High Molecular Weight (HMW) DNA Extraction – Digestion & Phenol-Chloroform
Updated: July 2019 (AEH edits to JFS and WLET toepad PC protocol)
Major HMW modifications are underlined

*** Use HMW tips, set centrifuge on soft brake ***
*** <u>DO NOT VORTEX ON HIGH</u> or pipette up and down as this will shear DNA ***

Sample Prep

1. Cut a 2 x 2mm chunk of tissue (usually liver or muscle) OR gently spin down (in benchtop centrifuge) the blood sample and pipette off ethanol (save in new tube in case you need to re-spin down).
    a. In general, for reference genomes, we prefer to sequence heterogametic individuals (males for mammals, females for birds etc.)
    b. We have found that fresh blood preserved in EtOH and stored at -80 performed well for birds
2. Place cut tissue or blood using pipette tip into labeled **<u>*LOW BIND*</u>** tube for digestion. Make sure the label includes what tissue type is being used.
    a. Bleach, wash in 95% EtOH, and then flame sterilize forceps between each sample

DAY 1
Digestion

1. Add the following to each tube:
    a. 180 µL Buffer ATL
    b. 20 µL Proteinase K
    c. 10 µL RNAse A (@ 10 ng/µL)
        i. The HMW, rapidly preserved tissues used for this protocol will often contain RNA. The addition of RNAse A will remove these fragments.
2. Manually rotate 10 times (can vortex on the lowest setting possible if needed) and spin down on a benchtop centrifuge, letting the centrifuge come to a full stop (e.g., do not forcefully stop with your hand). Place in pre-heated thermomixer at 56˚C
3. Incubate samples on a thermomixer at 56˚C at 200-300 rpm for ~2 hours
4. Remove from thermomixer and manually rotate 10 times (can vortex on the lowest setting possible if needed), and spin down on benchtop centrifuge **<u>*DO NOT VORTEX ON HIGH*</u>**
    a. Repeat steps 3-4 as needed until samples are completely digested.

DAY 1 Cont.
Phenol-Chloroform addition – PERFORM <u>ALL</u> REMAINING STEPS IN THE FUME HOOD PUTTING ALL LIQUID WASTE INTO THE PHENOL-CHLOROFORM LIQUID WASTE CONTAINER

1. Spin down Phase Lock Gel Light tubes at 12,000 rpm for 30 seconds
2. Transfer pre-spun sample to pre-spun Phase Lock Gel tube using **<u>*HMW TIPS*</u>**
3. Add 225 µL Phenol:Chloroform:Isoamyl Alcohol (24:25:1)
    a. Make sure PC is at room temperature, and manually invert bottle of PC 10 times to homogenize
4. Mix thoroughly by **<u>*SLOWLY*</u>** manually rotating tube for 10 minutes inside the fume hood
5. Open lid to each tube to vent gas that has built up in each tube
6. Centrifuge at 14,000 rpm for 15 minutes, set to **<u>*SOFT BRAKE*</u>**
7. Label **<u>*LOW BIND*</u>** tubes for final storage (include initial tube number). Make sure label includes what tissue type is being used.
8. KEEP supernatant (top solution) and transfer to final storage tubes being careful not to go near the interface between the two layers – ***we find it easiest to pour the supernatant into the final***

tubes rather than pipetting (if you do puncture the interface, return all liquid to the phase lock tube and repeat step 7)

9. Add 20 µL 3M NaOAc to each tube
10. Add 500 µL cold 100% ethanol (chilled in -80)
11. Manually rotate 10 times and store in -20˚C for 2-3hrs, up to overnight

DAY 2

Precipitation

1. Centrifuge at 14,000 rpm for 10 minutes, set to ***SOFT BRAKE***
2. Remove supernatant and keep the DNA pellet
3. Add 500 µL cold 70% ethanol to the samples without disturbing the DNA pellet
4. Centrifuge at 14,000 rpm for 10 minutes, set to ***SOFT BRAKE***
5. Remove ethanol and discard, being careful not to disturb the DNA pellet
6. Leave the tubes open and dry the DNA pellet in the fume hood (1-2 hours, until cannot smell EtOH)
7. Re-suspend pellet in 400µL 10mM Tris-HCl pH 7.5 (can go down to 200µL if low amount of starting tissue), manually rotate 10 times, then spin down on benchtop centrifuge ***DO NOT VORTEX HIGH***
    a. The addition of a large volume of Tris helps the sample to come into solution. You can concentrate the sample down later as needed.
8. Store in cool, dry, dark place (like bench drawer) overnight (12 hours minimum)
9. Store at 4˚C for 48 hours minimum (this wait time is crucial to allow all the DNA back into solution)

DAY 5+

Qubit

Getting HMW DNA back into solution and homogenized enough to get a clear reading on the concentration without fragmenting the DNA (e.g., by vortexing) is difficult because the DNA is in such long pieces. Thus, we recommend Qubiting each sample twice, and using 3µL of DNA. PC extractions on tissues will produce a huge amount of DNA, so it is more important to get an accurate concentration than to preserve the extra 1µL of DNA here.

1. Manually rotate the sample for ***5 MINUTES*** (can vortex on the lowest setting possible if needed) and spin down on a benchtop centrifuge
2. Proceed with Qubit protocols as normal
    a. 20 µL tips do not come in HMW so it is ok to use regular tips here

1.8x Bead Cleanup (Optional)

Sometimes your extractions will also contain short fragments of DNA that will clog up a long-read sequencer; this step allows you to remove any remaining impurities by re-eluting the DNA into fresh 10mM Tris-HCl. We use home-made speed beads, but there are other bead options available that are marketed for use on HMW DNA.

1. Manually rotate the sample 10 times (can vortex on the lowest setting possible if needed) and spin down on a benchtop centrifuge
2. Dilute the portion of the sample to be cleaned up to ~8-10 ng/µL
    a. e.g., add ~3500ng DNA using ***HMW TIPS*** (in 20 ng/µL) and ~380 µL Tris, total V = 400µL

   b. Diluting the sample lets you add more beads during the bead cleanup, which will help DNA adhere to the beads better and recovery to be higher
3. Proceed with bead cleanup protocols as normal, with the following modifications
   a. Always use **<u>*HMW TIPS*</u>** when pipetting DNA
   b. Increase amount of EtOH used to wash to make sure the large amount of beads don't dry out
   c. To elute the DNA off the beads, pre-heat the Tris to 56˚C and add 80µL, then incubate the mix of beads + DNA + Tris overnight at 30˚C at 300 rpm before putting on the magnet
   d. Qubit following the above protocol
   e. Because HMW DNA is "thick" and "stringy", some of it will never elute off the speed beads. We expect a ~45% return on a bead cleanup using this protocol. If preserving a low volume sample is important, it may be worth using different beads optimized for eluting HMW DNA.