Riboprobes (making and using) [or T7 in vitro transcription]

General Notes

Ref: J. M. Liu Post-doc, experiment 94

Always practice RNase-Free technique when working with RNA
- Use RNA-only pipet tips and tubes
- Wipe off counter tops and pipetmen with RNaseZap
- Filter-sterilize solutions prior to use
- Use either DEPC-H_2O or MQ-H_2O
- Keep RNA cold (4 °C or lower)

Reagents:

Forward and Reverse primers (Forward should have T7 sequence at 5' end: GGATCCTAATACGACTCACTATAGGG)

Reagents for PCR (buffer, polymerase, 2.5 mM dNTP mix)

T7 RNA Polymerase (Promgea 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)

DTR cartridges (Edge Biosystems)

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.

General procedure on Northerns with riboprobes (MtlS)

Run out RNA on 10% TBE-Urea gel in 1xTBE (200 V, 55 m)

Transfer RNA to nylon+ membrane in 1xTBE (200 mA, 1h)

Wash membrane with 6x SSC; crosslink; wash membrane with 1x SSC

Prehybridize membrane (standard size) with 6 mL UltraHyb-Oligo (Ambion) at **65 °C**, for at least 30 minutes

Hybridize overnight at 65 °C

Detection of probe (see appropriate protocol in BrightStar BioDetect kit (Ambion) or LICOR Odyssey Northern Blot Analysis)

Stripping the probe off the membrane
Riboprobes are pretty difficult to remove. 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.