Mayr lab
mRNA-Northern Blot Protocol

the protocol is based on the CSH protocol for Northern blots

Vortex all reagents
Use ONLY filter tips
Use ONLY sterile reagents/water

Chloroform Extraction (after Harvesting/Trizol)

1. Thaw the Trizol samples at RT.
2. Add 200 μL of Chloroform per eppi.
3. Mix the eppis for 15 seconds (For a small amount of samples, use your hands to shake the samples vigorously; for large amounts of samples, place another eppi block on top of the one holding your samples and hold it down tight, then shake vigorously).
4. After shaking allow the samples to sit 2-3 minutes.
5. Spin at 20,000 g at 4ºC for 20 minutes.
6. Transfer clear upper phase into fresh eppi's (for 1mL of Trizol this ends up being ~400 – 500 μL of the clear phase).
7. Add 500 μL Isopropanol to each eppi.
8. Vortex the eppis and store at -20°C for at least 2 hours (usually we do ON)

Washing the RNA

1. Spin the eppis at 20,000 g at 4ºC for 20 minutes.
2. Pour off the supernatant (the pellet should be stuck to the wall of the eppi).
3. Add 1 ml Et-OH (70%) to each eppi [50mL of 70% Ethanol = 35mL 200 proof Ethanol + 15mL Sterile Water].
4. Spin 10-15 minutes at 4ºC at 20,000 g.
5. Pour off the supernatant.
6. Remove liquid off of the pellet using first a P200, then a P2. Make sure you have removed ALL of the Ethanol!
7. Leave eppis open on the bench for 5 min, checking every so often to see if the pellet is clear. Sometimes this will take more than 5 minutes depending on the size of your pellet.
8. When the pellet is transparent, add sterile water to the eppi (the amount of water you add depends on the size of the pellet. For very small pellets, add 10 – 20 μL, for large pellets, add 40 – 60 μL of water).
9. Close the eppis and put on ice for at least 30 minutes (it is best if you allow the pellets to sit and dissolve over the day, before resuspending them).
10. Resuspend/lyse the pellet well by pipetting up and down for **at least** 1 minute (oftentimes this will take longer than a minute depending on the size of the pellet). If there is not enough volume to resuspend the pellet, keep adding water in 10 – 20 μL increments until you are able to dissolve the pellet well. Note the amount of water added.
11. Take 0.5 μL of dissolved RNA and put it in 4.5 μL of water (in a separate eppi or PCR tube).
12. Use the nanodrop to spec the starting amount of yotal RNA, and try to use at least 180 μg of total RNA for the Oligotex procedure.

Oligotex mRNA Spin-Column Protocol (Quiagen)*

*Before starting, make sure the heat block is set to 70°C, the water bath is set to 25°C, and 1mL tubes of OEB are heated up in the 70°C heat block.
*It is best to do Oligotex in batches of 8 samples, NO MORE than 8.

1. Pipette 180 μg of total RNA into 1.5 ml microcentrifuge tube and adjust the volume to 250 μL with sterile water.
2. Vortex the Oligotex Suspension (at room temperature), and make sure the beads are well suspended.
3. Add 15 μL of the Oligotex Suspension to each eppi containing RNA.
4. Add 250 μL of Buffer OBB to each eppi and mix well by pipetting up and down.
5. Incubate the eppis for 5 minutes at 70°C (in the heat block).
6. Remove the eppis and place them in a 25°C water bath for 15 minutes.
7. Centrifuge the oligotex:RNA complex for 2 minutes at max speed.
8. Pipette off the supernatant (the beads will form a pellet at the bottom).
9. Resuspend the pellet in 400 μL of Buffer OW2 by pipetting up and down.

10. Pipette the resuspended sample onto a small spin column placed in a 1.5 ml microcentrifuge tube.
11. Centrifuge the spin columns for 1 minute at max speed.
12. Transfer the spin column to new tube and apply 400 μL of Buffer OW2 to the column. Centrifuge the eppis for 1 minute at max speed and discard the flow-through.
13. Transfer spin column to a new eppi labeled with the sample name and date.
14. Place 4 eppis into the heat block and add 100 μL of hot buffer OEB (from the 70°C heat block).
15. Resuspend the beads in the OEB by pipetting up and down.
16. Spin the columns for 1 minute at maximum speed.
17. **Repeat** Steps 14-16 and discard the spin column; place the eppis on ice.

Precipitating mRNA

1. To each eppi (containing 200 μL mRNA and OEB), add the following:

    20 μL of 3M Sodium Acetate, pH=5.2 [sterile].
    1 μL Glycoblue
    550 μL 100% Ethanol (2.5X volume)

2. Vortex the eppis and put in the -20°C freezer overnight (or for a minimum of 2 hours).

Washing mRNA

1. Spin the eppis for 20 minutes at 20,000 g at 4ºC .
2. Pour off the supernatant.
3. Add 500 μL of 70% Ethanol.
4. Spin for 5-10 minutes at 20,000 g at 4ºC .
5. Pour off the supernatant and pipette off any remaining liquid 2X (first with a P200, then with a P2).
6. Let the pellet air dry 1-3 min (the pellet will be blue because of the Glycoblue; keep checking the pellet to make sure it doesn't over-dry).
7. Add 3 μL of sterile water on top of the pellet and lyse pellet well by pipetting up and down.
8. Spec the mRNA by making a 1:10 dilution (2.7 μL water + 0.3 μL mRNA).

Try to use over 2 μg of mRNA for a Northern blot (the "norm" is 2.8 μg). Make sure mRNA is even throughout the samples.

Final preparation of samples for the Northern Blot

1. In a new eppi, add the amount of mRNA you will be using for the blot (calculate how much you need to add for each sample and place that amount into a new eppi).
2. Make a separate eppi for the blot Marker, use 3 μL of the RNA Ladder (Fermentas Riboruler 0.2-6.0 kb) (in the -80°C freezer). TREAT THE MARKER LIKE ONE OF YOUR SAMPLES!
3. Add 10 μL of Glyoxal-mix to each eppi (including the Marker eppi) and mix by pipetting up and down.
4. Incubate the eppis for 1 hour at 55°C (heat block*). *Keep an eye on the temperature of the heat block to make sure that it stays essentially constant at 55°C.
    During this time, make a 1% gel [100 mL 1X TAE (RNA quality, NO ETHIDIUM BROMIDE!) + 1.0 g of GTG SeaKem Agarose]
        It is **essential that the surface of the gel is FLAT and EVEN**
5. After incubating for 1 hour at 55°C, transfer the eppis to ice and allow them to sit for 10 minutes.
6. Spin down the eppis to remove any condensation from their lids.
7. Add 2 μL of RNA loading dye [RNA Loading Buffer] to each eppi.
8. Load **the entire** sample on the gel and run the gel in 1X BPTE at 72 volts for 3-3 ½ hours **in the cold room**. [Run the gel until the darker dye band is 8 cm from the well].
9. Take a picture of the gel when done, note exposure time and **save** the image.

Transferring the mRNA to the Membrane using the Whatman Turboblotter

1. Set up two clean Pyrex dishes, one filled with a bit of sterile water, one filled with 20X SSC.
2. Soak the membrane in sterile water until it is completely soaked.
3. Soak the gel, membrane and Whatman papers (only those that need to be wet) in 20x SSC for 5 minutes.

4. Set up Whatman Turboblotter as denoted on the wick cover. *
   It is **essential that there are **NO** air bubbles between the gel and the membrane, as well as the gel and the Whatman paper above it. **
5. Leave the stack overnight.

After Transfer to Membrane*

*Set the Hybridization Oven to 80°C.

1. Set up two clean Pyrex dishes, one filled with 200 mL of 2X SSC (20 mL 20X SSC + 180 mL sterile water), and one with 200ml of 20 mM Tris HCL (pH 8.0).
2. Remove the upper Whatman papers and the gel from the stack, set the gel aside.
3. Cut the upper left corner of the membrane/blot (for orientation).
4. Put the membrane in the dish filled with 200 mL of 2X SSC and shake it for 5 minutes.
5. While the membrane is shaking, take picture of gel at same exposure time as the first picture from the previous day. Take another picture of the gel, but overexpose it (this shows how well the blot transferred).
6. After 5 minutes in the 2X SSC, place the membrane in the dish filled with ~200 mL of 20 mM Tris HCL (pH 8.0) and shake it for 25 minutes (this reverses the glyoxal reaction).
7. Remove the membrane/blot and place it on a piece of dry Whatman paper.
8. Cross-link the blot at 1000J.
9. Put blot in hybridization oven at 80°C for 30 minutes.
10. Once the blot is dry, write name of blot and date in bottom left corner of the blot in **pencil**.
11. Proceed either with Pre-Hyb (Ultra-hyb solution, Ambion) or save the blot in a drawer for later.

Hybridizing the Blot

Pre-Hyb*

*Set the Hybridization Oven to 42°C and pre-warm the ULTRA-Hyb buffer (Ambion) in the oven for at least an hour (or until the entire contents of the bottle are liquid and warm).

1. Roll the blot lengthwise (roll the long side) and place it inside the hybridization tube.
2. Add 16-17 mL of the warmed ULTRA-Hyb buffer to each hybridization tube.
3. Close the hybridization tube with its cap. Make sure that the gasket is inside the cap!
4. Roll the closed tube with the buffer inside until the blot sticks to the inside of the tube (the RNA side of the blot will be facing the inside of the tube).
5. Place the hybridization tube into the 42°C oven and set the rotation to "low".
6. Allow the blot to pre-hyb for **at least** 1 hour.

Labeling the Probe with ^{32}P (Megaprime Labeling Kit, Amersham)

*Make sure there is a 37°C water bath and 90°C heat block set up and that the ^{32}P alpha-dCTP is thawed (this can sometimes take up to 45 minutes).
We use DNA probes (PCR product: 300-700 bp, gel-purified).

1. Label two eppis: Eppi 1 = (Name of the probe + date), Eppi 2 = (Name of the probe + 2)
2. Into Eppi 1 add:
   25 ng of the probe
   5 μL of the Labeling Primer (Black cap)
   Sterile water for a total volume of 10 μL.
3. Into Eppi 2 add:
   10 μL of the Labeling Buffer (Blue cap)
   23 μL of sterile water
   2 μL Klenow Fragment Enzyme (Red cap)
4. Put Eppi 1 onto the 90°C heat block for 5 minutes.
5. Transfer Eppi 1 to ice for 2 minutes.
   **From this point on, work behind a shield due to radioactivity! **
6. While Eppi 1 is incubating, add 5 μL of radioactivity (32P alpha-dCTP) to Eppi 2.
7. Add all of the contents of Eppi 2 to Eppi 1.
8. Incubate Eppi 1 in a 37°C water bath for 1 hour.
9. After incubating, spin down the eppi to remove condensation.
10. Add 5 μL of 0.2 M EDTA to Eppi 1. *

11. Put Eppi 1 back on the 90°C heat block for 5 minutes.
12. Spin down Eppi 1 in the tabletop centrifuge to get rid of condensation on the lid, put on ice but immediately add to the blot.
13. **Mix the probe well** and add 27 μL (half) of the labeled probe (Eppi 1) to each Hybridization tube **or** you can add all of the probe to one hybridization tube, and then **save** the hyb buffer + probe in a falcon tube the next day to add to another blot). **When you add the probe, do not add it to the blot. Tilt the tube until the hyb buffer is above the blot and add the probe to the buffer by pipetting up and down. **
14. Close the hybridization tube well.
15. Return the hybridization tubes back to the oven (still at 42°C) and let them hyb overnight (on rotation speed "low").

Washing the Blots

*Make sure there are enough of Wash Buffers 1 and 2 before you begin washing. They should be in the oven in the hallway at ~45°C.

1. Pour off the UltraHyb + probe (approximately 17-20 mL) into a falcon tube (if you are going to re-use the probe), or into the liquid radioactive waste container*. *Pour the buffer like you are pouring wine, being very careful not to spill or let liquid drip on the outside of the hyb tubes. If you are saving the buffer/probe in a falcon tube, transfer it carefully and then put the falcon tube in the radioactive plexi box in the 4°C fridge.
2. Add 30 ml of Wash Buffer 1 (according to Ambion Ultra-hyb instructions) to the hyb tube and wash for **10 minutes** in the hyb oven, rotating at fairly high speed.
3. Pour off Wash Buffer 1 and repeat step 2.
4. Pour off Wash Buffer 1 and add 30 ml Wash Buffer 2.
5. Wash in Wash Buffer 2 for 20 minutes.
6. Pour off Wash Buffer 2.
7. Use forceps to take the blots out of the tubes and place them into a plastic sleeve for exposure. Make sure that the plastic sleeve is clean (no bits of dirt, paper, etc. stuck to it) before putting in the blot, and make sure that there are no wrinkles in the blot when you put it in the sleeve.
8. Expose the blot for 1 hour in a cassette (put down blots in their sleeves, then put film over the blots and close cassette, noting the date and time).
9. After 1 hour, take a picture of the blot and re-expose.

Wash Buffer 1 (1L)
100 mL 20X SSC
10 mL 10% SDS (Sterile)
Fill with sterile water to 1 L

Wash Buffer 2 (1L)
5 mL 20X SSC
10 mL 10% SDS (Sterile)
Fill with sterile water to 1 L

Scanning the Blots with Phosphorimager

Stripping the Blots

1. Warm 200-300 ml 0.5% SDS [50 mL 10% SDS in 1 L Water] to a boil in microwave (for 300 mL this takes ~2:30 minutes).
2. Allow the 0.5% SDS to cool for 10 sec (just so it's not boiling, or until all the bubbles have settled).
3. Put blot in a Pyrex dish on the shaker and carefully pour on the 0.5% SDS. **DO NOT POUR THE SDS DIRECTLY ONTO THE BLOT!!!!**
4. Shake for at least 20 minutes.
5. Pour off the 0.5% SDS.
6. Repeat steps 1-5 for at least another 2 times.
7. Remove the blot from the dish and let it dry for ~10-15 minutes on dry Whatman paper. Store in plastic sleeves or prepare for hybridization*. *Alternatively, you can expose the blot for 1 hour to see how well the stripping worked.

Recipes for Buffers:

BPTE Electrophoresis Buffer
100 mM PIPES
300 mM Bis-Tris
10 mM EDTA

The final pH of this 10x buffer is approx. 6.5. The 10x buffer can be made by adding 3 g of PIPES (free acid), 6 g of Bis-Tris (free base), and 2 ml of 0.5 M EDTA to 90 ml of distilled H_2O, treating the solution with DEPC (final concentration 0.1%) for 1 hour at 37°C, and then autoclaving.

Glyoxal-mix

6 ml of DMSO
2 ml of deionized glyoxal
1.2 ml of 10x BPTE electrophoresis buffer
0.6 ml of 80% glycerol in H_2O
0.2 ml of ethidium bromide (10 mg/ml in H_2O)

Divide into small aliquots and store at -80°C. Use each aliquot only once and then discard.

To deionize the glyoxal:

1. Immediately before use, mix the glyoxal with an equal volume mixed bed ion-exchange resin (Bio-Rad AG-510-X8). Alternatively, pass the glyoxal through a small column of mixed bed resin, and then proceed to step 3.
2. Separate the deionized material from the resin by filtration (e.g., through a Uniflow Plus filter; Schleicher & Schuell).
3. Monitor the pH of the glyoxal by mixing 200 µl of glyoxal with 2 µl of a 10 mg/ml solution of bromocresol green in H_2O, and observing the change in color. Bromocresol green is yellow at pH <4.8 and blue-green at pH >5.2.
4. Repeat the deionization process (Steps 1-2) until the pH of the glyoxal is >5.5.

Deionized glyoxal can be stored indefinitely at -20°C under nitrogen in tightly sealed microcentrifuge tubes. Use each aliquot only once and then discard.

RNA Gel-loading dye
95% deionized formamide
0.025% (w/v) bromophenol blue
0.025% (w/v) xylene cyanol FF
5 mM EDTA (pH 8.0)
0.025% (w/v) SDS