Protocol

Analysis of Small RNAs by Northern Hybridization

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This protocol describes how to use northern hybridization to detect 15- to 150-nt small RNAs. Total RNA is fractionated by electrophoresis through a denaturing polyacrylamide gel and then transferred to a nylon membrane by semidry electroblotting. After UV-cross-linking the RNA to the membrane, hybridization is performed in Church buffer using a ^{32}P -radiolabeled oligonucleotide probe followed by PhosphorImager analysis. The use of StarFire probes allows a substantial increase in the specific radioactivity of the hybridization probe. StarFire probes contain two domains. The target-specific domain at the 5' end hybridizes to the small RNA of interest. The universal template-binding domain at the 3' end is used to bind a universal template oligonucleotide that carries 10 deoxythymidines at its 5' end, providing a template for DNA polymerase to incorporate 10 [α - ^{32}P]deoxyadenosines at the 3' end of the StarFire probe.

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

Acrylamide:bisacrylamide (19:1, 40%, w/v)

[α - ^{32}P]dATP (6000 Ci/mmol, 10 mCi/mL)

Ammonium persulfate <R>

Prepare a 10% (w/v) solution.

Church buffer <R>

Formamide loading buffer for RNA gels <R>

[γ - ^{32}P]ATP (6000 Ci/mmol, ≥ 10 mCi/mL)

miRNA StarFire Nucleic Acid Labeling System (Integrated DNA Technologies; see Discussion)

StarFire Custom Probe (up to 50 nt, PAGE-purified)

StarFire Universal Template (HPLC-purified)

StarFire 10 $\times$ Buffer Mix (100 mM Tris at pH 7.5, 50 mM MgCl_2 , 75 mM DTT)

StarFire Stop Buffer (10 mM EDTA)

Exo $^-$ Klenow DNA Polymerase

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RNA samples and 5' ³²P-radiolabeled marker RNAs (e.g., RNA Decade Marker; Ambion, catalog no. AM7778)

SDS <R>

Prepare a 20% (w/v) solution.

SSC <R>

Prepare 20× and 1× solutions.

Synthetic oligonucleotide probes (DNA, RNA, or LNA-modified DNA)

T4 polynucleotide kinase (10 U/μL)

T4 polynucleotide kinase buffer (10×) <R>

TBE buffer <R>

Prepare 5× and 0.5× solutions.

TE buffer, 10× <R>

Prepare 10× and 1× solutions (pH 8.0).

TEMED

Urea

Equipment

Blotting paper, extra thick

Heating block (95°C)

Hybridization oven (e.g., Mini Autoblot Hybridization Oven; Bellco Glass, catalog no. 7930-10110)

Hybridization tubes (e.g., Autoblot tube, 35 × 150 mm; Bellco Glass, catalog no. 7910-35150)

Magnetic stirrer

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

PhosphorImager (e.g., FUJI FLA-5000) and software (e.g., ImageGauge v4.22)

PhosphorImager plate

Polyacrylamide gel apparatus (e.g., Bio-Rad Mini-PROTEAN, 8 cm × 7.3 cm × 0.75 mm)

Saran Wrap

Semidry transfer apparatus (e.g., Trans-Blot SD Semi-Dry Transfer Cell; Bio-Rad)

Sephadex G-25 spin columns UV cross-linker (e.g., Stratalinker UV Crosslinker; Stratagene)

METHOD

See Figure 1 for a flowchart of the procedure.

Preparation of a Denaturing Polyacrylamide Gel

1. Prepare a 15% denaturing polyacrylamide gel containing 8 M urea by mixing the following reagents:

TBE buffer (5×)	2 mL
Acrylamide:bisacrylamide (19:1, 40%, w/v)	3.75 mL
Urea	4.8 g
Deionized water	to 10 mL
2. Stir the mixture at room temperature with a magnetic stirrer until the urea dissolves.
3. Assemble the gel casting frame and casting stand.
4. Add 100 μL of 10% (w/v) ammonium persulfate
5. Add 10 μL of TEMED.

FIGURE 1. Flowchart for analysis of small RNAs by northern hybridization.

6. Mix the solution quickly, and then pour the gel into the mold of a minigel apparatus with a 10-well comb. Allow the gel to polymerize for 20 min at room temperature.

Preparation of Samples and Electrophoresis

7. Mix 5–20 µg of total RNA sample with at least an equal volume of formamide loading buffer to a final volume of 10 µL.
The amount of total RNA sample to be used varies according to the abundance of the RNA to be detected.
8. Heat the mixture for 5 min at 95°C, and then place the tubes on ice.
9. Wash the wells of the gel to remove urea. This is essential to produce uniform, straight bands. Then, carefully load 10 µL of each RNA sample into each well of the gel. Be sure to include 5' ³²P-radiolabeled RNA Decade marker. Label the marker by T4 polynucleotide kinase and [γ-³²P]ATP according to the manufacturer's instructions, and then dilute 200-fold to an activity appropriate for the small RNA to be detected by hybridization.
10. Run the gel in 0.5× TBE buffer at 200 V until the bromophenol blue reaches the bottom of the gel (~1 h).

Transferring RNA onto Membrane with a Semidry Transfer Cell

11. Cut a piece of Hybond N+ nylon membrane and two pieces of extra thick blotting paper to the dimensions of the gel, and soak them in 0.5× TBE buffer for 10 min at room temperature.
12. Place one piece of presoaked, extra thick blotting paper on the platinum anode. Remove air bubbles by rolling the paper with a clean plastic pipette.
13. Place the presoaked membrane on top of the blotting paper, and roll out the air bubbles.
14. Carefully lay the gel on top of the membrane, and roll out the air bubbles.
15. Place the other piece of presoaked, extra thick blotting paper on top of the gel, and roll out the air bubbles.
16. Connect the cathode to the sandwich stack, and then place the safety cover on the unit. Transfer at constant voltage of 20 V for 1 h.

17. After transfer, place the damp membrane on a piece of filter paper, and cross-link the RNA to the membrane by irradiation with 254-nm UV light (Stratalinker; autocrosslink setting of 120,000 μ J).
UV cross-linking immobilizes the RNA on the membrane, allowing blots to be reprobbed multiple times.
18. Place the cross-linked membrane into a hybridization tube with the RNA side facing the interior. Add 20 mL of Church buffer, place the tube into the hybridization oven, and incubate for 1 h to overnight at 37°C with rotation for prehybridization.

Preparation of 5' 32 P-Radiolabeled Oligonucleotide Probes

19. The probe is perfectly complementary to the small RNA to be detected with the same length. Set up the 5'-end labeling reaction by mixing the following reagents:

Nuclease-free water	6 μ L
DNA oligonucleotide probe (25 μ M)	1 μ L
T4 polynucleotide kinase buffer (10 $\times$)	1 μ L
T4 polynucleotide kinase (10 U/ μ L)	1 μ L
[γ - 32 P]ATP (6000 Ci/mmol, $\geq$ 10 mCi/mL)	1 μ L
20. Incubate the reaction for 1 h at 37°C.
21. Remove unincorporated [γ - 32 P]ATP with a Sephadex G-25 spin column according to the manufacturer's instructions.

Preparation of 3'-End 32 P-Labeled StarFire Oligonucleotide Probes (Optional)

22. Resuspend the StarFire Universal Template to a concentration of 12.5 μ M with 1 $\times$ TE buffer.
23. Resuspend the StarFire Custom Probe to a concentration of 100 μ M with 1 $\times$ TE buffer.
24. Dilute the StarFire Custom Probe to a concentration of 0.5 μ M with nuclease-free water.
25. Mix the following reagents in a 0.5-mL microcentrifuge tube:

StarFire buffer (10 $\times$)	1 μ L
StarFire probe (0.5 μ M)	1 μ L
StarFire Universal Template (12.5 μ M)	1 μ L
26. Mix by pipetting, and then incubate the tube for 1 min in a 95°C heat block.
27. Remove the tube from the heat block, and allow the reaction to cool to room temperature ($\sim$ 5 min). Briefly centrifuge the tube to collect the reaction at the bottom of the tube.
28. Add 6 μ L of [α - 32 P]dATP (6000 Ci/mmol, 10 mCi/mL) and 1 μ L of Exo⁻ Klenow DNA Polymerase. Mix by gently pipetting.
29. Incubate the tube at room temperature for 2 h, and then 40 μ L of StarFire Stop Buffer.
30. Remove unincorporated [α - 32 P]dATP with a Sephadex G-25 spin column according to the manufacturer's instructions.

Hybridization

31. After prehybridization (Step 18), add either the 5' 32 P-radiolabeled probe or the 3' 32 P-radiolabeled StarFire probe directly to the prehybridization solution, and incubate for 2 h to overnight.
These are alternative methods. The 3'-radiolabeled StarFire probe is generally more sensitive than the 5'-radiolabeled probe of the same sequence.
Two hours of hybridization usually yields at least 80% of the maximum signal. For highest sensitivity, perform hybridization overnight.
32. After hybridization, carefully transfer the hybridization solution into a 50-mL conical tube and store at -20°C.
33. Briefly rinse the bottle twice with 30 mL of 1 $\times$ SSC plus 0.1% (w/v) SDS.

34. Wash the membrane with 30 mL of 1× SSC plus 0.1% (w/v) SDS for 10 min at 37°C. Repeat wash twice.
35. Wrap the damp membrane with Saran Wrap, and expose it to a PhosphorImager plate overnight.
36. Read the hybridization signals with a PhosphorImager (e.g., FUJI FLA-5000), and analyze hybridization using appropriate software (e.g., ImageGauge v4.22).

See Troubleshooting.

Analysis of the Relative Abundance of Small RNA in Different Samples

37. Quantify the original hybridization signal and the background noise of the same sample according to instructions for using ImageGauge. Subtract the background noise from the original hybridization signal to get the specific hybridization signal of the small RNA.
38. Strip the probe from the blot by boiling the membrane in 500 mL of 0.1% (w/v) SDS for 10 min. Wrap the damp membrane with Saran Wrap, and expose it to a PhosphorImager plate overnight to check if stripping was successful. Repeat this step if the signals were not completely erased.
39. Hybridize the membrane with a probe that is specific to an internal control (e.g., a small nuclear RNA, tRNA, or 5S ribosomal RNA present in the same sample) to visualize specific hybridization signals of the internal control in each sample as described above.
40. Normalize the specific hybridization signal of the small RNA of interest to that of the internal control. The normalized values between different samples can now be compared to assess the relative abundance of the small RNA of interest.

Analysis of the Absolute Amount of Small RNA in a Sample

41. Order an RNA oligonucleotide with exactly the same sequences as the small RNA of interest, and make a series of dilutions (10^{-1} – 10^3 fmol).
42. Load the diluted RNA oligonucleotides and the samples on a denaturing polyacrylamide gel, and perform northern hybridization as described above (Steps 7–36).
43. Plot the specific hybridization signals (be sure to subtract background) of the diluted RNA oligonucleotides versus their concentrations to obtain a standard curve. Use this standard curve to determine the absolute amount of the small RNA in each sample.

In our experience, northern blots analyzed by PhosphorImaging can be linear over as many as five logs of signal.

Most miRNAs and some abundant endo-siRNAs and piRNAs are readily detected with 5' 32 P-radiolabeled DNA or RNA oligonucleotide probes. For low-abundance small RNAs, 3' 32 P-radiolabeled StarFire oligonucleotide probes or 5' 32 P-radiolabeled LNA-modified oligonucleotide probes are recommended.

TROUBLESHOOTING

Problem (Step 36): No signals are detected, but 5' 32 P-radiolabeled markers are.

Solution: Try the following.

- Check whether the probe sequence is perfectly complementary to the small RNA of interest.
- Check if the probe is properly labeled by resolving it in a 15% denaturing polyacrylamide gel followed by PhosphorImagery.
- Check the internal loading control.

- To increase sensitivity, use a 5'-end ^{32}P -labeled RNA oligonucleotide probe or a 5'-end ^{32}P -labeled LNA-modified DNA oligonucleotide probe, and increase the temperature to 60°C or 70°C, respectively.

Problem (Step 36): High background hybridization signals are detected.

Solution: Extend the prehybridization to overnight. Repeat the last wash twice more.

DISCUSSION

The highest specific activity that can be obtained using polynucleotide kinase to catalyze the addition of ^{32}P to the 5'-OH group of an oligonucleotide is $\sim 1.3 \times 10^7$ cpm/pmol. However ~ 10 -fold higher specific activity can be obtained using StarFire—a commercial system to add multiple radiolabeled deoxyribonucleotide triphosphates (dNTPs) to the 3' end of an investigator-defined oligonucleotide. These high-specific-activity probes are required to detect RNAs that are small in size and present in low abundance (e.g., miRNAs).

Starfire probes are generated as shown in Figure 2. In summary, an oligonucleotide is synthesized with a target sequence at its 5' end and a universal template sequence at its 3' end. A second oligonucleotide is then annealed to the universal template. The protruding single-stranded poly(T) tail of the second oligonucleotide serves as a template in a primer extension reaction catalyzed by the *exo*⁻ Klenow fragment of *E. coli* DNA polymerase I using α - ^{32}P -dATP as a substrate. As shown in

FIGURE 2. The StarFire oligonucleotide labeling protocol. (A) Two oligonucleotides are used in the reaction—a target-specific probe oligonucleotide and a universal template oligonucleotide. The oligonucleotides are annealed (B) and labeled in a primer extension reaction by DNA polymerase (C). Unincorporated nucleotides are removed by gel filtration (D). (Redrawn from Belhke et al. 2000, with permission from *BioTechniques*.)

Figure 2, up to 10 radiolabeled deoxynucleosides can be attached to an investigator-defined sequence, generating a single-stranded probe of specific size and very high specific activity.

RECIPES

Ammonium Persulfate

To prepare a 10% (w/v) solution: Dissolve 1 g ammonium persulfate in 10 mL of H₂O and store at 4°C. Ammonium persulfate decays slowly in solution, so replace the stock solution every 2–3 wk. Ammonium persulfate is used as a catalyst for the copolymerization of acrylamide and bisacrylamide gels. The polymerization reaction is driven by free radicals generated by an oxido-reduction reaction in which a diamine (e.g., TEMED) is used as the adjunct catalyst.

Church Buffer

1% (w/v) bovine serum albumin

1 mM EDTA

0.5 M phosphate buffer*

7% (w/v) SDS

*0.5 M phosphate buffer is 134 g of Na₂HPO₄•7H₂O, 4 mL of 85% H₃PO₄ (concentrated phosphoric acid), H₂O to 1 L.

Formamide Loading Buffer for RNA Gels

Deionized formamide	80% (w/v)
EDTA (pH 8.0)	10 mM
Xylene cyanol FF	1 mg/mL
Bromophenol blue	1 mg/mL

Purchase a distilled deionized preparation of formamide and store in small aliquots under nitrogen at –20°C.

Alternatively, deionize reagent-grade formamide by stirring on a magnetic stirrer with Dowex XG8 mixed bed resin for 1 h and filtering it twice through Whatman No. 1 paper. Store deionized formamide in small aliquots under nitrogen at –70°C.

SDS

Also called sodium dodecyl sulfate or sodium lauryl sulfate. To prepare a 20% (w/v) solution, dissolve 200 g of electrophoresis-grade SDS in 900 mL of H₂O. Heat to 68°C and stir with a magnetic stirrer to assist dissolution. If necessary, adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 L with H₂O. Store at room temperature. Sterilization is not necessary. Do not autoclave.

SSC

For a 20× solution: Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of H₂O. Adjust the pH to 7.0 with a few drops of a 14 N solution of HCl. Adjust the volume to 1 L with H₂O. Dispense into aliquots. Sterilize by autoclaving. The final concentrations of the ingredients are 3.0 M NaCl and 0.3 M sodium citrate.

T4 Polynucleotide Kinase Buffer (10×)

700 mM Tris-Cl (pH 7.6)

100 mM MgCl₂

50 mM DTT

Store at −20°C in small aliquots.

TBE Buffer

Prepare a 5× stock solution in 1 L of H₂O:

54 g of Tris base

27.5 g of boric acid

20 mL of 0.5 M EDTA (pH 8.0)

The 0.5× working solution is 45 mM Tris-borate/1 mM EDTA.

TBE is usually made and stored as a 5× or 10× stock solution. The pH of the concentrated stock buffer should be ~8.3. Dilute the concentrated stock buffer just before use and make the gel solution and the electrophoresis buffer from the same concentrated stock solution. Some investigators prefer to use more concentrated stock solutions of TBE (10× as opposed to 5×). However, 5× stock solution is more stable because the solutes do not precipitate during storage. Passing the 5× or 10× buffer stocks through a 0.22-μm filter can prevent or delay formation of precipitates.

TE Buffer, 10×

100 mM Tris-Cl (desired pH)

10 mM EDTA (pH 8.0)

Sterilize solutions by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle. Store the buffer at room temperature.

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