This protocol was optimized by Nelson Lau and is adapted from a Ruvkun protocol [B. Reinhart & A. Pasquinelli] which was adapted from the Ambros protocol

- **Kinase up RNA Markers (optional but necessary)**

Acquire RNAs of different lengths; any sequence will do. I have been using an 18'mer, 21'mer, 24-26'mer, 60'mer, 78'mer, and a 119'mer. I label with ^{33}P-γATP in a standard 5' kinasing reaction. Typically label up 10 pmoles of RNA with 50 μCi ^{33}P and gel purify, then resuspend pellet in 100 μl, load 1μl or less, and check on a practice transfer to see how hot the signal is. This can be titrated for optimal signal on blot exposure.

- **Running the Gel**

Make up 15% PAGE (Sequagel Mix) at Midi-Gel Thickness on Small Plates (19.5 cm x 16 cm glass plates). Pick the 20 well comb with wells of 4 mm x 15 mm x 0.8 mm. (30 μL)

Clean plates, don't over-silanize, and let polymerize at least for 30 min. Be careful pulling out combs, want very flat wells. Assemble gel onto the gel rig with an aluminum plate backing. The aluminum insures proper heat distribution during run and gives you more uniform mobility amongst the lanes.

15% PAGE – 30 mL from Sequagel Mix

18 ml	Concentrate	100 μl APS
9 ml	Diluent	30 μl TEMED
3 ml	Buffer	

Prepare samples of 20-30μg of total RNA (volume of 15 μl or less) mixed with at least 12 μl of Bartel Lab 8M Urea Loading Dye (contains BPB, XC, EDTA). Heat samples in 80^{o} C heat block for 5-10 mins, spin down, and use a beveled tip to load onto warm gel after urea from lanes have been washed out again. Run gel at 200 V for 1.0 hr (allows RNAs large and small to "gently" enter gel), and then raise voltage to 500 V for ~ 1.5 hr, until the Bromephenol blue dye just runs off the gel (Xylene cyanol dye should still be in the middle of the gel). Use 0.5x TBE for running buffer.

Separate gel onto Saran wrap, stain with 4 μg/ml EtBr in 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 snapshot on Gel Doc and save image.

- **Transfer Gel to Membrane**

Cut Nylon Membrane (Genescreen Plus from NEN works better than BioRad Zetaprobe GT) and 6 sheets of 3mm Whatman Chromatography filter paper to the size of the gel. Presoak membrane and filter paper in 0.5X TBE.

Place soaked membrane on the gel that sits on saran wrap, and position membrane to overlap the lanes containing loading dye. Now cut away any excess gel (remove wells, and gel from either sides). Place 3 sheets of soaked filter paper on top of membrane. Flip gel and membrane/filter over and place onto bottom platform of Semidry apparatus (Hoefer SemiPhor TE 70, Amersham Pharmacia). Now layer remaining 3 filter papers on top of gel. Use a cut plastic serological

pipette and roll along the gel sandwich to smooth out bubbles. Add a bit more 0.5X TBE buffer to make sure sandwich is quite moist.

Load on top lid of Semidry apparatus, and hook up to a Hoefer EPS 2A200 power supply that handles high current (Amersham Pharmacia). Transfer at constant current (3.3 mA/cm^2) for 35 minutes. The voltage should remain around 20V, but may get higher towards end of run. Do not let run longer, as the unit heats up and buffer will start to evaporate. You may use a bottle of liquid to press down on the lid. Positive electrode is on the base unit (pulls nucleic acids down).

Remove gel sandwich from apparatus, and flip sandwich back over so that membrane sits on top of gel. With gel facing down, locate the top of gel (look for the bumps from the wells). Take a syringe needle and poke through the membrane into the gel in the following pattern below. These marks will serve as guides to which side contains the RNA. Save the gel and restain. Take a picture of restained gel, all the RNAs <200 nt should be transferred, while larger stuff in the wells will be stuck. Name the pictures according to date and version (see Fig 1. for example)

While blot is still moist, UV crosslink with 1000 μJ of energy (Stratagene UV Crosslinker) RNA side up towards the lamps. Then bake for at least 30 min at 80°C. This will completely immobilize the RNA. When blot is dry, check with geiger counter for the side that contains the ladder marker. On that side, mark with pencil the date and version of the blot on both sides. Store baked blot dry in a Kapak sealed bag until you are ready for probing.

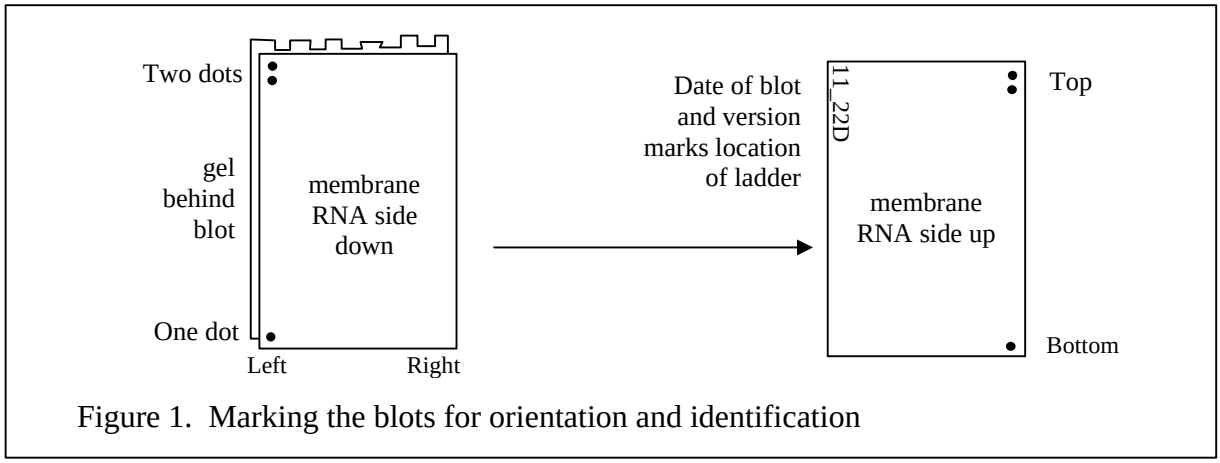

Figure 1. Marking the blots for orientation and identification

- **Prehybridization, Labeling, and Hybridization**

Note: You can carry out all these steps in one day by prehybing in the morning, do labeling 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.

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	10%	35 ml	280 ml
2X Denhardt's Solution	100X	1.5 ml	12 ml

For each blot, prepare 25 ml of warm pre-hyb solution (50°C). Denature 1 mg of sheared salmon sperm DNA (100 µl of 10mg/ml stock, buy from Sigma) by boiling for 5 min. Either immediately chill on ice or add to warm pre-hyb, and add to blot that is rolled up on a nylon mesh screen and place in a bottle. Place in hyb-oven and rotate at 50°C, making sure membrane unwinds onto bottle and is not creased. If the blot does not unwind properly and flatly onto tube, creases may form and cause regions of high background. Pre-hyb in oven for at least 2 hours, but can go overnight.

Labeling Reaction – 20 µL (oligo does not need to be gel purified)

2 µl 10µM oligo (~20mer, 20 pmoles)
2 µl 10X T4 PNKinase Buffer
2.5 µl ^{32}P γ-ATP, >7000Ci/mmole (ICN sells labeling grade stuff)
12 µl dH_2O
1 µl T4 Poly Nucleotide Kinase

Incubate 1 hr, 37°C

Kill kinase 10 min, 68° C

Note on radioactivity: The labeling grade radioactivity from ICN comes at a higher concentration and is much cheaper, but does not incorporate as efficiently as comparable radioactivity from NEN. NEN radioactivity incorporates better if you use a larger volume of their solution (12 ul ATP, 2 ul water), but the radioactivity from NEN is more expensive.

Add 30 ul water to heat-inactivated labeling reaction and purify probe from unincorporated label with G-25 MicroSpin Columns (Amersham Pharmacia). Follow manufacturer's instructions. After purification, expect about 1/3 label incorporation by comparing counts in column to eluant at a constant small distance from geiger counter.

Dump the Prehyb solution from the bottle. Prepare an additional 25 µl of Prehyb/Hyb solution that is warm and contains 1 mg of denatured sheared salmon sperm DNA. Add to blot, and now add 25 µl of eluted radiolabeled oligo to bottle. 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 – 4 washes) Store in 500 ml bottles

Final:	Start:	200 ml (>one blot)	500 ml (>three blots)
3X SSC	20X	30 ml	75 ml
25 mM NaH_2PO_4 pH 7.5	1M	5 ml	12.5 ml
5% SDS	10%	100 ml	250 ml
10X Denhardt's Solution	100X	20 ml	50 ml
dH_2O		45 ml	115 ml

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

Final:	Start:	200 ml (>two blots)	500 ml (>six blots)
1X SSC	20X	10 ml	25 ml
1% SDS	10%	20 ml	50 ml
dH_2O		170 ml	425 ml

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

- **Washing and Exposing**

Dump hot hybridization solution in a designated radioactive liquid waste container. Add 40 ml of 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. Mark radiolabel waste on sink tracking card and cards on waste containers.

Repeat Non-stringent wash, 40 ml, 30 min. 2 times. Dump wash in sink. Finally, wash once with Stringent Wash, 80 ml, 5 min.

Seal blots in Kapak Heat-Seal Bags. Stick sealed blots RNA side-up on a clean old autoradiogram with tape (generally 4 blots to an autorad). Place in cassette with a blanked phospohorimager screen. Expose in ON (at least 12 hrs).

Note: If after exposure you see lots of background, the following things might be happening:

Globally high background across entire blot:

- Is your blot dirty and dusty? Dry blots (newly made ones) should be stored wrapped in plastic, while recently probed blots should always be kept moist in heat-sealed bags so that SDS does not dry onto the entire blot.

- Still lots of unincorporated nucleotide in the probe mixture? In the past we have added 20 µl of 100 mM cold ATP during the two last Non-stringent washes (30 min. washes) thinking this could compete away leftover label. ATP during the washes is not necessary if the unincorporated label is removed efficiently during spin column purification.

- What sort of oligo probe are you using? We find short DNA probes (17-24nt) 5' kinased give acceptable background with this protocol. Longer DNA probes that are internally labeled or RNA oligo probes have been observed to give stronger signal, but are also more prone to higher background and crossreactivity. You may need to test different hyb temperatures to optimize different probes.

Localized regions of background:

- Is the blot and the nylon mesh hugging the bottle wall flatly and evenly? Creases, kinks or folds of an uneven blot can affect how well the blot is coated by prehyb and washes. Avoid these kinks or tugging on the blot with excessive force.

- Is the nylon mesh you are using clean? What about the plastic bag exterior? Residue on the nylon mesh will affect what radiolabel will stick to the blot. Residue, like dried SDS, on the plastic bag holding the blot can affect the exposure on the film or phosphor plate. Wiping the bag before exposing might help with this.

Stripping a Northern Blot Protocol

Wash blot in 80ml 1% SDS at 85C for 30 minutes, rotating. I generally use the Hybaid Oven (the smaller oven) with the snap-close caps (bigger glass bottles with a blue and a white snap-close caps). You can add an additional wash if desired, and thus shorten each wash to 10 minutes, but be sure liquid reaches to temperature.

When using GeneScreen Plus Nylon and doing correct UV crosslinking, 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.

Expose blot to phosphor screen for at least 12 hrs (Overnight) to ensure probe has been stripped. Store blot in Kapak Sealed bag, either at room temp or at -20C. Blots have pretty long shelf life (couple months), but your 33P marker may decay out.

A note on background and unstrippable probes:

RNA probes are very tricky to handle, even if you might assume higher sensitivity. They give much higher background and splotchy dots on your blot using the standard "N.Lau" protocol, and the splotches are extremely difficult to wash away (nearly impossible to strip). It has not been tried but perhaps a revision to the "N.lau" protocol by including Formamide in the Hyb solution and Nonstringent wash may help this, but no guarantees here.

As for DNA probes, these are generally quite easy to wash and strip. However, we have observed that for some mammalian microRNAs where the abundance is extremely high, the probe gets so concentrated into the hybridization spot that a small amount of covalent crosslinking occurs between the probe and the membrane (UV from ambient light, who knows...). This is especially crucial for tissue-panel and developmental northerns that are going to be probed multiple times. A good strategy is to examine other data suggesting the abundance of the microRNAs you are going to examine, and probe the least abundant miRNAs first, finally working to the most abundant miRNA last.

Protocol revised by Nelson Lau, 03/22/04

Wash blot in 80ml 1% SDS at 60C for 10 minutes, rotating.

Protocol by J. Graham Ruby, 2/4/04