**Genomic DNA extraction protocol for HMW DNA**

This DNA extraction protocol is applicable to a wide variety of cells and sample types. It is relatively "gentle" in both a physical and chemical sense, in that it typically does not result in excessive shearing of the genomic DNA. Although some shearing of DNA will result due to pipetting, we expect individual DNA fragments yielded by this procedure in the range of 20-40 kb. This will be useful for the Oxford Nanopore sequencing platform that we will use, because its biggest advantage is that it is able to produce very long reads (>30 kb); this is in contrast to Sanger and most other "second generation" sequencing methods that only produce shorter reads (~100-1000 bp), and therefore do not require long DNA fragments for sequencing. The library preparation method that we will be using for short read sequencing using the Illumina sequencing platform also requires long DNA fragments (~20-40 kb), so this DNA isolation procedure will be used for both of our sequencing objectives. Of course, long reads can only be obtained if the size of the starting DNA is of sufficient length (the reads can only be as long as the DNA fragments are!). Thus we will use this protocol, which is somewhat more laborious and time-consuming than other methods, but typically yields large DNA fragments. This will allow us to take advantage of the strengths of the Oxford Nanopore sequencing platform, and adhere to the Illumina library preparation protocol recommendations. This protocol is a modification of the bacterial genomic DNA isolation protocols suggested by the Department of Energy’s Joint Genome Institute (DOE-JGI): jgi.doe.gov/user-program-info/pmo-overview/protocols-sample-preparation-information/

**Basic outline of protocol:**

* Frozen cell pellets will be resuspended in a buffer containing deoxyribonuclease inhibitor (EDTA).
* Cell lysis will be achieved by treatment with enzymes (lysozyme to degrade bacterial cell walls, and proteinase K to degrade proteins) and detergent (sodium dodecyl sulfate, SDS).
* Nucleic acid will be separated from proteins and other impurities by treatment with CTAB, organic extraction (phenol/chloroform) and alcohol precipitation.
* Resuspended nucleic acid (DNA and RNA) will be treated with a ribonuclease (RNase) to degrade RNA.
* Alcohol precipitation will be repeated to yield pure DNA.

Subsequently, we will assess the concentration, purity and size of the isolated DNA using spectrophotometry and gel electrophoresis. Samples with sufficient quantity and quality of DNA will be prepared for sequencing.

**!!!!!!!!!!! USE WIDE BORE TIPS THROUGHOUT WHEN HANDLING DNA !!!!!!!!!!!**

**IMPORTANT NOTES ON SAFETY**

The organic extraction of nucleic acid from proteins and other impurities will involve use of phenol and chloroform, both of which are toxic and volatile**. ALL STEPS INVOLVING PHENOL AND CHLOROFORM MUST BE PERFORMED IN THE FUME HOODS, and you should wear proper protective equipment (safety goggles/glasses, lab coat, gloves)!!!** All waste (tips, tubes, organic phase, etc.) involved in phenol/chloroform extractions should be kept in the fume hoods. CTAB is also toxic, so take care when pipetting it.

**Materials and notes on what they are used for**

* 1xTE buffer (10 mM Tris and 1 mM EDTA, pH 8)
  + This will help to keep DNA stable and prevent nuclease activity…the ethylene diamine tetraacetic acid (EDTA) is a chelating agent, sequestering (“grabbing on to”) divalent cations like Mg2+ that are required for activity of deoxyribonuclease enzymes. The Tris will act as a buffer to stabilize the pH.
* 100 mg/mL solution of lysozyme (in 1x TE)
  + Lysozyme is an enzyme that will cleave bonds in the glycan chains present in cell walls of most Bacteria, weakening the cell wall and helping to promote cell lysis.
* 10% SDS (sodium dodecyl sulfate)
  + This is a detergent that will help to denature proteins and disrupt cell membranes, aiding in cell lysis.
* 20 mg/mL solution of proteinase K (in 1x TE)
  + This is an enzyme that will cleave a variety of peptide bonds in proteins, helping to promote cell lysis (for cells that have a protein component in their cell wall) and release DNA from DNA-binding proteins.
* 4.5 M NaCl
  + This, along with the CTAB, will assist in purification of DNA from carbohydrates and other polymers.
* 10% CTAB (hexadecyl-trimethylammonium bromide, in 4.1% NaCl)
  + CTAB will help to complex proteins, glycans, and other impurities and aid in their separation from DNA. 10% CTAB is quite viscous, so we will heat it to ~60 °C before using it to aid in pipetting.
* 24:1 solution of CHCl3:isoamyl (chloroform:isoamyl alcohol)
  + Organic solvent solution used for extraction of DNA from proteins and other contaminants.
  + The small amount of isoamyl alcohol prevents of foaming of the chloroform when mixed.
  + **NOTE: This solvent is toxic, and should only be used in the fume hood**
* 25:24:1 solution of phenol:CHCl3:isoamyl
  + Organic solvent solution used for extraction of DNA from proteins and other contaminants.
  + The small amount of isoamyl alcohol prevents of foaming of the chloroform when mixed.
  + **NOTE: This solvent is toxic, and should only be used in the fume hood**
* Isopropanol
  + Used for precipitation of DNA after organic extraction. This precipitation will help to separate the DNA (and RNA) from salts and other impurities.
* 70% ethanol
  + Used for precipitation of DNA after organic extraction. This precipitation will help to separate the DNA (and RNA) from salts and other impurities.
* 10 mg/mL solution of RNase A (ribonuclease A)
  + Used for digestion of RNA (but not DNA), allowing for eventual separation of RNA from DNA by precipitation

**Protocol**

* Add 400 µL of 1x TE to your frozen cell pellet and allow it to thaw completely at room temperature.
  + You should have approximately 500 µL of total cell suspension at this point.
* Add 20 µL of 100 mg/mL lysozyme, and mix well by inverting the tube several times.
* Incubate at 37 °C for 30 minutes.
* Add 40 µL of 10% SDS, and mix well by inverting the tube several times.
* Add 20 µL of 20 mg/mL proteinase K, and mix well by inversion.
* Incubate at 55 °C for 1-3 hours. If cells are not lysed (as seen by cleared solution with increased viscosity) incubation can proceed overnight (16 hrs). **I usually do 2 hrs. wait until you see that the cells are lysed!**
* Add 100 µL of 4.5 M NaCl, and mix well by inversion.
* Add 100 µL of pre-heated 10% CTAB solution and mix well by inversion.
* Incubate at 65 °C for 10 minutes.
* **Perform the following steps for organic extraction in the fume hood…Make sure to wear safety glasses and a lab coat when working with phenol and chloroform!**
  + Transfer lysed cell sample to a tube containing 500 µL of CHCl3:isoamyl alcohol. **WIDE BORE**
  + Mix well by inversion (30 times by hand…do not vortex, which may shear DNA).
  + Centrifuge at highest speed (~14,000 x g) for 10 minutes.
  + Transfer the **top layer** (aqueous layer…the organic phase is more dense than water) to a tube containing 500 µL of phenol:CHCl3:isoamyl **WIDE BORE**
    - NOTE: This step may be tricky, and is important for getting sufficient amounts of high-purity DNA. The DNA should be in the aqueous (top) phase, while most proteins should be in the organic (lower) phase or near the interface. At the interface you may see some white material. You want to remove as much of the top layer as possible (which contains the DNA that we want) WITHOUT disturbing the interface or the lower layer.
  + Mix well by inversion (30 times by hand…do not vortex, which may shear DNA).
  + Centrifuge at highest speed (~14,000 x g) for 10 minutes.
  + Transfer the **top layer** (aqueous layer…the organic phase is more dense than water) to a tube containing 500 µL of CHCl3:isoamyl (see NOTE above) **WIDE BORE**
  + Mix well by inversion (30 times by hand…do not vortex, which may shear DNA).
  + Centrifuge at highest speed (~14,000 x g) for 10 minutes.
  + Transfer the **top layer** (aqueous layer…the organic phase is more dense than water) to an empty tube, and return it to your lab bench. **WIDE BORE**
* Estimate how much volume you have using the P1000 pipetman. Add 0.6 volumes of isopropyl alcohol (isopropanol) to your extracted DNA.
  + i.e., if you have X µL of DNA, you will add (X \* 0.6) µL of isopropanol.
    - E.g. if you have 500 µL DNA extraction, you will add 300 µL (500 µL \* 0.6) of isopropanol.
* Mix well by inversion (30 times by hand…do not vortex, which may shear DNA).
* Incubate at -20 °C for 2 hours to overnight
  + In the presence of appropriate concentrations of salts and alcohol, DNA (and RNA) is not soluble and will precipitate out of solution. You may actually see white strands of DNA appear as precipitation occurs.
* Centrifuge at highest speed (~14,000 x g) for 15 minutes … **use cooled centrifuge set to 4 °C.**
  + This will pellet the precipitated DNA at the bottom of the tube. BEFORE starting the centrifuge, make sure that the hinge of the microcentrifuge tube is facing out (or in, if you prefer). This will help you remember where the DNA pellet will be, even if you can’t see it!
* Transfer the tube to ice in an ice bucket.
* Remove and discard supernatant (the DNA we want is in the pellet).
* Add 700 µL of ice cold 70% ethanol, and invert very gently a few times.
  + The DNA should still remain insoluble in this ethanol solution. This extra step will help to further remove salts and other impurities from the DNA.
* Centrifuge at highest speed (~14,000 x g) for 5 minutes … **use cooled centrifuge set to 4 °C.**
* Remove and discard supernatant (the DNA we want is in the pellet).
* Centrifuge at highest speed (~14,000 x g) for 10 seconds … **use cooled centrifuge set to 4 °C.**
* Remove and discard any remaining supernatant (the DNA we want is in the pellet).
  + This extra spin will help you remove the excess ethanol, and will shorten the amount of time required to allow residual ethanol to dry before resuspension.
* Allow the pellet to dry at room temp in the fume hood to remove residual ethanol. May take 20 min.- hours.
* Add 100 µL of 1x TE to the pellet containing DNA, mix by gently flicking the tube to help solubilize the DNA.
* Add 2 µL of RNase solution, and gently mix by stirring with pipet tip or gently flicking/inverting tube.
* Incubate at 37 °C for 1 hour.
  + This incubation will allow the DNA to resolubilize. Also, the RNase will degrade any RNA that may have co-purified with the genomic DNA (which usually happens with this method).
* Transfer the tube to ice in an ice bucket.
* Add 1/10 volume of 3M Sodium Acetate to your sample. (~10 µL)
* Add 2.5 volumes of 100% ethanol. Mix gently by inversion. (~275 µL)
* Place at -80°C for 30 min (alternatively, can place at -20°C for 2 hrs. to overnight)
* Centrifuge at highest speed (~14,000 x g) for 20 minutes … **use cooled centrifuge set to 4 °C.**
* Remove and discard supernatant (the DNA we want is in the pellet).
* Add 700 µL of ice cold 70% ethanol, and invert very gently a few times.
* Centrifuge at highest speed (~14,000 x g) for 5 minutes … **use cooled centrifuge set to 4 °C.**
* Remove and discard supernatant (the DNA we want is in the pellet).
* Centrifuge at highest speed (~14,000 x g) for 10 seconds … **use cooled centrifuge set to 4 °C.**
* Remove and discard any remaining supernatant (the DNA we want is in the pellet).
* Allow the pellet to dry at room temp for 5 -15 min. in the fume hood to remove residual ethanol.
* Resuspend pellet in 60 µL of DNase-free water. Do not pipet up and down-just mix by flicking and spin down.
* Store @ -20°C.

Run on a 0.8% agarose gel overnight (have been experimenting with this, and a stepwise gel works just about as well, and saves time) to check quality.

Notes and precautions.

* In step 1, do not use too many bacterial cells (an OD600 of not more than 1.2 is recommended), or DNA does not separate well from the protein. **THIS IS VERY IMPORTANT!!!**
* Inverting several times is sufficient to mix well. Shaking too hard will shear the DNA.

