mRNA Extraction/cDNA Preparation

What's needed:
- Fresh tissue or tissue in **RNAlater** (STORE RNALATER/TISSUE IN THE FREEZER)
- Dynal magnetic beads (**Dynabeads M-280**) (STORE IN REFRIGERATOR)
- Magnet – Dynal or other strong magnetic device
- Large (1.5-1.8 mL) centrifuge tubes
- Dissecting kit (scalpel/razor blade, fine tweezers)
- Ice bucket with ice
- **100 uM oligo-dT** (X(biotin)-T_{25})
- Binding/washing buffer (0.14M NaCl, 1.5 mM $MgCl_2$, 10mM Tris HCl, pH 8.6) (=**BWB**) (KEEP ON ICE)
- Extraction/lysis buffer (BWB + 0.5% NP40) (=**BWB-X**) (KEEP ON ICE)
- dNTPs (KEEP ON ICE)
- reverse transcriptase (**RT**) (ALWAYS KEEP ON ICE OR IN FREEZER – MINIMIZE WARMING!)
- **5X reverse transcriptase buffer** (KEEP ON ICE)
- autoclaved H_2O
- Heating block set to 42°C

Useful tips:
- mRNA is supposed to be very labile and is particularly susceptible at room temperature or above (when RNases are active). Thus when working with solutions containing mRNA or which will come into contact with mRNA, keep them on ice and do procedures as quickly as possible when mRNA containing solutions cannot be kept on ice.
- Tissues can generally be extracted from many times. Place extracted tissue back in RNAlater (the same tube) for later use
- Label all tubes prior to use.

PROCEDURE

1. Prepare oligo-dT/beads.

- Rinse 10-40 uL of beads (per sample) with 10-40 uL of **BWB** (per sample). During first rinse resuspend beads – the next two rinses beads do not need to be resuspended (i.e., leave tube in magnet).
- To "dry" rinsed beads add 95 uL of **BWB** and 5 uL of **100 uM oligo-dT** (per sample)
- Gently spin or agitate for 30 minutes – resuspend beads by flicking tube several times.

2. Prepare reagents, etc. (while beads are incubating with oligo-dT).

- Aliquot 250 uL of **BWB-X** in large centrifuge tube on ice (per sample). Use smaller volume (*e.g.*, 100 ul) if you will be extracting mRNA from a small volume of tissue.
- Prepare the reverse transcriptase cocktail (per sample -- if doing N (>2) samples, prepare N+0.5 times the following quantities):
  - 15 uL **H_2O**
  - 5 uL **8 mM dNTPs** (i.e., the normal working stock of dNTPs)
  - 5 uL **5X reverse transcriptase buffer**

  (keep on ice – the RT enzyme will be added just prior to use)
- Make sure heating block is set to 42°C

3. Finish preparation of oligo-dT/beads after 30 minute spin.

- First, place tube in centrifuge and briefly spin in a small tabletop centrifuge.

- Place tube in magnet, allow beads to pellet on side of tube (5 seconds or so), and remove all liquid being careful not to remove any of the beads.
- Rinse three times with 100 uL of **BWB** (per sample) as before (resuspending only the first rinse).
- Add 100 uL of **BWB** to "dry" beads (per sample) and place 100 uL of the **BWB**/beads mixture in new large centrifuge tubes. Place tubes on ice.

4. mRNA extraction

- Place small section of tissue (e.g., a few mm of venom duct) into the tube containing 250 uL of **BWB-X**.
- Quickly vortex tube (2-3 seconds).
- Place tube on ice and start timer. Incubate for 5 minutes.

5. Combine mRNA extraction with beads/oligo-dT solution.

- Vortex tube again (2-3 seconds).
- Add all of the mRNA extraction solution to the tube containing the 100 uL beads/oligo-dT mixture.
- Place tube on ice and start timer. Incubate for 5 minutes. Once or twice during the 5 minutes, flick tub to mix solution (i.e., resuspend beads).

6. Rinse beads/oligo-dT/mRNA mixture.

- Quickly centrifuge the tube containing the beads/oligo-dT/mRNA mixture in the small tabletop centrifuge.
- Place tube in magnet and after beads have pelleted on the side of the tube, remove all of the liquid (or as much as possible).
- Rinse beads three times with 250 uL of **BWB** (resuspending only the first time).

7. Incubation of beads and RT cocktail.

- Add 25 uL of the reverse transcriptase cocktail to the "dry" beads.
- Add 0.7 uL of **AMV reverse transcriptase** to tube.
- Gently flick tube to mix contents.
- Place in heating block at 42°C for 1 hour. Flick tube every 10-15 minutes to resuspend beads.

8. Cleaning of the cDNA (OPTIONAL – this step is only necessary if you are going to tail the cDNA or digest the oligo-dT if it contains a restriction site. This step is not needed if you are only going to amplify from cDNA)**.**

- Place tube in magnet and once beads have pelleted, remove all liquid.
- Add 100 uL of **100 mM NaOH** to "dry" beads. Incubate on benchtop for 10 minutes.
- Place tube in magnet. Remove solution.
- Wash beads three times with **BWB** (resuspending only the first time).
- Wash beads once more with **TE**.
- Add 25 uL of **TE** to "dry" beads.

9. Storage of cDNA.

- Label the tube with proper name of sample (if necessary) and store the cDNA at 4°C.