PAGE NORTHERN FOR MicroRNAs

Based on method optimized by Nelson C. Lau from a Ruvkum (Bartel Lab sanctioned) protocol.
Further modified by Pekala lab 2006.

Kinase up RNA markers/ladder

Acquire oligo's of different lengths to span size product of interest; any sequence will do. I used following oligos/primers from our primer inventory:

BGLII 1389	62 mer
BGLII 1288 antisense	37 mer
GLUT1 3'UTR REV 2320	26 mer
GLUT1 D1 FOR	21 mer
HuR CF	15 mer

Combined 2 pM (1 ul) of each in 1 vial for end labeling.

5 ul oligos
2 ul γ ^{32}P ATP, 6000 Ci/mmole, 10 mCi/ml (Perkin Elmer #Blu502Z)
2 ul 10X PNK buffer (from Ambion KinaseMax kit)
2 ul T4 PNK (from KinaseMax kit)
4 ul water (from KinaseMax kit)
14 ul

Incubated 1 hr in 37°C water bath. Heat sample 95°C for 2 minutes. Add 20 ul water (from Ambion kit); remove unincorp. ^{32}P on MicroSpin G-25 spin columns (GE Healthcare) per instructions.

Count 2 ul (in duplicate): combine with 298 ul water and add 3cc Ecoscint H; vortex; count.

- ✓ To check ladder:
  Poured 15% acrylamide/8M urea mini-gel using National Diagnostics reagents (see below). Polymerize 45 minutes, assemble gel apparatus, use 0.5X TBE as running buffer; rinse wells with 0.5X TBE.

  Make 2-fold serial dilutions of ladder 250,000 – 1953 CPM in 2 ul. Combine each with 2 ul TE + 4 ul Use Lau Urea Gel Loading Buffer (see recipe below). Heat 3 min at 95°C, put on ice, loaded gel. Run gel at 200 constant volts for ~1.5 hrs (until BPB ~1 cm from bottom). Put down on film for 1 hour at -80°. Also for 18 hr -80°C. I typically load 15,600 and 4000 counts in 2 lanes for a 6 hour or 18 hour exposure at -80°C.

  Make 6 ul aliquots; store in plexiglass box at -20°C. Will have to recount aliquot as needed….will maintain sufficient activity for minimum 4-6 weeks.

Running the Gel

Make up 15% PAGE (National Diagnostics Sequagel Mix) using ATTO apparatus and mini-gels (1 mm thickness).

Clean plates well. Siliconizing plates optional; I found it wasn't necessary. Assemble gel apparatus.

15% PAGE – 30 ml from Sequagel Mix

18ml Concentrate	240 ul 10% APS
9 ml Diluent	12 ul TEMED
3 ml Buffer	

Prepare samples of 20-30 ug total RNA (volume of 10 ul or less) mixed with at least 8 ul of Bartel Lab 8M Urea Loading Buffer (see recipe). Heat samples in 80°C heating block (5S-11 on high) for 5-10 mins, spin down, and use a beveled tip to load gel after urea from lanes have been washed out again. Run gel at 200 V for 30 min to 1 hr (allows RNA's large and small to "gently" enter gel) and then raise voltage to 400 V for 30 min – 1 hr, until the BPB dye just runs off the gel (Xylene cyanol should still be in the middle of the gel). Use 0.5x TBE for running buffer.

Separate gel onto Saran Wrap, stain with 4 ug/ml EtBr (1:2500 of 10mg/ml stock) in 25 ml 0.5x TBE for 5 min, and look for tRNA and 5S rRNA bands (78 and 120 nt) for quality of RNA prep and for visual loading control. Take digital picture on Shewchuk's camera and save image.

Transfer Gel to Membrane

Cut Nylon Membrane (Genescreen Plus from Perkin Elmer) size of gel; cut 2 sheets of 3mm Whatman Chromotography filter paper to fit semi-dry transfer apparatus (was a package of precut filter paper in drawer 5S-11 with Genescreen Plus membranes). Presoak membrane and filter paper in 0.5x TBE.

Assemble the Ideal Genie Electrophoretic Semi-Dry Blotter per instructions in order listed inside the tray:

1. cathode plate (banana connector should be in upper left) on bottom
2. bubble screen
3. Scotch-Brite pad presoaked in 0.5x TBE (add 0.5x TBE to level even with Scotch-Brite pad)
4. piece of filter paper presoaked in 0.5x TBE
5. prewetted Genescreen Plus membrane
6. place gel **on top** of membrane
7. prewetted filter paper (roll cut plastic pipette along gel sandwich to flatten and drive out bubbles)
8. Scoth-Brite pad presoaked in 0.5x TBE
9. bubble screen
10. anode plate (banana connector should be in upper right)

11. plastic 2-hole cover on top

Keeping tray level, slide the tray into tray holder. The sandwich should need to be compressed about 2 mm to slide into holder. If not, another Scotch-Brite pad should be added to the sandwich. Slowly tip the Genie to its vertical running position. Add buffer it it appears final buffer level will not cover the blotting area (of gel).

Connect Genie to power supply and begin blotting. Left-hand connection is (+) anode, right-hand connection is (-) cathode (because the membrane is BEHIND the gel here requires the positive plate to be on the bottom so nucleic acids will move from front to back…run to red). Transfer at constant current (200 mA) for about 1 hr, 15 min.

Remove gel from sandwich; do not separate gel from membrane. Use a syringe needle to mark the lanes on the membrane by punching small holes through top of membrane. May save the gel, restain with EtBr and take a picture to be sure all the RNA's <200 nt should be transferred; larger stuff will be stuck in the gel.

While blot is still moist, UV crosslink with 1000uJ of energy (Stratagene Crosslinker 5S-11; use auto setting) with RNA side up. Then bake for at least 30 minutes at 80°C (oven in 5S-18). This will completely immobilize the RNA. Can check blot with Geiger counter for side that contains the ladder. Mark date on this side with pencil. May store baked blot in sealed Kapok bag at 4°C until needed.

Prehybridization, Labeling, and Hybridization

Note: you can carry out all these steps in one day by prehybridizing in the morning, do labeling in the afternoon and start hybridization ON, or you can prehyb the night before. Also, the volume amounts listed here are for small hyb bottles (50 ml capacity). If doing large Northerns requiring larger bottles, double all volumes.

1. PreHyb/Hyb Solution – 50 ml per blot (25 ml for prehyb, 25 ml for hyb)

Final:	Start:	50 ml (one blot)	400 ml (eight blots)
5X SSC	20X	12.5 ml	100 ml
20 mM Na_2HPO_4 pH 7.2	1M	1 ml	8 ml
7% SDS	20% SDS	17.5 ml	140 ml
2X Denhardt's Solution	100X	1.5 ml	12 ml
DEPC Water		17.5 ml	140 ml

For each blot, prepare 25 ml of warm prehyb solution (50°C). Denature 1 mg of sheared salmon sperm DNA (100 ul of 10mg/ml stock, buy from Ambion or InVitrogen) by heating for 5 min in 100°C heat block (5S-19, high setting) or by boiling. Either immediately chill on ice or add to warm prehyb, and add to blot that is placed in hyb bottle. Place in hyb oven and rotate at 50°C, making sure membrane unwinds flat onto bottle and is not creased (if creased, may cause background problems). Prehyb in oven for 5 hours but can go overnight.

2. Labeling Reaction – 20 ul (**Note: oligo does not need to be gel purified**)

2 ul 10 uM oligo (~20 mer, 20 pmoles)
2 ul 10X T4 PNKinase Buffer (KinaseMax kit from Ambion)
30 uCi γ ^{32}P ATP, 6000 Ci/mmole, 10 mCi/ml (Perkin Elmer #Blu502Z)
1 ul T4 Poly Nucleotide Kinase (Ambion kit)
DEPC water to bring to 20 ul (Ambion kit)

Incubate 1 hr, 37°C water bath.
Kill Kinase 10 min, 68°C (5S-11 heat block, low setting)

Add 30 ul water (Ambion kit) to heat-inactivated labeling reaction. Remove 0.5 ul to scintillation vial containing 295 ul TE; set aside. Purify probe from unincorporated label with Microspin G-25 columns (GE Healthcare). Follow manufacturer's instructions. After purification, remove 2 ul to a second scintillation vial containing 298 ul TE. Add 3 cc Ecoscint H to each scintillation vial, vortex well and count. Calculate CPM/ul; expect between 33% and 50% incorporation.

Determine amount of labeled probe to add to blot; want 1.5 – 2 x 10^7 CPM total.

3. Hybridization

Dump the Prehyb solution from bottle/blot. Prepare an additional 25 ml Prehyb/Hyb solution that is warm (50°C) and contains 1 mg denatured (heat 100°C, 5 min) sheared salmon sperm DNA. Add to blot, and now add correct amount of purified labeled probe to bottle. Discard any unused probe. Close tightly and rotate in hyb oven at 50°C overnight. Meanwhile, prepare the following wash solutions:

Non-Stringent Wash Solution – 160 ml per blot (40 ml per wash for 4 washes) Store in 500 ml bottles.

Final:	Start:	200ml (>one blot)	500ml(>three blots)
3X SSC	20X	30ml	75ml
25mM NaH_2PO_4 pH 7.5	1M	5ml	12.5ml
5% SDS	20%	50ml	125ml
10X Denhardt's Solution	100X	20ml	50ml
dH_2O (DEPC not necessary)		95ml	240ml

Stringent Wash Solution – 80 ml per blot (1 wash only) Store in 500ml bottles

Final:	Start:	200ml (>one blot)	500ml(>three blots)
1X SSC	20X	10ml	25ml
1% SDS	20%	10ml	25ml
dH_2O		180ml	450ml

Place bottles in 50°C oven to warm up overnight and to get the SDS to dissolve back into solution.

Washing and Exposing

All washes to be done at 50°C. Dump hot hybridization solution into designated radioactive liquid waste container. Add 40 ml Non-Stringent Wash to each bottle. Cap and rotate in Hyb Oven for 10 min. Dump this first wash also in radioactive liquid waste container. Repeat Non-stringent wash, 40 ml, 10 min. Dump this second wash down the sink.

Repeat Non-Stringent Wash, 40 ml for **30 min**, dump; repeat 30 min wash. Dump wash in sink. Finally, wash once with **Stringent Wash, 80 ml, 5 min**.

Seal blot in Kapak Heat-Seal bag. Stick sealed blots RNA side up in film cassette and expose film 6 hrs and/or overnight -80°C.

Stripping a Northern Blot Protocol

Wash blot in 80ml 1% SDS at 85°C for 30 minutes, rotating (easiest to use hyb oven). You can add an additional wash if desired, and thus shorten each wash to 10 minutes, but be liquid is prewarmed to 85°C.

When using GeneScreen Plus Nylon and doing correct UV crosslinking and baking, RNA can be quite hardy on these blots, so multiple washes can be done with very minor loss of signal. Do avoid excessive wrinkling of the blot or letting it dry out before complete stripping of blot.

After stripping, heat-seal blot in Kapak bag and expose film for 18 hours -80°C to ensure probe has been stripped. Store in Kapak-sealed bag, either at room temp or at -20°C (we have also successfully stored sealed blots at 4°C). Blots have pretty long shelf life (couple of months) but your ^{32}P ladder marker may decay out.

REAGENTS AND BUFFERS FOR PAGE NORTHERNS (MICRO RNA ASSAY)

Bartel/Lau 8M Urea Loading Buffer

Makes 50 ml:

24 g urea
2 ml 0.5 M EDTA, pH 8.0
0.1 ml 1M Tris, pH 7.5
q.s. to 50 ml with dH_2O (nuclease-free not necessary)

*Check pH (7.5) but is usually ok. Filter-sterilize and store at 4°C.

To use: combine 1 ml of 8M Urea Loading Buffer + 12 ul of xylene cyanol dye stock and 4 ul of bromophenol blue dye stock.

Dye Stocks:

Xylene cyanol: dissolve 140 mg in 1 ml 8M Urea Loading buffer; store @ RT.
Bromophenol blue: Dissolve 140 mg in 1 ml 8M Urea Loading buffer; store @ RT.

100x Denhardts Solution:

To 200 ml nuclease-free water, add:

5 g ficoll (Sigma #F2637)
5 g polyvinylpyrrolidone (Sigma #PVP10-100G)
5 g fatty acid-free BSA (Sigma #A6003-10G)

Stir to dissolve. Ficoll may take a while. Q.S. to 250 ml with water.
Filter sterilize (do not autoclave).
Store in 25 ml aliquots in Abyss @ -80°C.

1 M Na_2HPO_4, pH 7.5 MW of anhydrous Na_2HPO_4 = 142

Add 14.2 g/ 90 ml nuclease-free water
pH to 7.5.
q.s. to 100 ml with water.
Filter sterilize. Store @ RT.

1 M Tris, pH 7.5 Trizma base MW = 121.1

Add 12.1 g to 90 ml nuclease-free water.

Correct pH to 7.5. Q.S. to 100 ml with water.
Filter sterilize. Store @ RT.

0.5 M EDTA Dihydrous MW = 372.2

Add 93.05 g to 450 ml water while stirring.
Add few drops of 10 N NaOH to ease into solution.
Q.S. to 500 ml with water. Filter sterilize. Store @ RT.

20x SSC (3 M NaCl, 0.3 M NaCitrate)

Makes 1 liter

Add to 800 ml dH_2O:
175.3 g NaCl
88.2 g NaCitrate dihydrate (MW 294) ***OR*** 57.6 g citric acid (MW 192)

pH to 7.0 w/ 1 N HCl. Q.S. to 1 L with water. Filter sterilize. Store @ RT.

20% SDS

Add 50 g SDS to 200 ml nuclease-free water.
Stir until dissolved (can heat slightly on hot plate while stirring to hasten).
Filter -sterilize. Store @ RT.