RNA BLOTS: Formaldehyde method

Note: Formaldehyde is toxic and a potential carcinogen. Handle it in the fume hood.

A. Preparation of gel

1. The lab has RNAse-free gel units dedicated to RNA blot analysis. If one of these is not available, prepare an RNAse free gel unit (including the tray and combs) by soaking in 1% SDS overnight (or commercial product like RNAse-Off from CPG)
2. Decide on the proper concentration of agarose. The standard range is 0.8 to 1.4% (w/v), depending on desired size range). An "average" gel is 1.2%.
3. Pour and run the gel in a hood to avoid formaldehyde vapors To make the gel:

   i. use the microwave to melt agarose in sterile water. Later you will need to add MOPS buffer and formaldehyde, so plan your volumes accordingly! Using DEPC-treated "RNASe-free" solutions for making the gel is not essential, since the formaldehyde will inactivate RNAse.

   ii. Cool the agarose to approximately 60C in a water bath.

   iii.Quickly add 10X MOPS running buffer to 1X final concentration, and formaldehyde to 0.7 M. The formaldehyde stock is a 37% solution, or 12.3 M, so dilute 1:17.5. *This concentration of formaldehyde is 1/3 of the "standard" protocol—but works fine!*

   100 ml gel recipe: 1.2 g agarose in 81 ml water
   10 ml 10X MOPS
   5.8 ml of 37% formaldehyde

   10x MOPS (500 ml)
   21 g MOPS
   3.4 g sodium acetate (mw 136.08)
   10 ml 0.5 M EDTA
   400 ml H2O
   pH to 7.0 w/ NaOH, raise vol. to 500ml

   10X MOPS is: 0.2 M MOPS **pH 7.0,** 10 mM EDTA, 50 mM NaOAc. Autoclave and store in the dark at 4C. It is normal for 10x MOPS to turn a little yellow after it is autoclaved (Don't overautoclave!), and the yellowing will increase over time. Do not use it once it becomes strongly yellow, and do not "overautoclave" since this will increase the yellowness.

B. Sample preparation

1. Typical gels include 5-10 µg of total RNA or 1 µg polyA+ RNA per lane.
2. Add RNA (your samples and RNA standards, if desired) to RNAse-free tube. Adjust volume to 4 µl with DEPC-treated water; if the original volume of RNA is too high, lyophilize or ethanol precipitate. Add:
   - 10 µl formamide (deionized by passage through mixed bed resin)
   - 4 µl formaldehyde
   - 2 µl 10 X MOPS buffer
   - 2 µl 0.4 mg/ml ethidium bromide (the general lab stock is 10 mg/ml).
   - 1 µl 0.2% bromophenol blue dye mix (0.2% bromophenol blue, 10mM EDTA, 50 % glycerol in DEPC-treated water)

   (The total volume is 23 µl; you can scale up or down).
3. Heat at 65C for 10 min, spin briefly to collect condensate, and place on ice (load the gel within about 10 min).

   Note: adding ethidium bromide allows direct visualization of the RNA after electrophoresis, which is a big advantage for quantitation. However, it does inhibit transfer of the RNA a bit during subsequent blotting. Don't add too much!

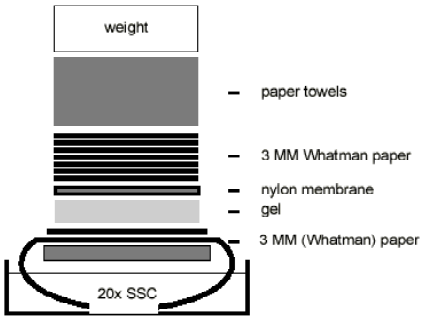
C. Electrophoresis

1. The gel should be cast, loaded, and run in the hood.

2. Place a stir plate under the cathode and anode buffer chambers to mix the running buffer. Stirring while running is not essential, but helps prevent a pH gradient from building up, keeps the gel cooler, and increases resolution.
3. Cover the gel with 1-2 mm of 1X MOPS buffer (no formaldehyde). Try to load the gel within an hour, to avoid dilution of the formaldehyde in the gel.
4. Load the gel and run for at least ten minutes at 5 V/cm in 1X MOPS buffer, without stirring. *Note: Filling lanes flanking your samples with loading buffer improves the running of the gel. Do not view the gel on the light box in mid-run ;EtBr nicks nucleic acids, so this will increase smearing.*
4. After about 10-20 minutes, once the samples have entered the wells, start the stir bars (a modest speed, ca. 60 rpm, is adequate).
5. Electrophorese at 5 V/cm maximum until the running dye has run 9-10 cm (2/3 to 3/4 of the way down the gel). Take a photo to document.
6. It is normal for the gel to become a little warm during the run.
7. Photograph the gel to document the concentration and integrity of the RNA. However, note that formaldehyde gels are much weaker than normal agarose gels, particularly when warm (cooling will "firm them up" a bit). So handle the gel carefully!!

D. Blotting to nylon membranes (Hybond N^+)

1. Rinse the gel in autoclaved water with gentle agitation for 15 min.
2. *Optional step: ONLY if you intend to probe of RNAs larger than 3 kb, soak the gel in 0.05N NaOH-1.5M NaCl for 15 min with gentle shaking, then 0.5 M Tris-HCl (pH 7.4)-1.5 M NaCl for 30 minutes.*
3. Immerse the gel in autoclaved 20X SSC or SSPE for 30-45 min, with gentle shaking. Use 200 ml for every 100 ml of gel.
4. Set up standard capillary blot as pictured:
   i. Fill tray or glass dish with blotting buffer (20 x SSC or 20 x SSPE; 10X may also be used).
   ii. Make a platform and cover it with a wick made from three sheets of Whatman 3MM paper, saturated with blotting buffer.
   iii. Place the gel on the wick (upside down).
   iv. surround with plastic to prevent "short circuits."
   v. overlay with dry Hybond membrane, cut to the size of the gel. Eliminate any bubbles!
   vi. cover with 3 sheets of 3MM paper, cut to the size of the gel, and soaked in blotting buffer.
   vii. cover with a stack of paper towels, a stiff piece of plastic, and a 0.5-0.75 kg weight.



5. Allow transfer to proceed for about 16 hours.
6. Carefully dismantle the apparatus, and mark the membrane with pencil to allow the lanes to be identified later on.
7. Fix the RNA to the blot. Rinsing the membrane following transfer is <u>not</u> advised. Fix the RNA UV crosslinking, RNA side up (use the program on the UV oven). You can also dry the membrane at 80°C for 60 minutes with or without vacuum, but this is inferior to UV treatment and is not recommended).
8. <u>Seal</u> the gel and paper towels in a plastic bag, seal the bag, and dispose.
9. Blots should be stored in the dark, wrapped to avoid contamination, at room temperature. Do not store in the refrigerator: condensation and fungi will destroy the membrane.