miRNA Northern Blot

Adapted from McManus Lab Protocol (MIT)

Reagents

30% Acrylamide/Bis 29:1 (BioRad #161-0156)
Temed (BioRad #161-0800)
10% APS (store at 20°C, only use for one week once thawed. Sigma #A7460)
Urea (electrophoresis grade, Fisher #BP169-500)
Formamide loading buffer (Ambion Gel Loading Buffer II #8546G)
Hybond-N+ nylon membrane (Amersham #RPN3038)
StarFire Nucleic Acid Labeling System (IDT)
α^{32}P-dATP redivue (6000Ci/mmol, 10mCi/ml) (Amersham - use within 3-4 days of reference date)
NucAway Column (Ambion #10070-30 – or an equivalent G-25 column)

5X TBE

54.0 g Tris Base
27.5 g Boric Acid
20.0 ml EDTA (pH 8.0)
bring to 1 liter with DEPC-treated H_20
filter and store at room temp

Hybridization Solution

7% SDS
200 mM Na_2HPO_4 (pH 7.2)
filter and store at 4°C

20X SSPE

175.3 g NaCl
27.6 g NaH_2PO_4
0.02 M EDTA
pH to 7.4 with NaOH
bring to 1 liter with DEPC-treated H_2O
filter and store at room temp

Prepare glass plates for gel

- Wash glass plates (17.5 cm x 17.5 cm) well with liquinox and rinse well
- Rinse with 95% EtOH and allow to dry
- Wash 0.75 mm spacers and 0.75 mm comb and wipe down with RNase Away
- Seal plates with Permacel tape or 0.5% agarose

Prepare 12% Denaturing Urea Polyacrylamide Gel

- 8 M Urea
- 12% Acrylamide/Bis 29:1
- 1X TBE

Filter through 0.2 um CA filter
Prepare ~ 15 ml per gel

- Add APS (50 ul 10% APS and 10 ul Temed per 10 ml)
- Pour gel with 20 ml pipette, position comb and allow to polymerize at room temp (30-60min)
- Pre-chill 3 L 0.5X TBE to 4°C for later transfer

Pre-run gel

- Wet comb and wells with 1X TBE and carefully remove comb
- Clamp plates to gel box with the notched plate towards top resevoir
- Add 1X TBE to both reservoirs and rinse out wells with a syringe and 20g needle
- Pre-run gel at 400 V (~25 mA) for 60min. Tape digital thermometer probe to middle of plate to monitor temperature. Ideally plates should reach about 50°C.

Load and run gel

- Prepare equivalent volumes of sample or ladder with 1X formamide loading buffer
- Heat at 70°C for 5 min, move to ice and quick spin samples
- Turn off power on gel box, rinse out wells and load samples
- Run gel at 350 V for 60min or until BPB reaches about 2 cm above bottom of gel (BPB runs around15 nt and cyanol about 60 nt)

Stain gel

- Pry apart glass plates and cut upper right corner of gel
- Place gel on plate into large dish with 0.5 ug/ml EtBr in DEPC-H_20 and stain 5-10 min
- Remove gel from dish on plate, cover top with saran wrap, and take picture of gel next to ruler
- Wash/equilibrate gel in 0.5X TBE for 10 min

Transfer gel and generate blot

- Pre-cut nylon membrane to size of gel with clean gloves and razor
- Build transfer sandwich on black side of cassette: sponge, 3 pieces Whatman filter paper, gel, membrane, 3 pieces Whatman filter paper, sponge
- Always pre-wet components in 0.5X TBE
- Put a piece of pre-wet filter paper on gel and flip over onto cassette with sponge and 2 pieces of filter paper
- Put pre-wet Hybond-N+ nylon membrane on gel and one piece filter paper on membrane
- Roll out bubbles with pipette and finish assembling sandwich
- Transfer cassette to BioRad Trans-Blot Cell and fill with pre-chilled 0.5X TBE
- Turn on cooling coil tank and place cooling coil in Trans-Blot cell
- Place Trans-blot cell on stir plate with stir bar positioned in middle
- Transfer at 80 V for 60 min in cold room (amperage should not go above about 1.5 A)
- Disassemble, mark blot, and rinse in 1X TBE

- Place damp membrane, RNA-side up, on dry piece of Whatman filter paper and UV crosslink at 1200 mJ in Stratagene UV Stratalinker 1800
- Stain gel carcass to assess transfer efficiency

Probe Generation

- Generate StarFire Probe using manufacturer protocol
  - Add 6ng (2 ul of 0.5 uM dilution) StarFire Custom AntiSense Probe, 1X reaction buffer, and 2 ul of 12.5 uM StarFire template oligo in 1.5 ml microfuge tube with screw cap
  - Heat tube 94°C for 1min in heat block with cap loosened, remove from heat block, and allow to cool to room temp (~15 min)
  - Add 2ul Klenow and 12ul α^{32}P-dATP (6000 Ci/mmol, 10 mCi/ml), leave at room temp for 2-4 hours, and stop reaction with StarFire stop buffer
- Remove unicorporated nucleotides using NucAway column using manufacturer's protocol
  - Do not overload column, we usually only load ~75 ul per column that have a recommended max volume of 100 ul
  - Measure total volume of probe eluted from column
- Determine specific activity using scintillation counter (Beckman LS6500)
  - Pipette 1 ul probe into 0.2 ml PCR tube and place tube into plastic scintillation vials
  - Use empty scintillation vial as a blank
  - Transfer vials into rack that has the program "1" card inserted in the back, blank always goes into first space
  - Start automatic counting – machine will detect the correct isotope (^{32}P) based on the program card in rack
  - Use counts per minute (CPM) of 1 ul to calculate specific activity (SA) of total amount of probe

    $$SA = \frac{(CPM/ul)(total\ volume)}{ug\ DNA\ template}$$

  - Should get SA of at least 1 x 10^9 cpm/ug
  - Use in probe in hyb buffer at final concentration of at least 1 x 10^9 cpm/ml

Pre-Hyb and Hybridization

- Wet blot in 1X TBE, roll membrane with RNA side in, and insert into hyb bottle
- Add ~25 ml (1 ml/10cm^2) hyb buffer and prehyb at 37°C for at least 30min in hybridization oven
- Add probe to appropriate volume of hyb buffer, mix well and place in bottle
- Hybridize 12-24 hours at 37°C in hyb oven (hybridization conditions vary and should be optimized for each probe)

Wash and Detection

- Remove hyb solution (can reuse some probes – save in a well-labeled falcon tube)
- Wash blot in tube with ~50ml pre-warmed wash buffer – this part is also variable Start with 3 X 10 minute washes at 37°C with 2X SSPE, 0.1% SDS

To increase stringency :
increase temp and number of washes (try this first)
or can also decrease SSPE and SDS concentration

- Remove blot from tube with clean forceps and wrap in saran wrap
  - Do not let blot dry out!
- Expose to phosphor imaging screen at room temp for 1 hour, if needed remove excessive background and re-expose (up to 48 hours if needed)
- Can store blot in wash or prehyb solution
- Dance a jig to celebrate the success of your experiment

Re-probing Blot

- Blots can be stripped and re-probed up to 3 or 4 times – depends on quality of blot
- To strip, quickly (~45sec) wash blot in boiling 0.1% SDS
- Check on phosphor screen