MMLV Reverse Transcription Bray Lab Style

RNA Extraction from Cells

1. Only need one well of a 6 well plate (around 6.5 million cells).
2. Remove media on cells and add 500uL Tri reagent solution (in tissue culture cabinet or fume cupboard). **750**
3. Leave the plate 10 mins **5** rocking at room temp.
4. Take liquid up into eppendorfs and add 110uL chloroform. *Do this in the fume cupboard and dispose of tips, plate and eppendorfs* **150** *in the "phenol-chloroform solid waste" in the bug room.*
5. Shake vigorously to mix. **Incubate 2-3 min**
6. Centrifuge, max speed, 10 mins, 4°C. **15 min, 12k g, start second round**
7. Take top layer (aqueous) into new eppendorf and add an equal volume of isopropanol – usually around 300uL. **450ul**
8. Put at -20°C to precipitate RNA.

Leave this at least two hours for the RNA to precipitate, or overnight is easier. Note that the RNA is most stable when precipitated in this way, so it is best to leave it here until you are ready to reverse transcribe it into cDNA.

RNA Extraction from Tissues

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Resuspending the RNA

1. Pellet the RNA at max speed, 15 mins, 4°C. **12k G**
2. Wash the pellet in 70% ethanol (30% DEPC water), 1mL, and pellet again for 5 mins. **750 uL, vortex**
3. Remove as much liquid as possible and leave to dry. When the pellet dried, it will go from white to more transparent. **5-10 minutes**
4. Dissolve in DEPC water in a volume of your choice. Usually use 50uL with cells but go lower if you think your pellet is small.
5. Nanodrop on the RNA setting to measure concentration.

Notes on the locations of the things you need

- Ambion DNase kit is kept in the cardboard packet in the molecular Biology freezer shelf.
- Ribolock RNase inhibitor is in the Enzymes box and does not need to be defrosted – treat like an enzyme.
- Oligo(dT)s and MMLV buffer/enzyme are Promega and found in the Enzymes box.
- dNTP aliquots are the same as you would use for PCR and located in Stock box 1.

DNase Treatment

We perform a DNase treatment at this stage to make sure that all DNA produced in the reverse transcription has come from RNA. We use the Ambion DNA-free kit.

1. Set up the following DNase reactions:

Water (from kit)	To total 25uL
RNA	10ug (adjust as needed if low yield)
Buffer	2.5uL
DNase enzyme	0.5uL
Ribolock RNase inhibitor	0.5uL

2. Put at 37°C for 30 mins in PCR machine.
3. After this, the DNase enzyme can be inactivated with the inactivation beads from the kit.
4. Add 2.5uL of beads to each sample.
5. Put tubes on their side and leave for 5 mins, flicking the tube a few times to distribute the beads.
6. Spin at 10,000xg, 1.5 mins. Can use adaptors in benchtop centrifuges for PCR tubes.

MMLV Reverse Transcription

1. Take the equivalent of 2ug of RNA from the DNase reaction into a new PCR tube. This will be 5uL if you have used 10ug of RNA in the DNase reaction. *Be very careful not to take any beads and not to touch/scrape the side of the tube with the pipette tip, as the beads could inhibit the reverse transcription.*
2. Add water to a total of 12.5uL and 1uL of Promega oligo(dT)s. *You can prepare these tubes with water and oligo(dT)s while you wait for the 30 min DNase reaction.*

Water	6.5uL (usually)
Oligo(dT)s	1uL
RNA from DNase reaction	2ug (5uL usually)

3. Put these tubes in the PCR machine at 65°C for 5 mins.
4. Take the tubes directly from the PCR machine onto ice to cool quickly.
5. Add the following to each to tube to complete the reaction. *You can prepare this in advance as a mastermix.*

Promega MMLV 5X buffer	4uL
dNTPs	2uL
Promega MMLV RT enzyme	1uL
Ribolock RNase inhibitor	0.5uL

6. Put these in the PCR machine with the following program:

- 25°C for 5 mins
- 42°C for 60 mins
- 70°C for 10 mins
- 10°C forever

7. We usually then dilute the 20uL reaction to around 100uL to give more material for qPCR and to make sure the cDNA is not too concentrated for highly expressed genes.

Next, perform qPCR…