# Oxford Nanopore Technologies

## Notes for GA LRA Workshop

## Vertebrate Genome Lab (Nivesh Jain, Tatiana Tilley, Jennifer Balacco)

Note: ligation kit/sequencing => duplex reads

STEPS:

1. DNA extraction
2. DNA Quantification by Qubit Broad Range
3. Fragment Length Analysis
4. DNA Shearing
5. Elimination of Short Fragments
6. Fragment Length Analysis
7. Library Preparation
    a. Ligation
    b. Ultra long

1.) <u>DNA Extraction</u>

Below mentioned are the kits that we use for DNA extractions. Most of our testing has been performed on blood samples only, although we did have success with gharial heart tissue. We are yet to do a proper "test" with tissues. We don't have a lot of experience with tissues. (We tried with the wallaby and stoat but the samples are not the best quality) We can do DNA extractions, we just haven't tested them on PromethION (YET!, Snow Crab will be on it soon)

1. Monarch HMW DNA Extraction Kit for cells and blood by NEB (borosilicate glass-bead based extraction): We use this kit for HMW and UHMW DNA extractions. The only changes suggested by NEB to use this protocol for UHMW DNA is the changing the speed of thermomixer from 2000rpm (for ligation) to 300rpm (for UL DNA) during lysis.

2. MagAttract Manual purification of High-Molecular-Weight Genomic DNA from Whole Blood by Qiagen (paramagnetic-silica beads): This protocol has been modified for nucleated blood samples because that is what we mostly work with. Used for extraction of DNA that goes into ligation sequencing library prep. THIS CANNOT BE USED FOR UHMW DNA EXTRACTIONS, DNA too short.

3. TEST: Bionano Prep SP-G2 Frozen Human Blood DNA Isolation Protocol by Bionano (paramagnetic-nanobind disc): We are currently testing this protocol for ONT sequencing as it did wonders for Bionano and gives really long DNA as well. ONLY FOR UHMW DNA EXTRACTION, NOT FOR DNA UPSTREAM OF LIGATION SEQUENCING.

Other plans include working with MagAttract HMW DNA Isolation for Tissue by Qiagen (because it works great for PacBio sequencing). This DNA will solely be used for ligation sequencing as the

DNA obtained by any MagAttract protocol is <165kb fragment length whereas with a nanobind disc and a borosilicate glass bead we can achieve fragment lengths of over 1mb.

2.) DNA Quantification by Qubit Broad Range

Triplicate readings are taken to get the closest most accurate number (CV%<25 is preferred). Additional sonication step is added to this protocol to get the most accurate DNA concentration reading.
In case of UMHW DNA, it is never accurate. CV's are always really high and never trustworthy.

3.) Fragment Length Analysis

This varies depending on what type of DNA we are working with.
UHMW DNA: PFGE (Pulsed-Field Gel Electrophoresis)
HMW DNA (for ligation sequencing): FEMTO Pulse System

4.) DNA Shearing (ONLY FOR DNA THAT HAS TO GO FOR LIGATION SEQUENCING)

DNA is sheared using Diagenode Megaruptor 3 to the 20-30kb fragment length.
ONT says we can go as high as 40kb but we haven't done a successful test for this (YET!) But 20-25kb fragment long DNA seems to work for us.

5.) Elimination of Short Fragments (<10kb) (ONLY FOR DNA THAT HAS TO GO FOR LIGATION SEQUENCING)

This is done through Circulomics (now PacBio) SRE-XS Size Selection kit. This helps in elimination of fragments shorter than 10kb (theoretically).

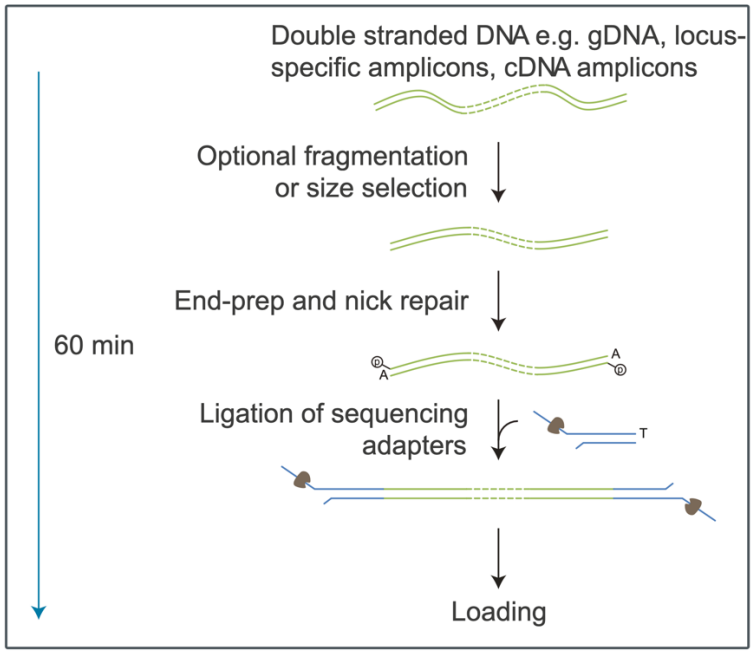
6.) Fragment Length Analysis (ONLY FOR DNA THAT HAS TO GO FOR LIGATION SEQUENCING)

We run another fragment length analysis to make sure that MOST of the short reads have been eliminated by this point.

7.) Library Preparation

7.1. LIGATION SEQUENING (this protocol/sequencing generates duplex readS)

For Ligation Sequencing we currently use Ligation Sequening Kit V14 (SQL-LSK114) in combination with R10.4.1 marathon flow cells.
ONT recommends input of 100-200fmol (~1ug) DNA per library. We do these calculations and might go to as high as 1.5 to 2ug DNA. Since, the fragment lengths are longer (mind you eventhough we shear our DNA at times we still get some longer DNA fragments in our library, between 40-100kb) this calculation is an approximation. 1ug is usually a good number to start with.




In brief, for ligation sequencing library prep we: 1. Repair the DNA, and prepare the DNA ends for adapter attachment. 2. Attach sequencing adapters supplied in the kit to the DNA ends. 3. Prime the flow cell, and load DNA library into the flow cell.

NO MAJOR CHANGES/MODIFICATIONS IN THE PROTOCOL THAT WE USE. At times we increase the adapter ligation time from recommended 10 mins to 15mins, and increase the elution time after washing with large fragment buffer (LFB) from recommended 10 minutes to 20 minutes.

Sequencing run length is 72 hours. We load a single library in three batches (most of the time), because they suggest loading at 10-20fmol and typically the library in full is more than that, so it needs to be split up/diluted so as to not overload the flow cell. Each load is preceded by a flow cell wash to get rid of any DNA from previous library. If the pore occupancy continues to look good and high after the first 24 hours, ONLY THEN we skip re-loading our library and let the run continue without any interruption. IF the sequencing pore occupancy decreases MAJORLY, then we wash our flow cells and load the second part of our library. FOR CHICKEN, SINCE IT IS A BLOCKY SAMPLE (CONFIRMED BY ONT) WE HAVE TO ALWAYS WASH AND RE-LOAD OUR LIBRARY OVER A PERIOS OF THREE DAYS.

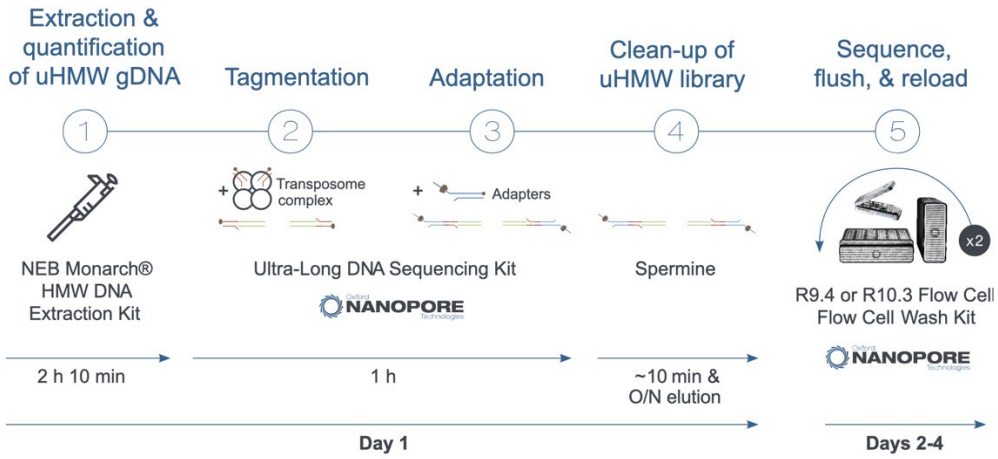
7.2 ULTRA LONG DNA SEQUENCING (no duplex reads): ultra-high molecular weight is highly viscous, fragile, and difficult to handle. It cannot be quantified like high molecular weight DNA. This DNA cannot be sheared or size selected.

We have worked with Ultra-Long DNA Sequencing Kit protocol (SQK-ULK001) with R9.4.1 flowcells and will be moving on to Ultra-Long DNA Sequencing Kit protocol (SQK-ULK114) with R10.4.1 flowcells. R10.4.1 flowcells did perform better in case of ligation sequencing and we are expecting they'll do the same in case of ultra-long sequencing as well.

Circling back to SQK-ULK001, which we have worked with, we go on with a relatively higher mass of DNA (35-40ug or more), they just give a better sequencing pore occupancy.

For library prep: 1. We tagment DNA using a diluted fragmentation mix. 2.Attach Rapid Adapters to the DNA ends. 3. Precipitate DNA and incubate overnight. 4. Prime the flow cell and load DNA library into the flow cell (over three days- ALWAYS)

MAJOR CHANGES WE MADE: we double the incubation time after adapter ligation step. From 30 minutes to an hour (even 1.5hours sometimes).




FUTURE PLANS:

1. We are now qualified for R10.4.1HD flow cells. HD- High Duplex
   These are developer access only flow cells and will help us get more than 50% duplex data per flowcell. Compatible only with ligation sequencing.
2. Switch to SQL-ULK114 sequencing kit in combination with R10.4.1 flow cells for Ultra long sequencing. ☺

I HOPE THIS HELPS.

I hope you can ignore all the grammatical errors, typos, misspellings, etc.