PROTOCOL EXCHANGE | *COMMUNITY CONTRIBUTED*

Northern Blot

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Abstract

Protocols of northern blot used to detech mRNA miRNA, or lncRNA expression.

Subject terms: Cell biology

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Reagents

Autoclaved, DEPC-treated water: all the solution should be steriled and RNASE free. For those cannot autoclaved, use only DEPC-treated water.
Agarose (Biowest, cat. no. 111860)
Formaldehyde (Merck, cat. no. 344198)
MOPS buffer (10×): 200 mM MOPS, 50 mM sodium acetate, 20 mM EDTA, pH 7.0. This solution can be diluted to 1×MOPS buffer with DEPC-treated water. 1×MOPS buffer is the Running buffer for electrophoresis.
RNA Loading buffer: Takara, cat. No. 9168
SSC buffer (20×): 3 M NaCl; 300 mM sodium citrate, pH 7.0. This solution is the transfer buffer and can be diluted to other concentration with DEPC-treated water during the later process.
5’ digoxin labeled DNA probe: Exiqon. MiRCURY LNA Detection Control Probe U6 hsammurno (cat. No. 99002-01): /5DigN/CACGAATTTGCGTGTCATCCTT. MiRCURY LNA Detection Probe has-miR-142-5P (cat. No. 38514-01): /5DigN/AGTAGTGCTTTCTACTTTATG. MiRCURY LNA Detection Probe has-miR-130a (cat. No.38029-01): /5DigN/ATGCCCTTTTAACATTGCACTG.
Nylon Membranes, Positively Charged: GE healthcare, cat. No. RPN303B
DIG Easy Hyb: Hybridization buffer (Roche, Cat. No. 11603558001)
Low stringency buffer: 2× SSC, containing 0.1% SDS
High stringency buffer: 0.1× SSC, containing 0.1% SDS
Maleic Acid Buffer: 0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5
Blocking Solution (10×): Blocking reagent (Roche, cat. No. 11096176001) is dissolved in Maleic Acid Buffer to a final concentration of 10% (w/v) and autoclaved and stored at 2 to 8°C. When used, dilute 10× Blocking Solution 1:10 with Maleic Acid Buffer freshly.
Washing buffer: 0.1 M Maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20

Anti-Digoxigenin-AP Solution: Dilute Anti-Digoxigenin-alkaline phosphatase antibody (Roche, cat. No. 11093274910) 1:10000 in Blocking solution not early than 2 hours before the incubation.
Detection Buffer: 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5
CSPD (Roche, Cat. No. 11363514910): a chemiluminescent substrate for alkaline phosphatase that enables sensitive and fast detection of biomolecules. Dilute CSPD 1:100 in dectection buffer in the dark.
Stripping buffer: 0.2 M NaOH, 0.1% SDS

Procedure

Separating RNA Samples on an Agarose Gel

1. Prepare a 1% × 2% agarose gel: Melt 1.0 g agarose in 84.6ml DEPC water and boil in a microwave. Let it cool down to 60°C, and add 10ml of 10 x MOPS buffer and 5.4ml 37% formaldehyde (total 2% (vol/vol))and fill the apparatus. Insert the comb and solidify for 30minutes.
2. Mix at least 10 µg total RNA with Loading Buffer (1:1).
3. Denature the RNA/Loading Buffer mixtures at 65°C for 10 min. Immediately chill the denatured samples on ice for 1 min.
4. Load the samples and RNA markers onto a dry gel. Fill the slot carefully with running buffer (1× MOPS buffer) only up to the top of the gel (but not over the sample slots).
5. Run the gel at a higher voltage (i.e., >34 V/cm) for about 10 min to get the samples into the gel. Then add extra running buffer to submerge the gel completely.
6. Run the gel at 34 V/cm for at least 2 h, until the RNAs are well separated.
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Transferring RNA to a Membrane (Capillary Transfer Method)

1. After the electrophoresis, soak the gel twice (2 × 15 min) in 20× SSC to remove formaldehyde (which can inhibit transfer).
2. Set up a blot transfer as follows, roll a sterile pipette over the sandwich to remove all air bubbles that formed between any two parts of the blot “sandwich”:
 Place a piece of Whatman 3MM paper that has been soaked with 20× SSC atop a “bridge” that rests in a shallow reservoir of 20× SSC.
 Place gel, facing down, on top of the soaked sheet of Whatman 3MM paper.
 Cut a piece of Positively Charged Nylon Membrane to the size of the gel and place the dry membrane carefully on top of the gel.
 Complete the blot assembly by adding two dry sheets of Whatman 3MM paper, cut to the size of the gel, a stack of paper towels, a glass plate, and a 200 – 500 g weight.
3. Let the RNA transfer (at least 6 h, preferably overnight) under RNase-free conditions, with sterile, RNase-free 20× SSC as transfer buffer.
4. The next day, disassemble the transfer stack. Place the membrane (RNA side facing up) on Whatman 3MM paper that has been soaked in 2× SSC.

5. Expose the wet membrane to UV stratalinker at 120 mJ/cm^2 for 1 minutes to fix the RNA to the blot.

Prehybridizing and Hybridizing the Blot

1. Place the correct amount of DIG Easy Hyb (about 10-15ml for 100cm^2 membrane) in a sterile tube, then place the tube in a water bath set at 50°C.
2. Place the blot into DIG Easy Hyb and incubate the blot for 30 minutes at 50°C.
3. During the prehybridization incubation, prepare the hybridization solution with 5'Dig-labeled DNA probe (final concentration: 20-50ng/ml or 1.5-4nM) in prewarmed DIG Easy Hyb.
4. Pour out the prehybridization buffer and immediately replace with prewarmed hybridization solution containing DIG-labeled probe.
5. Incubate the blot with probe at 50°C for 6 – 16 h, with gentle agitation.

Tips: the hybridizing temperature can be optimized to your own target RNA. For example, to get the best result of mir-142-5p, we hybridized the blot at 42°C overnight.

Strict washes

1. After the hybridization is complete, submerge the membrane in an RNase-free plastic container (tray, dish, etc.) tray containing Low Stringency Buffer (2× SSC containing 0.1% SDS).
2. Incubate the tray at room temperature for 5 min with shaking.
3. Pour off the used buffer and immediately cover the membrane with fresh Low Stringency Buffer.
4. Incubate the tray an additional 5 min at room temperature with shaking. During the process, preheat High Stringency Buffer(0.1× SSC containing 0.1% SDS) to 50°C.
5. Pour off the used Low Stringency Buffer. Immediately add the preheated High Stringency Buffer to the tray containing the blot.
6. Incubate the blot twice (2 × 15 min, with shaking) in High Stringency Buffer at 50°C.

Chemiluminescent Methods for Detection of Probes on a Blot

1. Transfer the membrane to a plastic container containing enough Washing Buffer and incubate for 2 min at room temperature, with shaking.
2. Discard the Washing Buffer and add enough Blocking Solution to tray.
3. Incubate membrane for 30 min at room temperature, with shaking. This blocking step can last up to 3 hours without affecting results.
4. Discard the Blocking Solution and add 20 ml Anti-Digoxigenin-AP Solution to the tray. Incubate the membrane for 30 min, with shaking.
5. Discard the Antibody Solution.
6. Wash membrane twice (2 × 15 min) with enough Washing Buffer.
7. Equilibrate membrane 3 min in Detection Buffer.
8. Place the membrane (DNA/RNA side facing up) inside a container. Apply enough CSPD

working solution over the surface of the blot until the entire surface is evenly soaked.
9. Incubate membrane for 5 min at room temperature.
10. Incubate damp membrane for 10 min at 37°C to enhance the luminescence reaction.
11. Expose the sealed envelope (containing the membrane) at room temperature to Lumi-Film X-ray film (15 – 25 min) and adjust the exposure time to get a darker or lighter band pattern.

Techniques for Stripping and Reprobing a Membrane

1. After detection of the first target on the membrane, rinse membrane thoroughly with double distilled water for 1 min.
2. Wash membrane twice (2 × 15 min) at 37°C in Stripping Buffer.
3. Rinse membrane in 2× SSC for 5 min.
4. Reprobe membrane with a second DIG-labeled probe: repeat the hybridization and detection procedure with a different DIG-labeled probe.

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Competing financial interests

All the authors declare no conflict of interest.

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