This is a revision of the previous small RNA northern protocol to detect over-expressed miRNAs (used in Fang and Bartel, MolCell, 2015).

• Extract total RNA

For detecting over-expressed miRNAs, a 6-well transfection of HEK293T cells is enough. 36-48hr after transfection, wash each well with 1 ml PBS, aspirate, and add 0.5 ml tri-reagent to each well. Rock at 4 °C for 20min, and follow the protocol exactly. Dissolve pellets in 30 ul of RNase-free water.

• Running the Gel

Make up 15% denaturing PAGE gel (UreaGel-SequaGel-System, National Diagnostics). For most applications we use medium thickness spacers (0.8mm) with 19.5 cm^2 large glass plate and 19.5 cm x 16 cm small plate and a comb with well volumes of ~30 µl (LABREPCO). Clean plates, spacers and comb thoroughly and dry. Plates can be silanized if desired.

15% PAGE – 30 mL from Sequagel Mix (enough for 1 gel):
- 18 ml Concentrate
- 9 ml Diluent
- 3 ml Buffer
- 100 µl APS (from 10% stock)
- 30 µl TEMED

Pour gel and let it polymerize for 1 hour. After set, **be careful pulling out combs so that wells remain very flat**. Assemble gel onto the gel rig, pour running buffer (0.5 x TBE) from an angle, and remove remaining bubbles at the bottom. An aluminum plate backing can be used on the rig to insure proper heat distribution during run and more uniform mobility between the lanes. **Rinse wells with running buffer to displace any urea that may have accumulated**. Pre-run the gel at 5W for at least 20 min.

Prepare samples of 5 -10 µg of total RNA (volume of 15 µl or less) mixed with an equal volume of 2x Loading Dye (Load dyeII from Ambion). Heat samples in 80 °C heat block for 5-10 min, spin down, and use a beveled tip to load onto warm gel after **urea from wells has 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 ~2 hr, until the BB dye just runs off the gel. (XC dye should still be in the middle of the gel.)

Separate gel onto Saran wrap, remove wells, and cut the bottom corner to indicate the first lane. Stain with 4 µg/ml ethidium bromide (36 ul of EtBr stock in 90 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. Save image.

• Transfer Gel to Membrane (revised, Dec. 2009)

Cut Amersham Hybond-NX membrane (GE Healthcare) and 5 sheets (4 used for transfer, 1 for

crosslinking) of 3mm Whatman Chromatography filter paper to size. The gel will swell slightly during the Ethidum staining, so be generous with sizing the membrane and filter paper. I usually cut **15 cm x 18.5 cm** from a 30 cm wide roll (one width can be cut into two gels). Label membrane with a pencil (date and title) and cut a corner.

Remove gel from staining solution but leave it on saran wrap. Presoak membrane and filter paper in 0.5X TBE. Place soaked membrane on the gel so that it overlaps the lanes containing loading dye. Note orientation of membrane on gel (I align two corners that mark the bottom of first lane). Cut away any excess gel that extends beyond the edges of the membrane. Place 2 sheets of soaked filter paper on top of membrane. Flip gel/membrane/filter sandwich over and place onto bottom platform of Semidry apparatus (e.g., Bio-Rad Trans-Blot SD Semi-Dry Transfer Unit). Layer remaining 2 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 top lid of Semidry apparatus, and hook up to a power supply that handles high current (e.g., Hoefer EPS 2A200, Amersham Pharmacia). Transfer at constant current (4 mA/cm^2 of membrane, I use 900mA for a whole gel) 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. Positive electrode is on the base unit (pulls nucleic acids down).

During transfer, prepare EDC solution for cross-linking the RNA to membrane. First, make 1x methylimidazole stock: add 30 ml of dH_2O to 408 µl of 12.5 M 1-methylimidazole. pH to 8 with 1 M HCl (I usually make a batch of 250 ml). Bring total volume up to 40 ml with dH_2O and filter sterilize. Stock solution can be kept at RT. For each membrane, make fresh EDC solution by adding 0.466 g of EDC (1-ethyl-3-(3-dimethylaminoprophy) carbodiimide) to 15 mL of the 1x methylimidazole stock. This is enough for 1 membrane of a whole gel.

After transfer is complete, disassemble the gel sandwich and soak the gel in the ethidium stain for ~ 5 min. 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. Save image.

Place in order on top of one another: a piece of saran-wrap large enough to completely wrap the membrane, the membrane face down, Whatman paper cut to size of membrane. Pour the freshly prepared EDC solution onto the Whatman paper and completely saturate it. Wrap up the membrane sandwich with the wrap and incubate at 60°C for 15 min to 2 hours (I usually do 1 hr). Disassemble sandwich and rinse membrane briefly several times with dH_2O. ~~Bake membrane at 80°C for 10 min. if saving membrane for later.~~ Otherwise membrane can go directly into pre-hybridization buffer.

• Pre-hybridization, Labeling, and Hybridization

Note: You can carry out all these steps in one day by pre-hybridizing in the morning, do labeling the afternoon and start hybridization ON, or you can pre-hyb the night before. For most blots (~16 cm^2 or less), 15 ml of solution is sufficient.

Heat the Oligo-hyb (Ambion, stored at 4 degree) solution up to 60°C to get everything into

solution.

Roll up blot with the transferred RNA on the inside of the roll and place in a hybridization bottle. Make sure that there are no creases as they can cause regions of high background. For each blot, prepare ~15 ml of pre-hyb solution warmed to 42-60°C. Place bottle in hybridization oven and rotate at 42°C for at least 30min, but can go overnight.

Labeling Reaction – 10 µL (oligo does not need to be gel purified)

1 µl 10µM oligo (~20mer, 20 pmoles)
1 µl 10X T4 PNKinase Buffer
1 µl ^{32}P γ-ATP, >7000Ci/mmole (~150 µCi/µl)
6 µl dH2O
1 µl T4 Poly Nucleotide Kinase

Incubate at 37°C for 1 hour.

Add 50 µl water to labeling reaction and purify probe from unincorporated label with P-30 Columns (BioRad). Follow manufacturer's instructions. After purification, expect about 1/3 label incorporation by comparing counts remaining in column to counts in eluate at a constant small distance from geiger counter.

For one blot, add 0.5 ml of Oligo-hye buffer to the probe and heat at 85°C for 5 minutes, then add **directly to the pre-hyb solution** (without touching the membrane) and hybridize overnight. Incubating for longer than 16 hours may result in high background levels.

• Washing and Exposing

I use a relative low stringent buffer for washes, but it works fine. The buffer contains 2 x SSC, and 1% SDS. I make a bottle of 1 L (add 100 ml of 20 x SSX, and 100 ml of 10% SDS).

Collect hybridization solution in a conical tube, and store at -20°C (probe can be re-used). Wash with 30 ml of wash buffer twice for 5 min. Then wash with 30 ml of wash buffer once for 30 min.

Make a support for the membrane with Whatman filter paper (label the back too) by wrapping it with saran wrap. Place the blot on the wrapped filter paper, and use another saran wrap to wrap it. Survey with a Geiger counter. If you can detect some signal, expose for 30 min to 1 hr, and scan at high sensitivity. I usually expose and scan twice, one after a few hours, and one after ON exposure.

• Stripping a blot

This approach works well for stripping most DNA oligos: bring 500 ml of 0.04% SDS (2 ml of 10% SDS to 500 ml of dH_2O) to a boil in the microwave. Be careful not to over-boil, as it is easy to get spill. Pour over used blot, RNA side up, in a glass dish. Shake gently for 20 minutes, and repeat twice. Seal and expose blot to phosphorimager plate for as long as you anticipate exposing your next probe.