Supplementary figures and tables

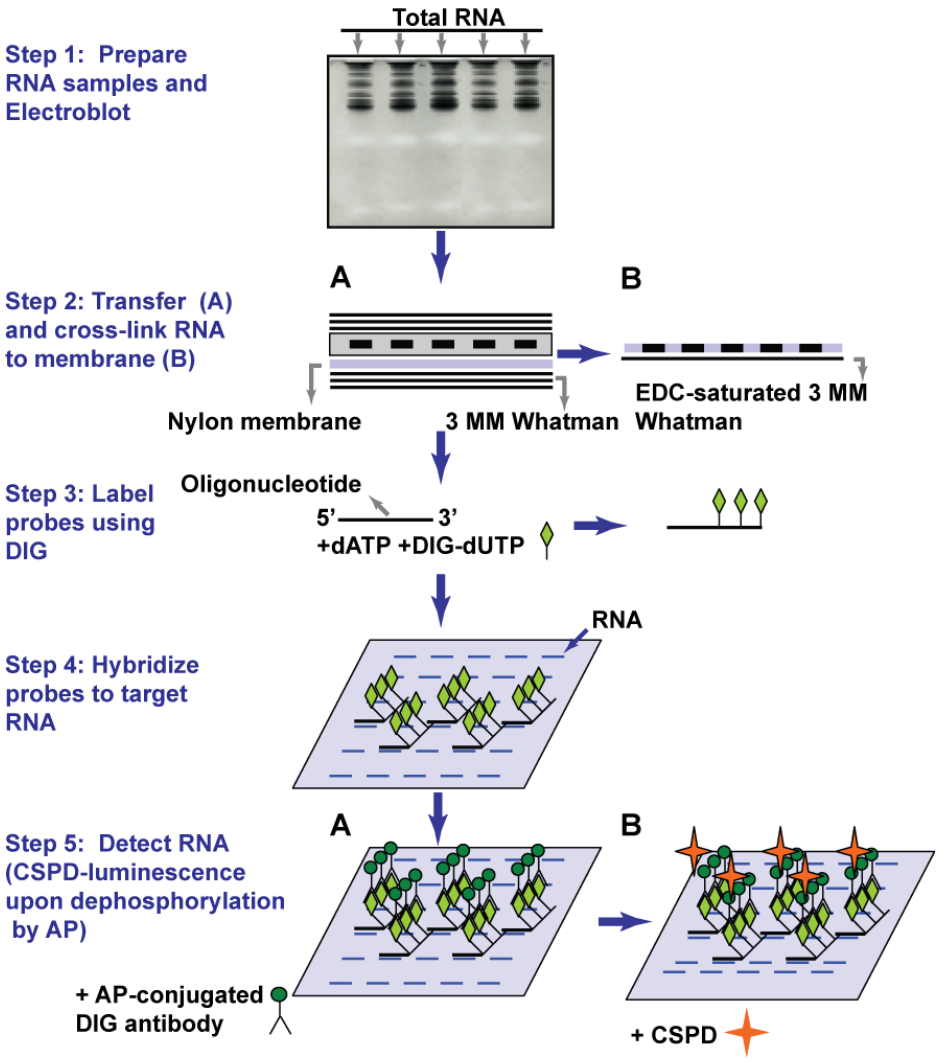

Figure S1: Overview of the LED northern blot protocol. The RNA samples are prepared and electrophoresed (Step 1). The migrated RNAs are transferred (Step 2A) and EDC-cross-linked (Step 2B) to a membrane. LNA-DNA-mixed oligonucleotide probes are labeled with DIG (Step 3) and hybridized to the target RNA on the membrane (Step 4). RNA-bound DIG-labeled probes are targeted by alkaline phosphatase (AP)-conjugated DIG antibody (Step 5A). The luminescence of CSPD, a substrate of AP, is subsequently used to detect the presence of target RNA (Step 5B).

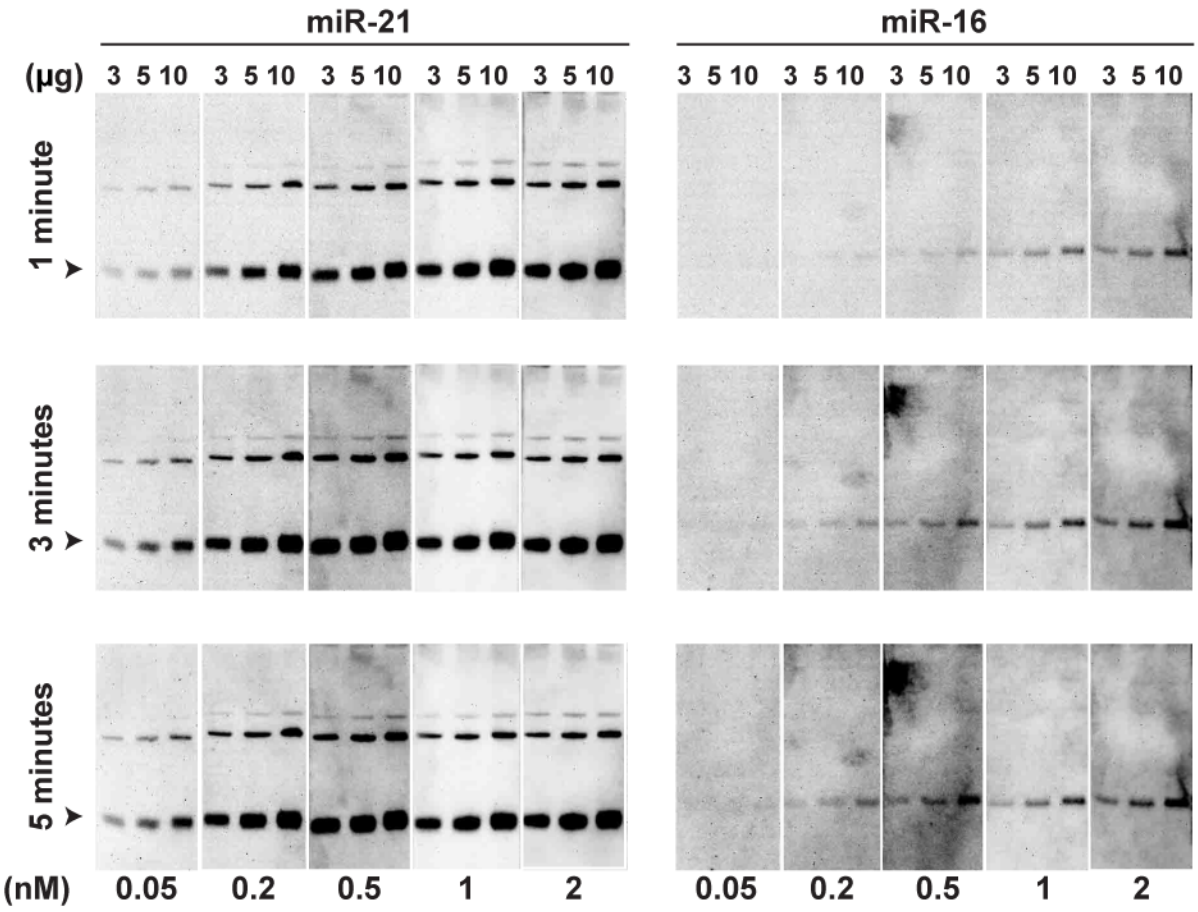

Figure S2: Effect of probe concentrations on the LED protocol. Five probe concentrations (0.05, 0.2, 0.5, 1, and 2 nM) were used to assess their influence on LED. Varying amounts of total RNA (3, 5, and 10 µg) were used to detect mature miR-21 and miR-16 (arrowheads) for each probe concentration, and the corresponding photo-luminescence was recorded over varying lengths (1, 3, and 5 minutes) of time. The upper bands may correspond to the precursor and primary transcripts of the miRNAs.

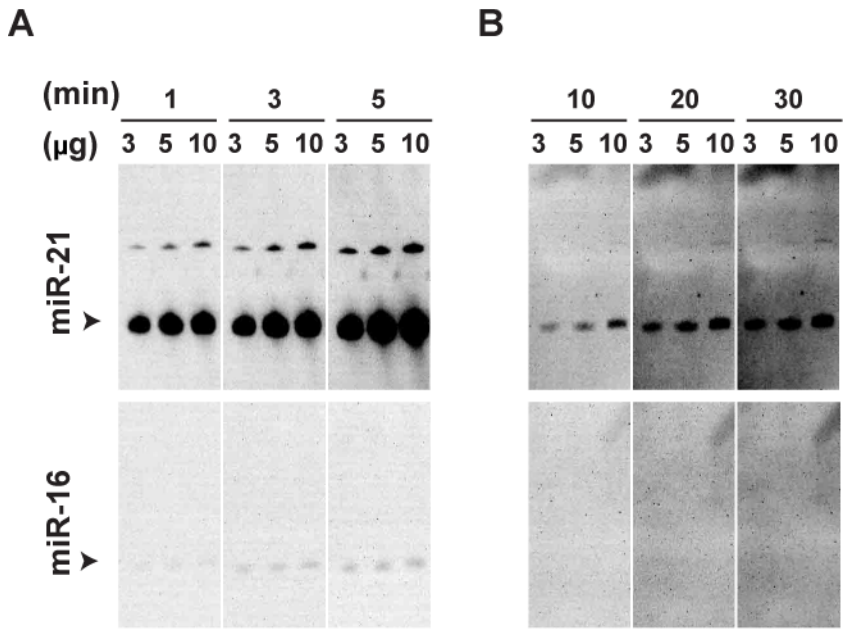

Figure S3: Comparison of the performances of ULTRAhyb (A) and modified Church–Gilbert buffer (B) containing 50% formamide (37 °C). Amount of total RNA (3, 5, and 10 µg) and duration of photo-exposure (1, 3, and 5 minutes) are identical to that in previous figures.

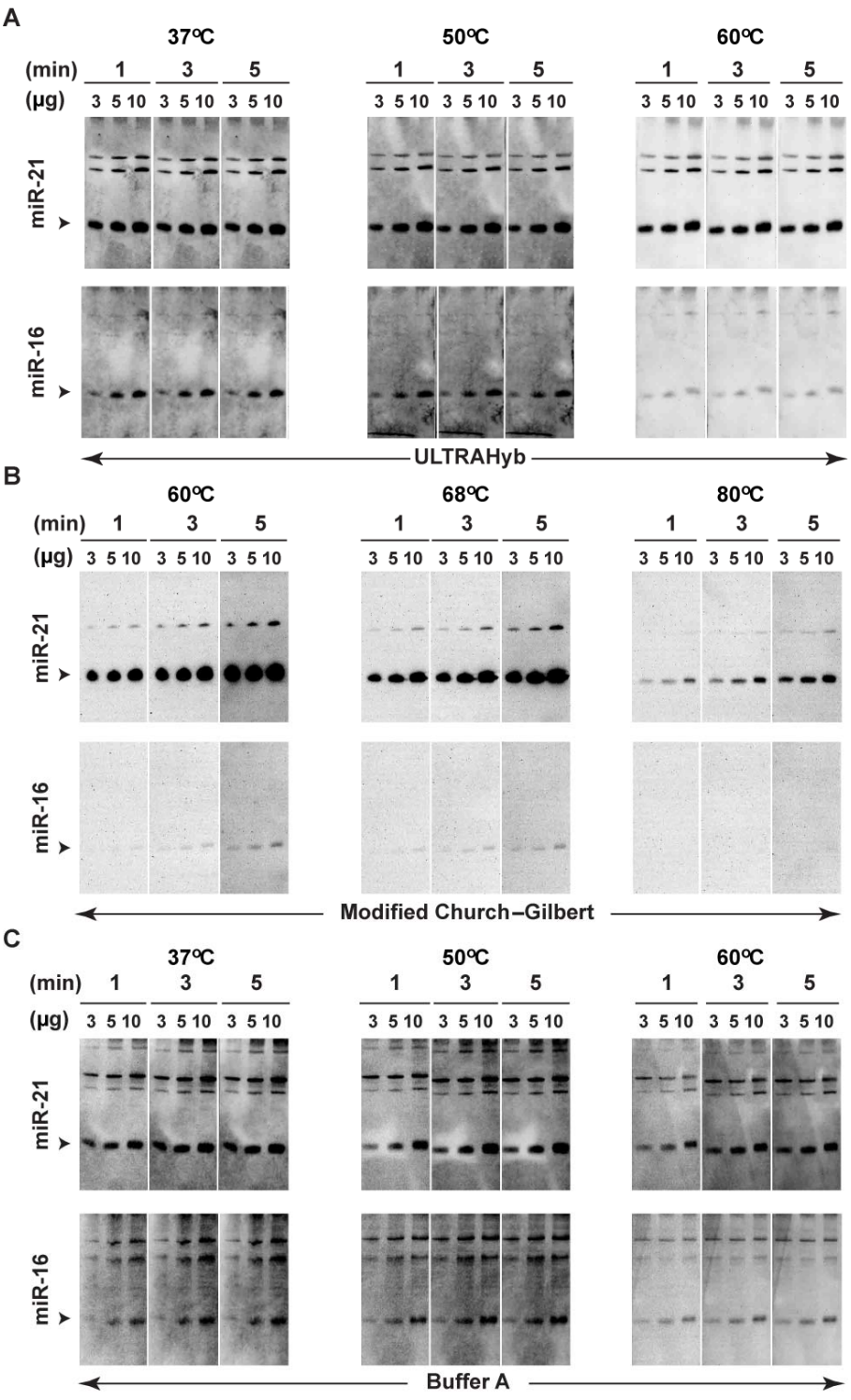

Figure S4: Temperature optimizations for LED protocol using ULTRAhyb (A), the modified Church–Gilbert buffer (B), and buffer A (C). The amounts of total RNA (3, 5, and 10 μg) and the durations of photo-exposure (1, 3, and 5 minutes) are identical to those in previous figures.

	Ambion	Roche (in-house preparation)			Roche	Church–Gilbert	Modified Church–Gilbert
		Buffer A	**Buffer B**	**Buffer C**			
Hyb. Condition	ULTRAhyb	5× SSC, 0.1% Lauroyl sarcosine, 0.02% SDS, 1% BBR	5×SSC, 50% deionized formamide, 0.1% Lauroyl sarcosine, 0.02% SDS, 2% BBR	7% SDS, 50% deionized formamide, 5× SSC, 50mM Sodium Phosphate, 0.1% Lauroyl sarcosine, 2% BBR	DIG Easy Granules	0.25 M Sodium Hydrogen phosphate, 1mM EDTA, 7% SDS	0.25 M Sodium Hydrogen phosphate, 1mM EDTA, 10% SDS, 0.5% BBR
Temperature	37 °C	50 °C	50 °C	50 °C	50 °C	68 °C	68 °C

Table S1: **Hybridization buffers used in this study and the corresponding temperatures used for hybridization.** ULTRAhyb (Ambion), and buffers B and C (Roche) contain formamide which reduces melting temperature of probe–target hybridization and hence the temperatures used are lower than Church–Gilbert protocols. Abbreviations: Saline-sodium citrate (SSC), sodium dodecyl sulfate (SDS), Boehringer blocking reagent (BBR), and Ethylene Diamine Tetra-acetic Acid (EDTA).

Problem	Possible reason	Solution
No Bands	• Lack of 5’ phosphate on RNA	• Substitute EDC cross-linking by UV cross-linking
	• Poor quality of RNA	• Check A_{260}/A_{280} ratio
	• Low RNA concentration	• Increase the amount of total RNA
	• Low probe concentration	• Increase the amount of probe
	• Poor RNA transfer to membrane	• Check northern blotting setup and extend transfer if necessary
	• Insufficient exposure	• Increase exposure time
High background	• Membrane contamination	• Handle the membrane carefully Membrane must not completely dry
	• Bubbles during blotting	• Remove all bubbles during blotting
	• Partially washed membrane	• Increase stringency of washing buffer and washing time
	• Membrane dried out during hybridization	• Cover membrane completely with hybridization buffer
	• Insufficient blocking time	• Increase period of blocking
Many bands	• Nonspecific probe	• Redesign probe based on different LNA spiking patterns

Table S2: Potential problems, reasons for failures and possible solutions.

Detailed LED protocol

RNA isolation and preparation

Trizol (Invitrogen) or TRI-reagent (Sigma) are widely recommended for extracting total RNA. We generally use Trizol. We recommend following manufacturer-recommended procedures for using Trizol and other reagents. The quality and quantity of the extracted RNAs can greatly affect the final results and hence RNA samples must be prepared meticulously. Mini column-based methods should be avoided since small RNAs are frequently eliminated during elution.

Probe design

Since probes composed of either LNAs or traditional DNA oligonucleotides are chemically very similar, standard DNA labeling reactions are applicable to LNA-modified probes. LNA-modified oligonucleotides can be designed and purchased from Exiqon (http://www.exiqon.com). For novel small RNA detection, LNA-DNA mixed oligonucleotide probes can be directly synthesized (Integrated DNA Technologies, IA). Since the position ("spiking pattern") of the LNA bases on the probe can greatly affect the structure and hybridization dynamics of the oligonucleotide(1), testing several spiking patterns (lnatools.com) of LNA nucleotides along the probes, may help improve both sensitivity and specificity. For instance, for a few miRNAs that we tested, a stretch

of eight LNA bases at 5'-positions 8 to 15 of small RNA probes, a new pattern that we have developed, yields better performance than currently marketed probes.

Use of size markers

Sizes of the small RNAs that are detected using LED protocol can be easily determined using standard radioactive labeling methods or using a radiation-free method. To implement a non-radioactive method, a set of synthesized single-stranded RNAs of varying sizes (10–30 bases) or pre-synthesized microRNA markers (New England BioLabs), run parallel to the target RNA should be stained by SYBR-Gold- or ethidium bromide (5–20 minutes). The stained gel should be subsequently photographed alongside a ruler using a transilluminator. The positions of markers on the membrane can be readily identified by superimposing the resulting photograph with that of the final film containing the RNA bands.

Reagents

- 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma, cat. no. 7750)
- 1-Methylimidazole (Sigma, cat. no. M50834)
- 3MM Whatman chromatography paper (Whatman, cat. no. 3030917)
- Agarose UltraPure (Invitrogen, cat. no. 15510-019)

- Ammonium persulfate (Sigma, cat. no. A3678)
- Anti-Digoxigenin-AP, Fab fragments (Roche, cat. no. 11 093 274 910)
- Chloroform (Sigma, cat. no. C2432)
- CSPD (Roche, cat. no. 11 655 884 001)
- Diethylpyrocarbonate (DEPC; Sigma, cat. no. D5758)
- DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche, cat. no. 03 353 583 910)
- DIG Wash and Block Buffer Set (Roche, cat. no. 11 585 762 001)
- Ethanol (Merck, cat. no. 1.00983.1000)
- Ethidium bromide (EtBr; Invitrogen, cat. no. 15585-001). Note: Mutagenic
- Gel Loading Buffer II (Ambion, cat. no. AM8546G)
- Gel Loading Solution (Ambion, cat. no. AM8556)
- Isopropyl alcohol (Sigma, cat. no. I9030)
- Nylon Membranes, positively charged (Roche, cat. no. 11 209 299 001)
- SequaGel Kit (National diagnostics, cat. no. EC-833). Note: Neurotoxic
- TBE buffer, 10× solution (National diagnostics, cat. no. EC-860)
- Tetramethylethylemediamine (Sigma, cat. no. T9281)
- Trizol (Invitrogen, cat. no. 15596-026) Note: Trizol is toxic and corrosive.
- ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion, cat. no. AM8670)

- X-ray film (many suppliers). We generally use Kodak BioMax XAR Film.

Equipment

- ChemiDoc-IT imaging system (UVP) or Phosphor imaging/intensifier screen
- Electrophoresis system for polyacrylamide gel electrophoresis (PAGE). Note: We use Mini-PROTEAN Cell from Bio-Rad, but vertical protein gel electrophoresis systems are also suitable.
- High-capacity power supply for PAGE
- Hybridization oven and bottle
- Tabletop coolable centrifuge (~12,000 g, 2–8 °C)
- Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, cat. no. 170-3940)
- UV transilluminator

Reagent setup

DEPC-treated water Add DEPC (1 ml) to distilled water (1,000 ml). Mix thoroughly and use a fume hood to incubate overnight at room temperature.

Denaturing PAGE Follow manufacturer's (National diagnostics) instructions.

EDC cross-linking solution Add 245 µl of 12.5 M 1-methylimidazole to 9 ml DEPC-treated water, maintaining a pH of 8.0 using 1 M HCl(2); the solution can be prepared up to 2 hrs in

advance and stored at room temperature. Immediately before use, add 0.753 g EDC and bring the volume up to 24 ml with DEPC-treated water. The solution is sufficient to saturate a typical (20 × 16 cm^2) 3 MM Whatman chromatography paper.

High stringent buffer 0.1× SSC with 0.1% (wt/vol) SDS

Low stringent buffer 2× SSC with 0.1% (wt/vol) SDS

Washing buffer 1× SSC

Blocking buffer DIG Wash and Block Buffer Set (See manufacturer's instructions)

Development buffer 1× Detection buffer from DIG Wash and Block Buffer Set

DIG Washing buffer 1× Washing buffer from DIG Wash and Block Buffer Set

Procedure

An overview of the described scheme is presented in Figure 1.

RNA extraction (1–2 h)

1. Isolate total RNA using Trizol reagent following manufacture's instructions (steps 2–12,).

2. Homogenize samples (5–10×10^6 of cells) in Trizol (1 ml) reagent.

3. Incubate the homogenized samples (5 min) at 15–30 °C.

4. Add 0.2 ml chloroform, corresponding to 1 parts of chloroform for 5 parts of Trizol that was used for initial homogenization.

5. Shake the samples vigorously by hand (15 sec) and incubate (2–3 min) them at 15–30 °C.

6. Centrifuge (~12,000 g, 15 min) the samples at 2–8 °C.

7. Transfer the aqueous phase to new RNase-free tube.

8. Add 0.5 ml of isopropyl alcohol per 1 ml of Trizol reagent used for initial homogenization.

9. Incubate (10 min) samples at 15–30 °C.

10. Centrifuge the samples (~12,000 g, 10 min) at 2–8 °C.

11. Remove the supernatant and then wash the RNA pellet once with 80% ethanol.

12. Mix the sample by shaking and centrifuging (~7,500 g, 5 min) at 2–8 °C.

13. Using a small tube (1.5 ml), dissolve the resulting RNA pellet in 30–50 µl of DEPC-treated water.

Sample quantity and quality check (1–2 h)

14. We typically use SmartSpec 3000 (Bio-Rad) to measure RNA concentration by spectrophotometry at 260 nm. Note: The required quantity of RNA depends on the abundance of the target RNA. We generally use between 5 and 20 µg of total RNA per lane of the denaturing PAGE.

15. Mix agarose (~1.0–1.2% wt/vol) with 1 × TBE and heat in a microwave oven until boiling.

16. Allow the agarose solution to cool down and pour it into a gel holder with a comb.

17. Add 1/10 volume of gel loading solution (10×) to sample RNA (~1–3 µg).

18. Run the gel at 80V (~45 min) and visualize the quality of the RNA samples on a UV transilluminator. The clear observation of distinct, 18S and 28S, rRNA and tRNA bands is a good indicator that the RNA sample is intact.

Prepare denaturing PAGE (2–3 h)

19. We use the Bio-Rad PROTEAN 3 vertical gel system with 10.1 × 7.3 cm^2 plates and 1.5 mm spacers. Clean the glass plates with DEPC-treated water and assemble the plates following manufacturer's instructions.

20. Prepare 10 ml of 15% denaturing gel using 6 ml SequaGel Concentrate, 3 ml SequaGel Diluent, 1 ml SequaGel Buffer, 4 µl TEMED, and 40 µl 10% ammonium persulfate. Note: Decreasing the acrylamide content may yield poor resolution of final bands corresponding to different sizes of RNAs.

21. After gel polymerization, remove the comb carefully and apply the gel onto the PAGE apparatus. Note: Gels require at least one hour to set.

22. Rinse the wells off any excess acrylamide and urea with 1× TBE using a syringe and a needle. Ensure that the wells are clean and devoid of pieces of gel or urea.

23. Pre-run the gel at ~200–400 V for 60 min.

Sample and marker preparation (0.5 h)

24. Add Gel Loading Buffer II to RNA samples (~5–20 µg) and the previously synthesized size markers. Denature (95 °C, 5 min) the RNA samples and chill the denatured samples on ice.

25. Add ethidium bromide to the size markers at 10–50 µg/ml final concentration before loading.

Electrophoresis (1.5–2 h)

26. Immediately before loading samples onto the gel, rinse the wells of the warm gel thoroughly.

27. Load samples with marker in each wells and run the gel at about 200V for 50 min or until bromophenol blue (BPB) from the loading solution reaches about 1 cm above bottom of gel. BPB and cyanol from the loading solution runs around 15 bases and 60 bases, respectively.

28. To enable the estimation of the RNA sizes of the final bands, use a transilluminator to image the gel along with a ruler placed parallel to the gel.

29. Disassemble the gel apparatus and soak the gel in 1×TBE for 10 min with shaking.

Transfer gel to membrane (0.5–2 h)

30. Cut nylon membrane and 6 sheets of 3MM Whatman papers to the size of the gel and soak them in 1×TBE for 5 min with shaking.

31. Starting from the side of the positive electrode of semi-dry transfer cell, place three sheets of Whatman papers, nylon membrane, the gel, and an additional three sheets of Whatman papers to form a paper–membrane–gel–paper sandwich. With each layer added, roll out any bubbles with plastic sterile pipette between the gel, membrane and papers. Add another three sheets of papers on top of the gel (**Figure S1**). Note: Ensure that the sandwich is moist by adding a small but adequate amount of 1× TBE buffer.

32. Transfer the RNA to the membrane at 10–15V (60–90 min) in a cold room. The majority of the BPB should remain on the membrane after completion of transfer, an indication that transfer has occurred.

Cross-linking (1.5–2.5 h)

33. Saturate a Whatman paper with freshly prepared cross-linking EDC reagent. On top of the Whatman paper, place the membrane on the side void of RNA(2).

34. Wrap Whatman paper and the membrane in Saran and incubate at 60 °C for 1–2 h to facilitate RNA–membrane cross-linking.

35. Remove residual cross-linking solution by thoroughly rinsing the membrane with distilled water. Note: The membrane can be now wrapped in Saran and stored at -20 °C for later use.

Preparation of DIG-labeled LNA probe (0.5 h)

36. In a reaction vial, add 2 µl containing 50 pmol of LNA probe to 7 µl of DEPC-treated water.

37. In a reaction vial kept on ice, mix reaction buffer (4 µl), $CoCl_2$-solution (4 µl), DIG-dUTP solution (1 µl), dATP solution (1 µl), and 400 U Terminal transferase (1 µl) that are available from DIG-labeling kit. Centrifuge briefly, incubate (15 min) at 37 °C, and then place on ice.

38. Stop the reaction by adding 2 µl 0.2M EDTA (pH 8.0).

Hybridization (3–12 h+)

39. After completion of cross-linking, roll membrane with RNA side in and insert into hybridization bottle.

40. Add 5–15 ml of ULTRAhyb hybridization buffer and pre-hybridize at 37 °C for at least 30 min in hybridization oven.

41. Denature the probe at 95 °C for 1 min and add the probe to the hybridization buffer to yield a final concentration of 0.5 nM.

42. Hybridize at 37 °C overnight with slow rotation.

Washing, blocking, and detection (2–6 h+)

43. Discard hybridization buffer from bottle.

44. Wash the membrane twice with Low Stringent Buffer at 37 °C for 15 min.

45. Wash the membrane twice with High Stringent Buffer at 37 °C for 5 min.

46. Briefly rinse the membrane with Washing Buffer at 37 °C for 10 min.

47. Incubate the membrane in Blocking Buffer for 3 hrs at room temperature.

48. Replace the blocking buffer in hybridization bottle with DIG antibody solution prepared by mixing DIG antibody solution and blocking buffer at a ratio of 1:15,000.

49. Incubate the membrane at room temperature for 30 min.

50. Wash the membranes in DIG Washing buffer four times for 15 min each.

51. Incubate membrane in development buffer for 5 min. Note: Do not allow the membrane to dry if it must be reused to detect additional RNAs.

52. Prepare substrate solution by adding CSPD (see Reagents) to development buffer at a ratio 1:100.

53. Remove blot from bottle with clean forceps and place on Saran wrap. Apply the substrate solution to the surface of the membrane, incubate for 5 min.

54. Put the membrane in a heat-sealable plastic bag, squeeze out extra buffer from membrane surface, and seal the bag.

55. Place the sealed membrane in the dark at 37 °C for 15 min.

56. Expose the membrane to X-ray films using phosphor imaging screen at room temperature or using intensifier screen at -80 °C. Note: While a few seconds of exposure is sufficient to get a

strong signal for highly expressed small RNAs, poorly expressed small RNAs will require more exposure time. Alternatively, the ChemiDoc-IT Imaging System could be used to detect photo-emissions from the membrane. ChemiDoc-IT Imaging System has several advantages over X-ray films including low-background noise and capability to detect a wide range of signal intensities.

References

1. Valoczi,A., Hornyik,C., Varga,N., Burgyan,J., Kauppinen,S. and Havelda,Z. (2004) Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. *Nucleic Acids Res.*, **32**, e175.

2. Pall,G.S. and Hamilton,A.J. (2008) Improved northern blot method for enhanced detection of small RNA. *Nat. Protoc.*, **3**, 1077-1084.