Northern for Detecting Big RNAs separated in Agarose Gels

A. E. Pasquinelli (see Bagga et al., 2005)

Running Gel:

1. Make fresh 0.5M MOPS, pH 7.0 (52.3g/500ml) (filter sterilize and store in dark - good for ~1 month)

Make 800mL of gel running buffer:
- 64ml .5M MOPS (40mM)
- 3.2ml .5M EDTA (2mM)
- 732.8ml ddH2O

2. Prepare gel: for 1% gel in ~12X14cm casting tray dissolve 1g agarose in 100ml gel running buffer by heating; when cool add: 2ul of 10mg/ml EtBr, swirl to mix, pour gel and add comb that will hold at least 30ul volume (1.5mm teeth work well).

3. Prepare RNA samples:
- 5-20ug total RNA in 10ul volume
- 15ul MOPS/EDTA gel running buffer
- 5ul formaldehyde

____

30ul total volume - mix well

heat at 70C for 10-15 minutes and then ice 1'; add 6ul of 6X glycerol loading buffer with dyes (300ul 100% glycerol + 25ul 10% BPB/XCFF dyes + 675ul DEPC H2O)

- include an RNA (or DNA) size marker lane for orientation and sizing

4. Run at 80V for 2 hours to 2.5hrs

5. Photograph gel under UV - you should easily see the large ribosomal RNA bands as well as your size marker; smeary rRNA bands indicates RNA degradation or poor denaturation.

Transferring:

6. Trim gel to remove wells and then rinse gel gently in sterile water for 5' with gentle shaking 2X

7. Cut membrane (I recommend BIORAD Zeta Probe GT-tested - #162-0194) to size of gel (usually 12x13) and carefully wet by laying it on top of ddH2O in a tray, allow liquid to soak into membrane and then gently shake to immerse entire membrane, about 1min

8. Transfer membrane to another tray of 10X SSC and equilibrate with gentle rocking for at least 5'

9. In pyrex baking dish, place plastic gel casting tray upside down, fill tray with 10X SSC to just below top of tray and layer with:

    2 sheets whatman, wetted in 10XSSC, with ends dipped in 10X SSC to serve as wicks
    gel with bottom side up
    Membrane
    3 pieces whatman, wetted in 10X SSC, cut to size of membrane
    stack of paper towels 5-10cm high and cut to size of gel
    place light weight evenly on top of stack (e.g. glass plate)
    Transfer for 6-18hrs (typically overnight)

10. Rinse blot in 6X SSC for 2' to remove any agarose particles; check blot under UV and mark lanes, rRNAs (28S ~3500nt, 18S ~1750nt)

11. On a sheet of dry Whatman, place membrane RNA side up (not covered by Whatman) and X-link at 1200 microJ x 100 on Optimal-setting. Sandwich membrane between dry whatman paper and bake in oven at 80C for 30'. (You can store blot at RT like this covered in saran wrap).

Prehybridization:

1. Make prehyb solution: 12.5 mL 20X SSC
17.5 mL 20% SDS
18 mL water
1 mL 1M sodium phosphate (31.6ml 1M NaH2PO4.H2O + 68.4ml 1M Na2HPO4.8H2O)
1 mL 50X Denhardt's (5g Ficoll + 5g polyvinylpyrrolidone + 5g BSA/ 500mL, heat and stir to dissolve and filter sterilize, aliquot and store at -20C)
____
50mL - place at 58C to thin solution (this takes a while so make it ahead of time)
2. Gently re-wet membrane by laying it on 2X SSC and then agitate to fully immerse; place back between whatman to ease into seal a meal bag and then remove whatman
3. Seal bag and cut out corner for adding solution - add ~25mL of prehyb (place other 25ml back at 58C) + 250uL 10mg/ml sheared salmon sperm that has been boiled 5' and put on ice 1'
4. Squeeze out excess bubbles and seal and gently massage solution throughout
5. Place at 58C with shaking ON (or at least 4hrs.)

Hybridization:

1. Prime-It II Random Primer Labeling Kit (Stratagene, #300385) - use DNA fragment (typically gel purified PCR product) 500-1500bp

Mix: ___ul DNA for 25ng (gel purified!)
___ul DEPC H2O
10ul random oligo primers
____
34ul t.v.

Boil 5' (use screw cap tube!)

Briefly centrifuge to bring liquid to bottom of tube

Add: 10ul 5X Buffer (-dATP)
5ul alpha-32P dATP (3000 Ci/mmol)
1ul Exo(-) Klenow (5U/ul)

Incubate at 37C for 10'

Add 2ul Stop Solution

2. Purify probe by precipitation (you can try spin columns but there is less probe loss with ethanol ppt)

Add 240ul 5M NH4OAc + 750ul ethanol; vortex and dry ice/ ethanol bath 15'

Centrifuge at top speed 30' (does not need to be done at 4C and will likely contaminate your centrifuge with radioactivity)

Carefully remove ethanol and wash with 500ul 70% ethanol - spin 10'

Remove sup and let air dry

Resuspend pellet (you likely will not see anything but you should hear it) in 10ul Te

3. Denature probe: 10ul probe
   100ul .2N NaOH
   250ul 10mg/ml salmon sperm
   ____
   - boil 10' and then put on ice
4. Cut seal a meal bag and squeeze out prehyb; add remaining 25mL hyb solution
5. Pipet in probe solution
6. reseal and shake at 58C ON

Wash:

1. Cut corner of seal a meal bag and carefully pour probe solution out (can pour into 50ml conical tube and freeze at -20C and reuse); cut open rest of bag and place membrane in tray
2. Wash solution: 60ml 20X SSC
   80ml 50X Denhardt's
   100ml 20% SDS
   10ml 1M Sodium Phosphate
   150ml water
   ____
   400ml, place at 58C until in solution (this takes a while so make it ahead of time)
3. Use 200ml for first wash - shake at 58C for 10, flipping over membrane once after 5'
4. Pour off wash solution and add remaining 200ml and wash another 10' 58C

5. For final wash add 100ml 0.5XSSC, 1.5%SDS (pre-warmed at 58C) and shake 5' 58C - monitor with geiger counter - wash with another 100ml 1XSSC, 1%SDS 1 - 5' depending on strength and specificity of the signal.
6. Wrap membrane in saran wrap and expose to film with screen at -80C

Stripping:

1. DO NOT let membrane dry!
2. Bring ~500ml 0.1% SDS to a boil
3. Pour ~250ml over membrane and shake 5'; flip over and shake another 5'
4. Repeat with remaining 250ml
5. Expose to film to verify strip
6. Membrane can be stored in saran wrap at 4C and reused up to a year later (we have reprobed blots at least 4 times without loss in signal).