 1 L)	Final concentration
Tris base	121.1 g	1 M
Boric acid	61.8 g	1 M
EDTA (disodium salt)	7.4 g	0.02 M

Prepare with RNase-free H₂O. Dilute 100 mL to 1 L to make gel running buffer. Store for up to 6 mo at room temperature.

REFERENCES

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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot080838

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