Northern Blot (sRNA) – LICOR

General Notes:

Practice RNase-Free technique:

- *Use RNA-only pipet tips/tubes*
- *Use RNaseZap to clean counters and pipets*
- *Filter/sterilize solutions*
- *Use MQ-H_2O or UltraPure H_2O*
- *Keep RNA cold (-80 °C for long term storage; -20 °C for a few days; on ice while in use on the benchtop)*

Materials:

- *Mini-PROTEAN Tetra Cell (BioRad)*
- *TransBlot Turbo (BioRad) or Mini Trans-Blot Cell Assembly for Transfer (BioRad)*
- *Loading buffer [LBII (Ambion)- Glycerol, bromophenol blue, xylene cyanol, formamide], stored at -20 °C*
- ***10x TBE Stock (Dilute to 1x TBE as needed – F/S):***

  108 g Tris Base
  55 g Boric Acid
  40 mL 0.5 M EDTA (disodium salt), pH 8.0

  Bring to 1000 mL with MQ H_2O

- ***1x and 6x SSC – F/S (scale up as needed):***

  1x: 1 mL 20x SSC (Ambion)
  19 mL MQ H_2O

  6x: 6 mL 20x SSC (Ambion)
  14 mL MQ H_2O

- ***ULTRAhyb-Oligo (Ambion)****, stored at 4 °C, needs to be heated to go into solution prior to use (****Tip****: Place in rotisserie set at 65°C to help homogenize solution)*
- ***Odyssey Blocking Buffer (LiCor)***
- ***Wash Buffers (do not need to be filter sterilized)***

  Low Stringency: 50 mL 20x SSC
  2.5 mL 20% SDS
  Bring to 500 mL with MQ H_2O

  High Stringency : 2.5 mL 20x SSC
  2.5 mL 20% SDS
  Bring to 500 mL with MQ H_2O

Protocol (for detecting ~120 nt sRNA):

1. **Prepare a 10% TBE-Urea gel:**
   To a beaker ***add****:*
   a. 3.3 mL 30% acrylamide (Protogel) [**Read SDS and SOP before use!!**]
   b. 1.0 mL 10x TBE
   c. 2.136 mL water (MQ)
   d. 4.2 g urea

   Cover with Parafilm and Incubate *at 37˚C until urea dissolves. Then add*
   e. 330 µL 1.6% APS (stored at 4 °C, needs to be less than 1 month old)
   f. 5 µL TEMED

   Swirl *to facilitate polymerization and then pour into gel casting frame.*
   Allow gel to ***solidify*** *in a Mini-PROTEAN Tetra cell casting frame/stand for 45'. Gel can be stored overnight, wet with 1x TBE, at 4 ˚C.*

   This general video shows how to set up the gel casting frame (but we pour a different kind of plates): *https://youtu.be/EDi_n_0NiF4*

2. **Prepare RNA Samples:**
   a. **Prepare** RNA samples with equal volume of LBII, (try to load the same volume in each lane; adjust volume of RNA with UltraPure H_2O).
   b. **Heat** for 5' at 85 ˚C, **pico-centrifuge**.
   c. Keep RNA cold (on ice).

3. **Pre-Run and Run Gel:**
   a. **Pre-Run gel** in 1x TBE at 250 V for 20' in a Mini-PROTEAN Tetra cell tank.
   b. **Pipette** out gel debris from each lane before loading RNA samples.
   Note: This step is extremely important, since debris largely interferes with sample loading. Consider also pipetting gel debris immediately prior to loading each individual well, since debris can recollect if you leave the well to sit over time.
   c. **Run** gel in 1x TBE at 200 V for 45-60', (it is OK if the dark blue loading dye runs off gel; note, adjust the time so that your RNAs of interest are in the middle half of the gel).

****If you are planning to use the Trans Blot Turbo, begin preparation of Riboprobe while the gel is running (see Riboprobe section below)****

4. **Transfer Gel to Membrane:**
   Disassemble the Tetra tank; remove the gel from the glass plates, cut off wells and notch the TOP LEFT corner of the gel with a razor blade.

   a. Traditional Method
      i. **Equilibrate** sponges, filter paper and membrane (with TOP LEFT corner cut) in 1x TBE.
      ii. **Prepare** gel sandwich (black, sponge, filter paper, 10% TBE-Urea gel, a 60 cm^2 membrane, filter paper, sponge, white).
      iii. **Transfer** (black to black, clear to red) in Mini Trans-Blot Cell in 1x TBE at 200 mA for 60'. Add a stir bar and ice block to cell and place on a magnetic stirrer.
      ****Begin preparation of Riboprobe (see Riboprobe section below)****

b. **Trans Blot Turbo (recommended)**
   i. **Equilibrate** extra-thick filter paper and membrane (with TOP LEFT corner cut) in 1x TBE.
   ii. **Prepare** gel sandwich in the Trans-Blot Turbo “RNA” Cassette (cassette bottom, extra-thick filter paper, membrane, TBE-Urea gel, extra-thick filter paper, cassette top)
   iii. **Transfer** using turbo command on main menu. Select the number/size of gel(s) that you will be transferring with this program. This will take you to a screen showing the current conditions in the cassettes. Push the RUN button to start the transfer. Transfer will be finished in 7 minutes.

5. **Wash Membrane and cross-link:**
   Handle *membrane* ***gently****, avoid touching the bulk of the membrane, even with gloved fingers:*
   a. **Wash membrane** in ~20 mL of 6 X SSC for 2’ with rocking.
   b. UV **Cross link: “Autocross link”**.
   c. **Wash membrane** in ~20 mL of 1 X SSC for 1’ with rocking.

6. **Prehybridization and Hybridization:**
   a. **Pre-hybridize** (≥30’) standard membrane size in 6 mL of ULTRAhyb-Oligo (Ambion) at 60-65 ˚C.
   b. Add biotinylated riboprobe specific for RNA (10 µL of a 20 µL in vitro transcription reaction is PLENTY!) <u>and</u> DNA probe for 5S (2.5-5 µL, ~ 250 ng, 800-5S).
   c. Allow to **hybridize** overnight at 60-65 ˚C in the dark (since IR dye is light sensitive)

The following day….

7. **Stringency Washes**
   a. Remove hybridization solution and wash at room temperature (door to chamber cracked open is fine) in Low-Stringency Wash Solution for 5 minutes. Repeat.
   b. Wash 15 minutes at 50-65 °C (same temp as overnight hybridization is fine) in High Stringency Wash Solution. Repeat.

8. **Blocking**
   a. Add 1 ml of 20% SDS to 19 ml Odyssey Blocking Buffer for a final concentration of 1% SDS.
   b. In a black Licor container, cover blot with Odyssey Blocking Buffer plus SDS and gently shake at room temperature, in the dark, for a minimum of 60 minutes. For more sensitive detection, blocking for a longer time may reduce background.
      Do not pour blocking solution directly onto blot. Dribble the solution down the side of the container so it gently washes over the blot.

9. **Streptavidin Incubation**
   a. Dilute Streptavidin-IRDye 680 conjugate or Streptavidin- IRDye 800CW conjugate (whichever is appropriate for the riboprobe) with 10 mL Odyssey Blocking Buffer plus 1% SDS to a concentration of 1:10,000.
   b. Remove old blocking buffer and lightly cover the blot with the 1:10,000 streptavidin-IRDye 680 or Streptavidin-IRDye 800CW buffer, approximately 5 ml/10 cm2. Incubate

30 minutes at room temperature while gently shaking. [Avoid incubation for longer than 30 minutes, which may lead to high background.]
Do not pour blocking solution directly onto blot. Dribble the solution down the side of the container so it gently washes over the blot.

10. **Wash**
    a. Wash the blot 3 times in 10-15 mL 1x PBST (0.1% Tween-20) for 5-10 minutes each, shaking at room temperature. Follow with a rinse in 1x PBS for 5-10 minutes at room temperature. Wash steps must be performed in darkness. Use a black dish or cover container with aluminum foil.
    Longer washes will reduce background.

11. **Scan Blot!**
    a. Scan blot on Odyssey. Start with the **Intensity** parameter set to 5 for Northern blots. If necessary, scan again and adjust intensity.

12. **Storing and/or Stripping**
    a. The blot can be dried between two pieces of filter paper, in the dark, and then stored (still sandwiched between filter paper) at -20 °C and rescanned or even re-probed at a later time.
    b. If stripping of riboprobe is needed: I have had some luck with this protocol:
       - Incubate membrane with TE+1%SDS buffer (enough to cover membrane)
       - Rotate in a hybridization oven for 30 min at 85-95 °C.
       - Repeat this twice, each time with new buffer.
       - Expose membrane again to see if it worked.

Making Riboprobes

Reagents:

Forward and Reverse primers (Forward should have T7 sequence at 5' end:
GGATCCTAATACGACTCACTATAGGG) – See Appendix Below
Reagents for PCR (buffer, polymerase, 2.5 mM dNTP mix)
T7 RNA Polymerase (Promega P2075) with 5x Buffer and 100 mM DTT
2.5 mM ATP, CTP, GTP mix
2.5 mM UTP
2.5 mM biotin-16-UTP (Roche 11-388-908-910)
RQ1 DNase (Promega M6101)
Micro Bio-Spin 30 (BioRad)

Protocol:

Prepare DNA template (for MtlS riboprobe – 042208A and 042208B)

1. Set up the following reaction in a PCR tube:
   2 µL gDNA
   1 µL 50 µM Forward Primer
   1 µL 50 µM Reverse Primer
   5 µL 10x Buffer
   4 µL 2.5 mM dNTPs
   36 µL H_2O
   0.5 µL DNA Polymerase
2. Run PCR reaction 30 cycles, 55 °C 30s; 72 °C 30s
3. Run 5 µL reaction on a 2% agarose gel (product for MtlS runs around 120 nt)
4. Column purify PCR reaction (Qiagen PCR Cleanup) – elute with 50 µL 1 mM TRIS
5. Quantitate (5-10 ng/µL is good)
6. DNA can be stored at -20 °C

Make riboprobe

1. Set up the following reaction in a 1.5 mL RNase-free tube:
   2 µL PCR-generated DNA template
   4 µL 5x Buffer
   2 µL 100 mM DTT
   4 µL 2.5 mM rNTP mix
   2.4 µL 2.5 mM UTP
   1.6 µL 2.5 mM biotin-16-UTP
   3 µL H_2O
   1 µL T7 RNA Polymerase
2. Let reaction incubate at 37 °C for 1-3 hours
3. Add 1 µL RQ1; incubate at 37 °C for 30 minutes
4. Clean up reaction with BioRad Micro P-30 column (in fridge)
5. Add riboprobe* to hybridization buffer

* Amount of riboprobe will vary; 10 uL of a 20 uL reaction is PLENTY for detecting MtlS.

Appendix: Designing Primers to Make Riboprobes (or for in vitro transcription)

1. Identify the sequence that you want to transcribe
    a. For riboprobes, the PCR product should be ~ 100-500 bp long
    b. For riboprobes, the **forward** primer should be complementary to the transcript that you want to probe for.
2. Design the forward and reverse primers, following standard primer-design protocol (~ 55 °C T_m, 3' end is G/C)

Append to the 5' end of the forward primer the following sequence (T7 promoter):
GGATCCTAATACGACTCACTATAGGG

3. Order the primers from IDT
4. Resuspend primers in 1 mM TRIS-HCl, pH 8.0 to a final concentration of 50 μM