This is a revision of the protocol 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**

Acquire RNAs of different lengths; any sequence will do. We typically use synthetic 18'mer, 21'mer, and 24'mer along with in vitro transcribed, phosphatase treated 60'mer, 78'mer, and 119'mer. Label with ^{33}P-γATP in a standard 5' kinasing reaction. Typically label 20 pmoles of RNA with 50 μCi ^{33}P. For longer markers, it is necessary to heat and snap cool the RNA on ice to melt any secondary structure that may be occluding the 5´ end. Gel purify the markers, and resuspend the pellet in 25 μl and scintillation count. Mix a stock of marker with as high a specific activity as possible, and dilute as needed. Load approximately 3000 cpm/band.

- **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 an equal volume of 2x Loading Dye (Deinoized formamide plus BPB, XC, EDTA). Heat samples in 80° 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 2 W for 20 minutes (allows RNAs large and small to "gently" enter gel), and then raise to 12 W 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 well for us) and 4 sheets of 3mm Whatman Chromatography filter paper. The gel will swell slightly during the Ethidum staining, so be generous with the membrane and filter paper. Cut one corner of the nylon membrane to make it asymmetric, and label the blot with a pencil. 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 (4 mA/cm^2) for 45 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).

After transfer, disassemble the gel sandwich and leave the gel soaking in the ethidium stain. Place the membrane RNA side up on a wet whatman (from the transfer sandwich), and UV crosslink with 1200 x 100μJ energy (Stratalinker). Take a picture of the transferred gel to make sure that the RNA has transferred from the gel. It is common to see a smear of material remaining near the well, but all of the discrete rRNA and tRNA bands should no longer be visible.

- **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

Heat the solution up to 50°C to get everything into solution. Aliquot extra hyb solution into 50 ml falcon tubes and freeze at –20°C.

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 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 μl ^{32}P γ-ATP, >7000Ci/mmole (~150 μCi/μl)
13 μl dH_2O
1 μl T4 Poly Nucleotide Kinase

ICN and NEN sell high specific activity crude ^{32}P γ-ATP that works well for labeling.

Incubate at 37°C for 1 hour

Add 30 ul water to heat-inactivated labeling reaction and purify probe from unincorporated label with G-25 MicroSpin Columns (GE). 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.

Heat half of the desalted probe to 85°C for 5 minutes, then add directly to the prehyb and hybridize 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
dH_2O		65 ml	162.5 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

Add the SDS last to avoid precipitation with the salt from the SSC. Place bottles in 50°C incubator to warm up overnight and to get any precipitant to dissolve back in solution.

- **Washing and Exposing**

Dump hot hybridization solution in a designated radioactive liquid waste container. Rinse the tube briefly with 30 ml of Non-Stringent Wash, dispose and add another 30 ml of Non-Stringent wash and incubate at 50°C for 10 minutes. Repeat Non-stringent wash twice, 30 minutes per wash.

Wash with the Stringent Wash once for 15 minutes.

Place the blot in a Kapak heat-seal bag, or a piece of saran wrap. Survey with a Geiger counter. You should barely be able to detect the markers, and depending on the signal, you may be able to

detect your RNA of interest. If the blot seems hot in weird places, you may wish to continue to wash longer.

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 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 blot

Bring 250 ml of 0.1% SDS to a boil in the microwave. Pour over used blot, RNA side up, in a glass dish. Shake gently for 20 minutes, and repeat. This approach has worked well for stripping most DNA oligos. Seal and expose for as long as you anticipate exposing your next probe.