Fiber RNA extraction protocol, 4/4/19

**Materials Needed**

Sigma RNEasy Isolation reagents and tubes (in box)

15 mL tubes for grinding

Acid washed glass beads (#22)

Beta Mercaptoethanol

Optima water

DNAse I

DNAse digestion buffer

Phenol-chloroform

5M ammonium acetate

**Grinding**

1. Start hot water bath at 56oC
2. Remove DNAse I and its digestion buffer from the RNA freezer and thaw on ice.
3. Weigh out 0.75g/sample of acid washed glass beads
4. Add glass beads to 5-6 ovules in 15 mL tubes (1-2 bolls for younger fiber)
5. Add liquid nitrogen tube
6. Vortex for 1 minute on, rest in liquid N2 for 20 seconds. Refill N2 in tube every 10 minutes.
7. Repeat vortex/rest cycle for following times (resting doesn’t count):
   1. 5DPA—2 mins (2 vortexes)
   2. 10DPA—10 mins (10 vortexes)
   3. 15DPA—12 minutes (12 vortexes)
   4. 20DPA—15 minutes (15 vortexes)
   5. 25DPA—20 minutes (20 vortexes)

**Extraction**

1. Prepare 1.5mL Lysis buffer (kit) per sample and add 15uL BME (beta-mercapto, big plastic bucket in hood) per sample (10ul BME/1mL Lysis)
2. Add 1.5mL Lysis buffer/BME to each tube and vortex 1 min
3. Incubate in hot water bath (56oC) for 5 minutes
4. Prepare Filtration columns and collection tubes for each sample (2/sample)
5. Vortex 2-3 min
6. Centrifuge at maximum speed for 3 minutes (at 4oC for all centrifugation)
7. Pipette 750 uL of the supernatant onto each filtration column (each sample will have 2 Filtration columns with 750ul each)
   1. Getting floaters is OK but to be avoided. Do NOT take any pellet
8. Close cap and centrifuge at max speed for 2 minutes. Save the flow-through, discard Filtration Column.
9. Prepare Binding column and collection tubes for each sample (1/sample)
10. Add 750uL Binding Solution to flow-through and pipette 5 times to mix (or vortex briefly)
11. Add 750uL of sample to the Binding column and centrifuge at max speed for 1 min
12. Discard flow-through and tap collection tube upside down on absorbent paper to drain
13. Repeat 11-12 until each sample has been condensed down onto a single Binding column and all the Binding solution has been centrifuged out
    1. This should be 2x/Filtration tube (750uL filtrate + 750uL Binding) and 4x/sample (2 Filtration tubes each)

**On-column DNA Digestion**

1. Pipette 300uL Wash Solution I onto each binding column and centrifuge at max speed for 1 minute.
2. Discard the flow-through and tap the collection tube out on absorbent paper before returning the column to the collection tube.
3. Combine 10uL/sample DNAse I with 70uL/sample DNAse digest buffer in separate tube
4. Pipette 80uL DNAse mixture onto the center of each Binding column.
5. Incubate at room temp for 25 minutes
6. Prepare final collection tubes for each sample
7. Pipette 500uL Wash Solution I onto each Binding column and centrifuge at max speed for 1 min.
8. Discard flow-through and tap Collection tube onto paper towel to drain.
9. Repeat Steps 7-8 twice.
10. Pipette 500uL Wash Solution 2 onto each column. Centrifuge at max speed for 30 seconds
11. Discard flow-through and tap collection tube to drain.
12. Repeat Steps 10-11.
13. Centrifuge the Binding column and collection tubes at max speed for 1.5 minutes
14. Transfer Binding columns to final collection tubes and let stand in hood for 2 min
15. Pipette 180uL Optima water to center of Binding columns and let stand for 2 min
16. Centrifuge at max speed for 1 minute. RNA is in flow-through; store in -80 until needed or proceed directly to phenol-chloroform cleanup.

**Phenol-chloroform cleanup**

1. Add 20uL 5M ammonium acetate to eluted RNA (total volume 200uL now)
2. Add 200uL phenol-choroform to each tube
3. Vortex 10 seconds (solution will be milky white)
4. Centrifuge at max for 10 minutes
5. Remove top layer and add to new tube; discard bottom layer
6. Add 200uL isopropanol
7. Precipitate at -20oC overnight
8. Centrifuge at 12000G for 10 min
9. Remove and discard supernatant
10. Add 200uL 75% EtOH
11. Centrifuge at 7500G for 5 min
12. Remove and discard supernatant
13. Dry in hood for 20-30 minutes
14. Re-suspend with 50uL H2O
15. Store in -80