

**PAGE NORTHERNS TO DETECT SMALL RNAS**

Amy Pasquinelli (adapted from Lee et al., 1993) (see Pasquinelli et al., 2003)

**Running gel:**

1. Assemble 10x10cm glass plates with .8mm spacers, 10 well comb (holds ~30microliter volume/ well).
2. For ~11% PAGE: 4.65 ml Sequagel concentrate - 23.75% (National Diagnostics)  
4.35 ml diluent (Natl. Diag.)  
1 ml buffer (Natl. Diag.)  
90 ul 10% APS  
4 ul TEMED  

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~10ml
3. Allow to polymerize ~.5-1 hr. and then pre-run in 0.5X TBE ~30min. at 150V  
5X TBE stock:54 g Tris base  
27.5 g boric acid  
20 ml .5M EDTA  

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1 L with Millipore water - filter sterilize
4. During pre-run prepare RNA samples - mix 1X formamide load dye with RNA resuspended in water (i.e. 10ul RNA at 1ug/ul + 10 ul formamide load dye)  
Formamide load dye: 20 ul .5M EDTA  
800 ul 100% deionized formamide  
100 ul of 10mg/ml xcff/bpb dye mix  
80 ul RNase free water  

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1 mL (store at 4C)
5. Heat RNA samples at 65C 15min., pulse samples to bottom of tubes, place on ice while rinsing wells of gel and then load
6. Run at 150V until bpb dye is at bottom - usually 1.5-2 hours

7. Disassemble gel plates and with gel stuck to one plate cover with saran wrap and carefully transfer gel to saran wrap; place gel on saran wrap into tray and stain with .5ug/ml ethidium bromide in .5X TBE 5-10 minutes with gentle shaking. Pour out EtBr solution and carefully take a picture of stained gel on saran wrap - you should easily see the tRNAs (~80nt), 5S (~120nt) and 5.8S (~150nt) rRNAs) (see last page of this protocol for an example). Never reuse the EtBr solution for gels to be used in Northern.

**Electroblot Transferring:**

1. Cut blot membrane (Zeta-Probe GT 162-0194, BIORAD) and 4 sheets of whatman paper to same size as gel (~9x9 after removing wells and taking into account side spacers).
2. Gently wet blot in water then presoak blot ~5 min. in .5X TBE
3. Assemble: plastic holder
  - wetted sponge pad
  - 1 sheet wetted whatman
  - place next whatman directly on top of gel so that the gel sticks and then add to stack, gel side up
  - presoaked membrane (use a glass Pasteur pipet to gently roll out any bubbles)
  - 2 more sheets wetted whatman paper (roll out bubbles)
  - wetted sponge pad
  - plastic holder and lock together
4. Place into transfer tank (Owl or Biorad sell nice transfer tanks) and add enough .5X TBE to cover (~2L)
5. Stir slowly in cold room and transfer 2 hours at current of 200mAmps

**Fixing:**

1. Disassemble transfer and check wet membrane under UV light to verify that ethidium bromide stained RNAs transferred OK (can photograph too)
2. Place membrane RNA side up on dry whatman and X-link at 1200 microJ x 100 on Auto-setting (UV 1800 STRATAGENE)

3. Sandwich membrane between dry whatman paper and bake in oven at 80C for ~.5hr until dry (X-linking AND baking seem to be important for efficient cross-linking of very small RNAs).

**Prehybridization:**

1. Make prehyb solution:
  - 12.5 mL 20X SSC (175.3g NaCl + 88.2g sodium citrate/L, pH to 7.0 and filter sterilize) (5X f.c.)
  - 17.5 mL 20% SDS (200g SDS/L, pH 7.2, filter sterilize) (7% f.c.)
  - 18 mL water
  - 1 mL 1M sodium phosphate (31.6ml 1M NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O + 68.4ml 1M Na<sub>2</sub>HPO<sub>4</sub>.8H<sub>2</sub>O) (0.02M f.c.)
  - 1 mL 50X Denhardt's (5g Ficoll + 5g polyvinylpyrrolidone + 5g BSA/ 500mL, heat and stir to dissolve and filter sterilize, aliquot and store at -20C) (1X f.c.)

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50mL - place at 50C to thin solution

2. Wet membrane in 2X SSC and place back between whatman to ease into seal a meal bag and then remove whatman
3. Seal bag and cut out corner for adding solution - add ~10-25mL of prehyb (place remaining back at 50C to keep thin) + 250uL 10mg/ml sheared salmon sperm that has been boiled 5' and put on ice 1'.
4. Squeeze out excess bubbles and seal and gently massage solution throughout
5. Place at 50C with shaking ON (or at least 4hrs.)

**Hybridization (two methods (A or B) are described below – we recently switched to Starfire probes and column purification (B) which seem to work well and decrease experimental and detection time):**

**A – Original probe labeling and purification**

1. Kinase probe:      2ul DNA oligo 250ng/ul (~25pmol)  
                             5ul 10X buffer  
                             15ul  $\gamma$ 32P ATP 6000Ci/mmol  
                             26ul H<sub>2</sub>O  
                             2ul T4 polynucleotide kinase  
  
                             \_\_\_\_\_  
                             50ul - place at 37C 45-60'  
                             68C 10' to kill kinase
  
2. Precipitate:        240ul 5M NH<sub>4</sub>Oac  
                             750ul ethanol  
                             - vortex to mix and place in dry/ice ethanol ~15'  
                             - spin 30'; pipet out ethanol and monitor to make sure oligo ppt  
                             - add 500ul 70% ethanol and spin 10'; carefully remove ethanol  
                             - air dry ~10'
  
3. Resuspend in 10ul TE by pipeting and monitor to make sure counts in solution (there will always be lots of counts still stuck to tube too)
  
4. Denature probe:    100ul .2N NaOH  
                             250ul 10mg/ml salmon sperm  
  
                             \_\_\_\_\_  
                             - boil 5' and then put on ice
  
5. Cut seal a meal bag and squeeze out prehyb; add fresh hyb solution (10-25ml)
  
6. Pipet in probe solution
  
7. reseal and shake at 50C ON

**B – Alternative Starfire probe synthesis & column purification (column purification can also be used for kinased oligo probes)**

**StarFire PROBE HYBRIDIZATION**

**MATERIALS:**

StarFire® Complete Kit- from IDT DNA (www.idtdna.com)

Exo-Klenow DNA Polymerase (5 units/ul)

StarFire® 10X Buffer Mix (100 mM Tris pH 7.5, 50 mM MgCl<sub>2</sub>, 75 mM DTT)

StarFire® Stop Buffer (10mM EDTA)

One PAGE-purified StarFire® Custom Probe (up to 50 bases)

StarFire® Universal Template (sufficient for 25 labeling reactions)

$\alpha$ -<sup>32</sup>P-dATP (10mCi/ml, 6000 Ci/mmol)

TE (10mM Tris pH 8.0, 1 mM EDTA)

Illustra Autoseq G-50 Dye Terminator Removal Kit, #27-5340-01, GE Healthcare (50 columns)

250ul 10mg/ml salmon sperm

**1. RESUSPEND OLIGOS:**

- Briefly centrifuge oligos
- Resuspend StarFire template oligo in 25 ul TE (final concentration 12.5 uM)
- Resuspend StarFire probe to 100 uM in TE (stock solution)
- Dilute an aliquot of StarFire probe stock solution 1:200 in water (0.5 uM)
  
- Store all StarFire reagents in -20° C

**2. StarFire LABELING PROTOCOL:**

1 ul StarFire probe oligo

1 ul StarFire template oligo

1 ul StarFire buffer mix

3 ul – mix using pipette (do not vortex)

- Place tube in boiling water bath, or 95° C, for 1 minute

- Cool at room temperature for 5 minutes – centrifuge briefly

3 ul StarFire oligo/template/buffer mix (from above)

6 ul  $\alpha$ -<sup>32</sup>P-dATP

1 ul Exo-Klenow DNA Polymerase

10 ul – allow reaction to proceed at room temperature 1-1.5 hours

- Add 40 ul of stop buffer

### 3. Labeled Oligo Purification

- Vortex spin column briefly, snap off bottom of column & slightly loosen column cap
- Place column in 2 ml collection tube, spin at 2000 x g, 1 min
- Transfer column to new 1.5 ml tube
- Load entire labeled oligo sample from above to the center of the column
- Recap loosely, spin at 2000 x g, 1 min
- Column will turn green because of unincorporated  $^{32}\text{P}$ , but flow-through should be very hot

4. Cut seal a meal bag and squeeze out prehyb; add fresh hyb solution (10-25ml) and 250 ul salmon sperm

5. Pipet in purified probe solution

6. reseal and shake at 50C ON

### Wash:

1. Cut corner of seal a meal bag and carefully pour probe solution out (can pour into 50ml conical tube and freeze at -20C and reuse); cut open rest of bag and place membrane in tray containing Wash solution:

30ml 20X SSC (3X f.c.)

40ml 50X Denhardt's (10X f.c.)

50ml 20% SDS (5% f.c.)

5ml 1M Sodium Phosphate (.025M f.c.)

75ml water

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200ml, place at 50C until all in solution

3. Use 100ml for first wash - shake at 50C for 10', flipping over membrane once after 5'

4. Pour off wash solution and add remaining 100ml and wash another 10' 50C

5. For final washes add warmed 200ml 0.5XSSC, 1.5%SDS and shake 10' 50C with flipping at 5'; after this wash carefully monitor your blot- if counts are localized, add final 200ml 0.5xSSC, 1.5% SDS and briefly shake - shake the entire 10' if counts are still screaming and not localized.

6. Wrap membrane in saran wrap and expose to film with screen at -80C (or PhosphorImage screen)

7. After desired exposures, strip membrane by shaking in boiled water for 10' two times, with flipping at 5'; expose stripped gel to film + screen to verify strip worked; membrane can then be stored at 4C wrapped in saran; membrane can be reprobed at least 3X

11% Mini-PAGE  
(15ug RNA/ lane stained ~5min.  
with .5ug/ml ethidium bromide)



\*wells and large staining RNA cut off