Ribonuclease Protection Assay:

Making RNA probe

- Linearized DNA (transcribed 3'-5')
- Reaction for RNA probe
  - $^{32}$P-CTP 1mCi/25µl total per order
  - aliquot: 2.5 µl, 3.7 µl, 5.0 µl (commonly using 3.7 µl)
  - Note: fill out form for Isotope.

1. Mix all reagents in isotope aliquot tube (using microtubes)

<table>
<thead>
<tr>
<th>Rx'n</th>
<th>10 µl</th>
<th>15 µl*</th>
<th>20 µl</th>
<th>add order</th>
<th>stored place</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotope</td>
<td>2.5</td>
<td>3.7</td>
<td>5.0</td>
<td>origial</td>
<td>-20</td>
</tr>
<tr>
<td>Mix-C(A,G,T)</td>
<td>2.0</td>
<td>3.0</td>
<td>5.0</td>
<td>first</td>
<td>-20</td>
</tr>
<tr>
<td>5x buffer</td>
<td>2.0</td>
<td>3.0</td>
<td>4.0</td>
<td>first</td>
<td>-20</td>
</tr>
<tr>
<td>DTT</td>
<td>0.6</td>
<td>0.9</td>
<td>1.2</td>
<td>first</td>
<td>-20</td>
</tr>
<tr>
<td>RNasin(antiRNase)</td>
<td>0.3</td>
<td>0.4</td>
<td>0.6</td>
<td>second</td>
<td>-20</td>
</tr>
<tr>
<td>DNA</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
<td>third</td>
<td>-20</td>
</tr>
<tr>
<td>H$_2$O(RNase free)</td>
<td>0.6</td>
<td>1.0</td>
<td>0.2</td>
<td>first</td>
<td>-20</td>
</tr>
<tr>
<td>Enzyme*</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
<td>last</td>
<td>-20</td>
</tr>
</tbody>
</table>

* different reaction uses different enzyme--for rat OT mRNA, using SP6 RNA polymerase

The following protocol is for 15 µl reaction

2. Incubate in 37 C incubator for 45-60 min (For Northern blot assay, incubate 1.5-2 h)
   Start to prepare the gel

3. Add 0.6 µl rRNasin and 6.0 µl DNase I
   Incubate 37 C incubator for 20min

4. Add 100 µl TE, 150 µl mixture of phenol/chloroform (stored in frig), 4 µl tRNA(stored in -20 C)

5. Vortex and spin 5 min

6. Take top aqueous layer to a clean tube
7. Add 50 µl 10M Am Acetate, 50 µl TE and 800 µl EtOH

8. Incubate in -20 C or -70 C or dry ice for 30 min
   (can be kept in -20 C for overnight)

   Before starting any reaction, boil water
9. Centrifuge 15 min at RT

10. Take all the liquid out using tip and napkin
    Dry pellet in air for 5-10 min

11. Add 3 µl TE and mix with pipe twice and leave tip in the tube
    Add 16 µl loading buffer and mix well

12. Boil the samples for 5 min and remove to ice immediately

13. Quick spin

14. Load to gel
Ribonuclease Protection Assay:

**Preparation and running polyacrylamide-gel for probe purification:**

- Pour 100 ml acrylamide gel in flask with vacuum outlet
  - Vacuum for 30 min
- During acrylamide vacuum, clean the glasses using Contrad, water, and EtOH
- Put in spacers and tape at two sides and bottom
  - Tight sides with clamps
- Add 300 µl Ammonium persulfate (AP)(stored in frig) and 60 µl TEMED (stored in frig, brown bottle)
- Pour into plate set immediately and put the comb on (don't put in too deep)
- Set about 1-2 hr
- If the gel is not for same day experiment, cover wet paper towel and Saranwrap to protect from dry (leave on bench for next day)

Boiling water and turn on 37 C Shaking water bath (on radioactive area)
- Check or make the running buffer from 10x TBE buffer
  - 1 L of 1x TBE: 100 ml 10x TBE + 900 ml dd H₂O
- Fill in the top and bottom of gel cells up to edge of glasses
- Tight glass plates and gel cast with clamps in both sides
- Take out the comb and wash the wells several times
- Prerun 20-30 min
- Load samples after boiled and run at 350 v for 1-2 hrs
- Carefully take one glass from gel (starting on the corner)
- Keep big glass on the bottom and put the saran wrap on the top of the gel
Ribonuclease Protection Assay:

Isolation of probes

Preparing materials:
- Labeled microcentrifuge tubes (according to number of samples)
- Surgical blade (type: No.11)
- Marker
- Small size film
- Protecting glass
- Paper trawl

- Put film on top of gel, confirm by left hand, and mark top and right edges of film on the gel
- Keep film on the gel for few seconds
- Make a mark by folding a corner for orientation and develop film
- Cut a window on band site of film
- Fit the marks on gel and cut band through the window and save them into tubes
- Make another picture to make sure that the band cut completely
- Add 400 µl Gel Eluting buffer (gel buffer-stored in frig)
- Incubate in 37 C shaking water bath for 2 hrs (150-200 bases) or 3 1/2 - 4 hrs (>400 bases)
- Transfer supernatant to clean tubes and avoid gel pieces
- Add 1 ml EtOH
- Incubate on dry ice for 20-30 min (can be in −80 C freezer up to set 3 hrs)
  Starting prepare the RNA samples
- Spin the RNA probe for 15 min
Continue prepare the RNA samples

- Air dry pellets (set upside down and absorb the liquid on wall using napkin)

- Make Hybridization buffer (volume of buffer depending on the number of samples)
  - 500 µl buffer:
    - 100 µl 5x salt (stored in –70 C)
    - 400 µl formamide (stored in frig, working in chemical hood)

- Make working concentration of probe solution:
  - add 3 µl TE, and add 50 µl Hybridization buffer to probe tube
  - mix well
  - take 2 µl to scintillation vial with 2 ml scintiSafe Plus (Fisher Sci) to count
  - <10 µg RNA: 0.5-1 million cpm per sample
    - < 50 µg RNA: 2-3 million cpm per sample
    - 100 µg - 200 µg RNA: 10 million cpm per sample
  - counted number / 2 = count per µl
  - cpm/ml x total volume in probe tube = total count per purification
Ribonuclease Protection Assay:

**RNA protection assay**

- Total RNA sample preparation (prepare ahead or during probe incubating period)
  - calculate the volume of RNA for the assay
  - take proper volume of RNA to a clean microcentrifuge tube
  - make up all the samples to same volume using TE (usually 200 µl)
  - add 1:10 Na-Acetate to sample tube
  - add 3x amount EtOH of sample solution volume (600µl)
  - for small amount of RNA, add 5-10 µl tRNA (under 10 µl RNA)
  - Store in -20 C for over night, or dry ice or -70 C for couple of hours

- make negative control set--tRNA
  - 10 µl tRNA of 10 mg/ml stock (100µg/ml)
  - 90 µl TE
  - 10 µl Na-Acetate
  - 300 µl EtOH
  - keep on dry ice
- spin all samples and control for 10 min
- dry pellet (taking liquid off, put upside down, don't need very dry)
- add 10 µl TE to each sample and control
  - keep on dry ice
- dry pellet with Spin-Vacuum machine in 30 C heating for 10 min
  - after dry, put samples on dry ice immediately
- when probe ready, add 2 µl TE, 30 µl probe working solution to each sample
- denature by leaving in boiling water for 20-30 min
  - during boiling, dissolve the pellet
- after boiled, mix well by vortex and spin seconds
- wrap with parafilm
• incubate in 55°C water bath for 14-18 hrs or overnight (O.N.)

• after incubation, add 3 µl T1 RNase (cleave after G)

• add 300 µl RNase A mixture (cleave after C and U)
  RNase A mixture:
  5 µl RNase A of 5 mg/ml (stocked in –70°C)
  10ml digesting buffer (kept in RT)
  Final Concentration: 2.5 µl/ml
  keep on ice

  Ribonuclease digesting buffer:
  500µl 1M Tris (PH 7.5) (final 10mM)
  500µl 0.5M EDTA (final 5mM)
  3ml 5M NaCl (final 0.3M)
  make up to 50ml with DEPC water

• vortex to mix

• incubate in 30°C water (using hot tap water) for 30 min
  starting prepare the big gel

• add 3 µl Proteinase K (25µg/ml)(stored in -20°C)

• add 10 µl 20% SDS

• vortex to mix

• incubate at 37°C Incubator for 20-30 min--continue prepare the gel

• set up another set of clean tubes, and add 400 µl mixture of
  phenol/chloroform and 4 µl tRNA

• transfer samples to above tubes( because several times hot and cold,
  tubes may broken)

• vortex and spin 4-5 min

• transfer the aqueous layer to clean tubes
• If closing bottom layer is not clear, can take another tube and use tip
to take top liquid (don't bring bottom liquid to clean tube)

• add 400 µl isopropanol for precipitation

• incubate ½ -1 hr in room temperature

• spin 15 min

• take off supernatant and save it.

• air dry pellet

• add 2 µl TE and 6 µl loading buffer

• mix 2 µl unhybridized probe +200 µl TE (approximatly 3k at channel x10)
take 10 µl and mix to 20 µl loading buffer recoun 10 ml (expose to be in 2k-3k in x1)

• boil 15 min during boiling dissolve the pellet (make sure ppt. dissolved)

• put on ice and then quick spin

• load to gel(keeping on ice)
Note: after boiled, the samples must be loaded as soon as possible
Ribonuclease Protection Assay:

**Preparation and Running of acrylamide gel**

- put 100 ml 6-8 % acrylamide in flask with vacuum tube and vacuum for 30 min
during acrylamide vacuum, clean the glasses using Contrad, water, and EtOH

- coat Sigmacote on small glass plate

- put in spacers and tape the sides and bottom

- tight sides with clamps

- add 300 μl Ammonium persulfate (AP), 70 μl TEMED in to the gel

- pour gel between glasses immediately

- put comb in

- sit about 2 hrs

- take off bottom tape and set 1500-1600 volt and 50 watts

- pre-run about 30 min

- before load samples, re-wash the wells again

- run about 2-3 hrs (the bottom dye is on edge of glass plates)

- after running, drain the running buffer and lay the glass plates on table (small piece on top)

- take off tape and spacers

- pour some ice on glass for 1 min

- take off the top glass starting the corner (note: keeping gel on bottom glass--if having bubbles, don't worry because after filter paper cover the gel, the bubbles will be gone)
• use filter paper to cover the gel and confirm by hand (gel will stick on paper)

• cut off the extra paper

• cover saran wrap on top of gel

• lay the gel on gel-dry machine for 50 min - 2 hrs.

• after the gel completely dry, transfer it to film case and exposure to X-ray film in -70 C (different tissues and amount of RNA has different espoused time)
Ribonuclease Protection Assay:

**Chemical and Buffer:**

**6% acrylamide gel:**
- Urea 500 g
- ddH₂O 200 ml
- mix to dissolve may need to heat
- after completely dissolved and cool down a little bit
  - add 40% Acrylamide 150 ml
- make up to 900 ml with dd. water
- add ion exchange 10 g and mix for 15 min on stir
- filter
- add 100 ml 10x TBE
- store in a dark bottle at RT

**8% gel:**
same as 6% except 200 ml 40% acrylamide per liter

**40% Acrylamide:**
- acrylamide 380 g
- Bis 20 g
- make up to 1 liter with dd. water

**Mixture of nucleotides-without C:**
- ATP 50 µl
- GTP 50 µl
- UTP 50 µl
- Water 50 µl
- Total: 200 µl
- Aliquoted to 25 µl

**Proteinase K:**
- make a 25 mg/ml solution in sterile water and store in freezer in 60 µl aliquots.
20 % SDS:
  SDS  20 g
  dd. water  100 ml

10 M Ammonium acetate:
  Dissolve 385.4 g ammonium acetate in 150 ml H2O
  Add H2O up to 500 ml

10% Ammonium- Persulfate (AP):
  AP  10 g
  dd. water  100 ml

Ribonuclease digesting buffer:
  10mM(1M) Tris (PH 7.5)  500µl
  5mM(0.5M) EDTA  500µl
  0.3M(5M) NaCl  3ml (or 0.88 gram)
  make up to 50ml with DEPC water

Gel Eluting Buffer:
  50ml of Buffer:
  3.85 g Ammonium Acetate (2 M)
  1.25 ml 20% SDS (1%)
  0.125 ml tRNA (50 µg/ml)
  make up to 25 ml using DEPC water