SOP: RNA Blot Hybridization

By

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A. Electrophoresis of RNA

1. Preparative work:

   i) Clean the followings with laboratory detergent and soak in 3% hydrogen peroxide (H_2O_2) for 20 min:

      - electrophoresis apparatus
      - Erlenmeyer flask
      - measuring cylinders (50, 500, 1000 ml)
      - plastic container
      - blot transfer tray

      Rinse thoroughly with Milli-Q H_2O (or DEPC H_2O) and dry.

   ii) Deionize formaldehyde and formamide. It is a must when formaldehyde looks yellow in color or has a pH<4 (i.e. oxidized). Formamide in the deionized form can be available from Amresco. *Deionization*: Add 1 g of mixed bed resin (AG 501-X8, Bio-Rad or M-8032, Sigma) to 10 ml of formaldehyde/formamide. Swirl the mixture every 3-5 min within the 1 h-deionization. Add extra resin when the beads turn golden-yellow (i.e. an indication of saturation). Remove the resin by filtration. The deionized materials should be used immediately. Discard any unused portion.

iii) Prewarm appropriate amounts (see below) of the 10×MOPS buffer (0.2 M MOPS, pH 7; 50 mM NaOAc; 10 mM EDTA, pH 8.0; filter sterilize and store in a brown bottle) and 36.5 % formaldehyde in a 55-60°C waterbath. Reserve a heat-block or PCR machine if possible. Empty a place in the fume hood for pouring the gel.

2. Prepare 200 ml (50 ml) of molten agarose solution by melting 2.4 g (0.6 g) of agarose (use a non-foaming one, such as BioRad or Promega) in 170 ml (42.5 ml) DEPC H_2O in an Erlenmeyer flask in a microwave oven. Note: After adding agarose to water, wait for at least 1 min before melting (this can minimize foaming). Allow to cool to 55-60°C under running water and place in the preset waterbath (at 55-60°C). Equilibrate for 15 mins.

3. Add 20 ml (5 ml) prewarmed 10×MOPS buffer and 10 ml (2.5 ml) prewarmed 36.5% formaldehyde. This will produce a solution of 1.2% agarose in 1×MOPS buffer and 0.6 M formaldehyde.

4. Cast a 0.5- to 0.75-cm-thick gel (a thinner gel offers better resolution of the RNA) using the solution prepared in (3). This should be done in a fume-hood to minimize formaldehyde fumes exposure (formaldehyde is toxic and teratogenic). Allow the gel to solidify for at least 30 min.

5. Prepare a 1.25×RNA loading buffer as follows:

100% formamide	62.5 μl
10×MOPS	12.5 μl
36.5% formaldehyde	24.0 μl
4% bromophenol blue	1.0 μl
Total:	100 μl (for 10 RNA samples)

6. Prepare the RNA for denaturation by mixing the following in a sterile 0.2 or 0.5 ml thin-wall microcentrifuge tube:

   2.5 μl RNA (up to 20 μg; dissolved in DEPC H_2O)
   10.0 μl RNA loading buffer
   0.5 μl ethidium bromide (1 mg/ml; prepared with DEPC H_2O)

   Thorough pipetting is essential.

   Note:

   i) Do not exceed 20 μg RNA per lane to prevent overloading and losing resolution. If rare mRNAs are of interest, as much as 4-5 μg of poly(A)$^+$ RNA can be applied per lane. But in any case, use of mRNA (e.g. 1 μg for moderate expression) always produces better resolution and lower background.

ii) An appropriate RNA marker (e.g. Gibco/BRL 0.16-1.77 kb or 0.25-0.95 kb RNA ladder) should be included.

7. Denature all RNA samples and the RNA marker by heating at 65°C for 10 min using a PCR thermal cycler. Quench on ice for 2 min. Quick spin to collect the mixture at the bottom of the tube.

   During the incubation of the RNA, immerse the gel in running buffer (1×MOPS; without ethidium bromide). Note: Use the minimal amount of buffer necessary to completely cover the gel.

8. Electrophorese the samples at a maximum of 5 V/cm distance between the electrodes (usually 100 V is OK). At the end of electrophoresis, capture the image with inclusion of dimension (using a fluorescent ruler) under an UV transilluminator.

9. Soak the gel in plenty of DEPC-H_2O (or Milli-Q H_2O) and apply gentle shaking to remove formaldehyde. Typically, a 30 min-soaking in several changes of H_2O is adequate.

B. Northern Transfer

10. Capillary transfer can be a choice for RNA blotting. Transfer (5×SSC) should be allowed to take place overnight (i.e. at least 16 h). Baking at 80°C for 2 h is a good means for immobilization and storage (either at room temp. and away from moisture or at -20°C). Once the RNA has been immobilized, it is relatively resistant to degradation.

C. Hybridization

11. Hybridization using ExpressHyb (Clontech) is highly recommended. Hybridization can be performed at 60-68 °C for 2 h using a cDNA probe at 2×10^6 cpm/ml. Membrane is washed according to the manufacturer's instructions (i.e. initial wash: 2×SSC, 0.05% SDS, rm temp for 30-40 min with several changes; final wash: 0.1×SSC, 0.1% SDS, 50°C for 40 min with one change).

D. Autoradiography

12. Expose the membrane to the K-screen (Kodak) for overnight and capture the image using the Fx molecular imager (BioRad). Over-exposure should be prevented if quantification is required (the Fx software will indicate saturated pixels).

13. For normalization, strip the membrane accordingly and probe with a housekeeping gene (e.g. β-actin) or a rRNA gene.