

Northern blot analysis for microRNA

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Materials

1. 10~50 µg of total RNA extracted from HeLa cells treated with siRNA
2. RNA loading buffer
3. Probe: DNA oligonucleotide complementary to the microRNA of interest (6µM)
4. ^{-32}P -ATP (20µCi/µl, 800 mCi/mmol)
5. T4-polynucleotide kinase (T4 PNK, TAKARA)
6. RNA size marker (Decade RNA marker, Ambion)
7. Positive charged nylon membrane (Zeta-probe GT membrane, Bio-Rad)
8. 3M paper
9. Transfer unit: Hoeffer TE70 SemiPhor Semi-Dry Transfer unit
10. 0.5X TBE
11. Hoeffer gel apparatus (18 X 16cm) or equivalent, combs (1mm, 15 wells), spacers (1mm), and a power supply
12. Urea-polyacrylamide stock solution (12.5%): Dissolve 118.75g acrylamide, 6.25g Bis-acrylamide, 100ml 5X TBE (final 0.5X) and 420.42g Urea (final 7M) in water to make 1 L.
13. Ammonium persulfate (APS) solution: 20% dissolved in water
14. 3M sodium acetate, pH5.5
15. Glycogen
16. TEMED

17. UV-crosslinker
18. Hybridization chamber
19. Hybridization bottle
20. Hybridization buffer (Expresshyb solution, Clontech)
21. Salmon sperm DNA (10 mg/ml)
22. 20X SSC: Dissolve 175.3g NaCl (final 3M) and 88.2 g sodium citrate (final 0.3M) in water to make 1L. Adjust the pH to 7.0 with a solution of HCl and then sterilize by autoclaving.
23. 10% SDS solution
24. Washing solution I: 2X SSC, 0.05% SDS
25. Washing solution II: 0.1X SSC, 0.1% SDS
26. Glass tray
27. Kodak X-AR5 autoradiography films
28. Autoradiography cassettes with intensifying screens

Procedure

Gel electrophoresis and blotting

1. Assemble a gel cast.
2. Mix 30 ml of 12.5% urea-polyacrylamide stock solution, 100 μ l of 20% APS and 20 μ l of TEMED. Pour this mixture into the gel cast immediately and insert a comb as fast as possible because the gel solidifies in a few minutes.
3. Pre-run at 350V for at least 60 min using 0.5X TBE as the running buffer.
4. While pre-running the gel, prepare RNA samples. Add 10 μ l of RNA loading buffer to 10~50 μ g of HeLa total RNA dissolved in 10 μ l of TE buffer, and then boil

this at 95°C for 5 min.

5. Load the RNA sample (from step 4) on 12.5% pre-run urea-polyacrylamide gel and run at 350V until bromophenol blue reaches the bottom of the gel. For RNA size marker, we use Decade RNA marker from Ambion. The markers are end-labeled with ^{-32}P ATP and polynucleotide kinase according to the manufacturer's manual. The marker is loaded on a lane that is 2 lanes away from the samples. The amount of radioactivity of the marker should be 30~50 c.p.s.

6. Disassemble the gel cast and remove one of the glass plates from the gel. Place OHP film on the gel surface and detach the gel from the glass plate.

7. Transfer the gel to a glass tray filled with 200 ml of 0.5X TBE buffer.

8. Add a few drops of EtBr to the 0.5X TBE and stain the gel for 10 min on a rocker. Destain it with 200 ml of 0.5X TBE for 5 min. Examine the gel under UV for quality and quantity of the RNA.

9. Soak Zeta-Probe GT membrane in 0.5X TBE for 10 min. Soak 4 pieces of filter paper (3M papers) in 0.5X TBE.

10. Assemble the transfer unit in the following order: two pieces of 3M paper, Zeta-Probe GT membrane, gel, and two pieces of 3M paper on the top.

11. Transfer at 250mA at RT for 1 hr.

12. Crosslink the transferred RNA to the membrane by UV-irradiation for 1 min.

13. Sandwich the membrane between two dry 3M papers and bake at 80°C for 30 min.

14. Store at RT between filter papers until use.

Preparation of the probe

1. Mix the following: 3 μl of 6 μM DNA oligonucleotide probe, 2 μl of 10X T4 PNK buffer, 5 μl of ^{-32}P -ATP, 1 μl of T4 PNK and 9 μl of distilled water.
2. Incubate at 37 $^{\circ}\text{C}$ for 1hr.
3. Incubate at 68 $^{\circ}\text{C}$ for 10 min to inactivate T4 PNK. Add 180 μl of TE buffer, 20 μl of 3M sodium acetate (pH 5.5), 1 μl of glycogen and 800 μl of 100% EtOH. Incubate at -80 $^{\circ}\text{C}$ for at least 20 min.
4. Spin at 13,200rpm for 15 min at 4 $^{\circ}\text{C}$.
5. Wash the pellet by adding 500 μl of 75% EtOH and spin again for 5 min.
6. Carefully remove the liquid, air-dry the pellet, and store the pellet at -20 $^{\circ}\text{C}$.
7. Immediately before use, dissolve the probe in 50 μl of TE. We usually get $\sim 1 \times 10^7$ cpm in total. If this is used in 5 ml hybridization buffer, the final radioactivity is $\sim 0.2 \times 10^7$ c.p.m./ml.

Hybridization

1. Soak the membrane with 2X SSC.
2. Put the membrane into the hybridization bottle.
3. Put 5 ml of hybridization solution (pre-warmed to 37 $^{\circ}\text{C}$) into the bottle.
4. Add 50 μl of salmon sperm DNA (boiled at 95 $^{\circ}\text{C}$ for 3 min and quick-chilled on ice for 1 min) and mix.
5. Incubate at 37 $^{\circ}\text{C}$ for 30 min for pre-hybridization with rotation in a hybridization chamber.
6. Replace the solution with 5 ml of fresh hybridization solution pre-warmed at 37 $^{\circ}\text{C}$.
7. Add 50 μl of the radiolabeled probe and 50 μl of salmon sperm DNA (both

boiled and chilled) to the hybridization solution and mix well.

8. Incubate at 37°C for 1hr for hybridization with rotation in the hybridization chamber.

9. Rinse the membrane in the bottle with 5~10ml of washing solution I and repeat once.

10. Transfer the membrane to a glass tray filled with 200ml of washing solution I.

11. Shake the glass tray at RT for 30 min.

12. Repeat washing with washing solution I.

13. Wash with washing solution II for 15 min, twice.

14. Remove the residual liquid quickly and wrap the membrane in plastic wrap and expose to an X-ray film at -80°C. The bands corresponding to pre-miRNA and mature miRNA get weaker when Drosha or DGCR8 is successfully depleted (Fig. 5).

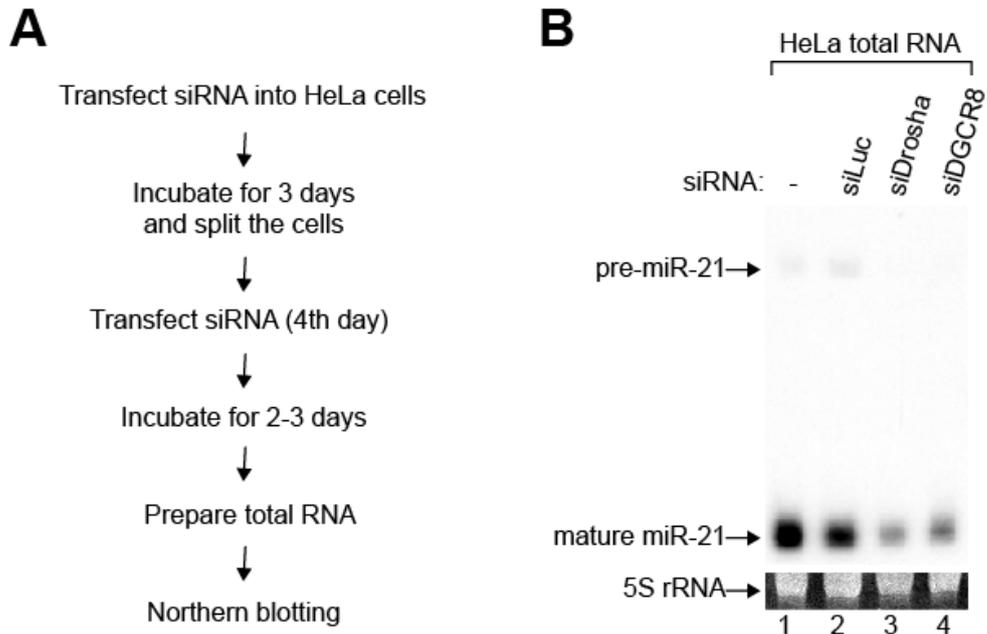


Figure 5. Northern blot analysis of pre-miRNA and mature miRNA. (A) Experimental scheme. (B) Typical results. RNAi was carried out by transfection of siRNA duplexes into HeLa cells. Both pre-miR-21 and mature miR-21 are detected by radiolabeled DNA oligo probe. 5S rRNA bands stained with ethidium bromide are presented as a loading control.