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Processing frozen cells for population-scale SQK-LSK114 Oxford Nanopore long-read DNA sequencing SOP

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ABSTRACT

Abstract

As part of the GP2 initiative we will generate long-read sequencing data for ~1000 samples to better understand the genetic architecture of Parkinson's disease. To generate this large-scale Nanopore data we have developed a protocol for processing and long-read sequencing frozen human blood samples, targeting an N50 of ~30kb and ~30X coverage.

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We are still developing and optimizing this protocol

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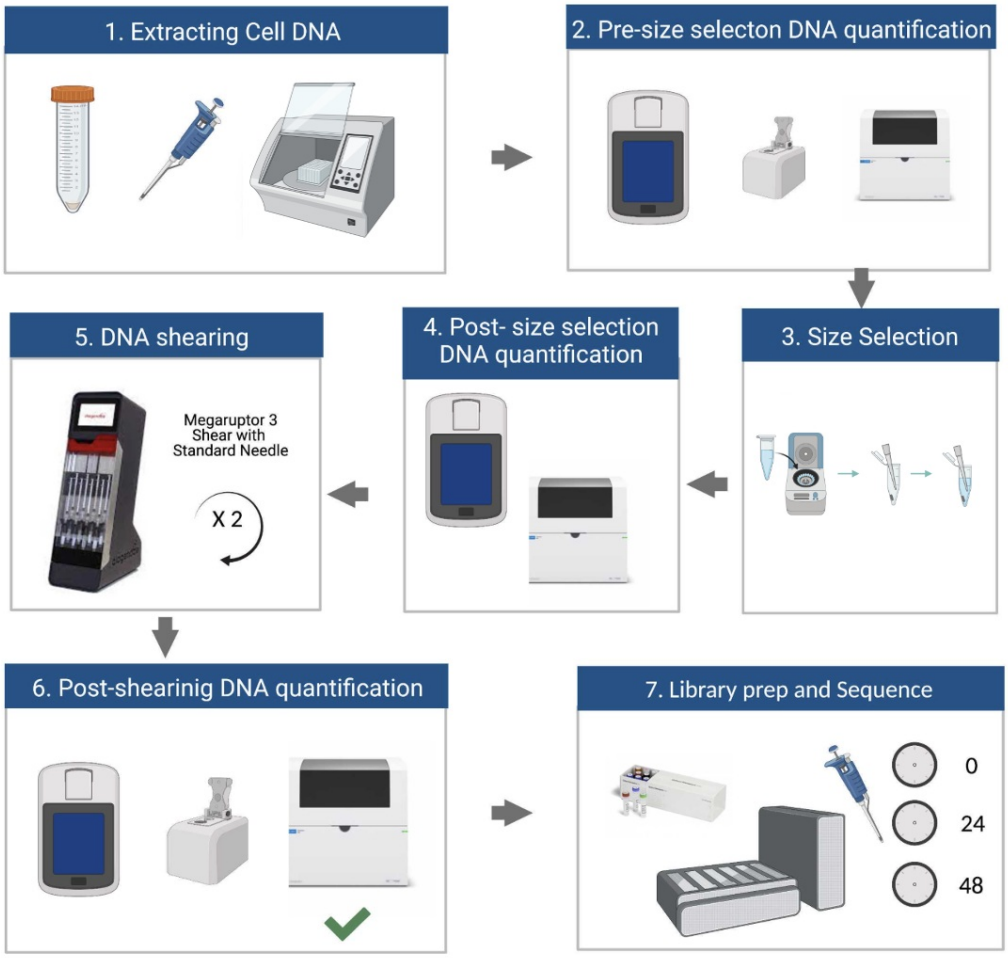

Figure 1. Overview of the HMW DNA Extraction and ONT Sequencing Protocol from Frozen Human Cells

MATERIALS

List of consumables / reagents / equipment needed

Consumables:

	A	B
	Description	Supplier
	1.5mL DNA LoBind tubes	Eppendorf
	KingFisher 96 deep-well plates, barcoded	Thermo Fisher
	KingFisher 96 deep-well tip combs, barcoded	Thermo Fisher

	A	B
	Ethanol (96-100%)	
	Isopropanol (100%)	
	3mm Nanobind Disks	PacBio
	1ml Luer-lock Synringes	BD
	1.5" Needles	SAI Infusion Technologies
	DNA Fluid+ Kit	Diagenode
	0.2ml thin-wall PCR tubes	
	PromethION Flow Cells	Oxford Nanopore Technologies

Reagents

	A	B
	Description	Supplier
	Nanobind HT CBB kit	PacBio
	TE pH 8	Millipore Sigma
	Isopropyl Alcohol	
	Agencourt AMPure XP beads	Agencourt
	NEBNext® Companion Module for Oxford Nanopore Technologies ® Ligation Sequencing	New England BioLabs
	Ligation Sequencing Kit (SQK-LSK 114)	Oxford Nanopore Technologies
	Flow Cell Wash Kit (EXP-WSH004)	Oxford Nanopore Technologies

	A	B
	Ultra Pure Water	KD Medical
	1x dsDNA BR Working Solution	Thermo Fisher
	Absolute Ethanol	Thermo Fisher
	BluePippin HPP Cassettes and Instrument	SAGE SCIENCE
	Agilent Femto Pulse System and Genomic DNA 165 kb Kit	Agilent
	Tapestation 4200 and Genomic DNA Green Tape	Agilent

Equipment

	A	B
	Description	Supplier
	KingFisher Apex/96 Deepwell plates for DW Magnets	Thermo Fisher
	Megaruptor	Diagenode
	NanoDrop 8000	Thermo Fisher
	Qubit 4	Thermo Fisher
	Vortex Genie-T	Scientific Industries
	Microfuge	
	Magnetic separator (suitable for 1.5mL Eppendorf tubes)	
	PromethION 24 or 48	Oxford Nanopore Technologies

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Part 1: Extracting Cell DNA (~2 hours per 8 samples)

From: PacBio HMW DNA Extraction Cultured Cells Protocol

- Cell Input Requirements: $1x10^6$ – $2{,}5x10^6$ diploid human cells or equivalent
- Cell counts should be accurately determined using a hemocytometer or cell counter.
- Cell pellets should be frozen dry with as much liquid removed as possible.
- For non-diploid or non-human cells, the cell input should be scaled appropriately to contain 5–25 µg of DNA.

1.1 Thaw frozen cell pellets on ice (about 5 to 10 minutes).

1.2 Prepare the Kingfisher 96 deep well plates as follows:

Plate 1: Elution: 100 µL Buffer EB
Plate 2: CW2 Wash 1: 700 µL Buffer CW2
Plate 3: CW2 Wash 2: 700 µL Buffer CW2
Plate 4: CW1 Wash: 100 µL Buffer CW1
Plate 5: Nanobind storage: 3mm Nanobind disks
Plate 6: Place KingFisher Apex 96 deep-well tip comb

Note: Nanobind disks do not need to be perfectly centered in the wells, but ensure they are at the bottom of the well and not stuck to the side walls.

1.3 Prepare the **Plate 7** (Lysis/Binding) 5m
1. Add 50 µL of 1x PBS to the pellet and pipette mix 10X with a standard P200 pipette to re-suspend cells, then add all in **Plate 7** (Lysis/Binding)*

- Mix until the cell pellet is fully resuspended without visible lumps. Sticky cell types may require additional pipette mixing or vortexing.
- Aggressive mixing at this step will not affect DNA size. However, incomplete resuspension will result in inefficient lysis and digestion which will lead to low yield, low purity, and high heterogeneity.

* The Kingfisher protocol specifies 50 µL PBS for 1x10^6 cells, however alternatively, per well it is possible to use 2.5x10^6 cell pellets with 50 µL PBS. Precise cell-counting is required, if the pellets contain more cells than predicted this can lead to either extremely viscous DNA or a failed extraction.

2. Add 20 µL of Proteinase K directly to each well.
3. Add 5 µL of Buffer CLE3 directly to each well.
4. Add 20 µL of RNase A directly to each well. Let rest on bench top for 00:05:00.
5. Add 150 µL of Buffer BL3 to each well against the side of the well.

Note 1: Adding BL3 directly to the solution may affect extraction performance.

Note 2: Sample and reagents in lysis binding plate **must** be added to the plate in the order listed above.

1.4 Select the Cell_Nanobind_HT_APEX script (102-998-100) on the KingFisher Apex instrument, press 'Start' and insert plates into the KingFisher Apex instrument as indicated on the display.

1.5 After ~00:21:00, when the program pauses do not press 'Next'. Add 250 µL isopropanol (IPA) to lysis binding plate. Re-insert the plate and now press 'Next' to resume the protocol. 21m

Note: Add IPA gently against side of the well. Adding IPA directly to solution may affect extraction purity.

1.6 When the program ends after ~01:05:00 (from the start), transfer eluate from **Plate 1** (Eluate Plate) to a 1.5mL Eppendorf DNA LoBind tube. 1h 5m

Note: The program is designed to leave the nanobind disk in the elution plate. If the nanobind disk ends up in the tip comb plate, this does not affect extraction performance. Use a P200 to remove any elution buffer remaining on nanobind disk.

1.7 Pipette mix the sample 10 times with a standard P200 pipette to homogenize and disrupt any unsolubilized "jellies" that may be present.

- Take care to disrupt any regions that feel more viscous than other regions.
- Limited pipette mixing will not noticeably reduce DNA size or sequencing read lengths but is important for accurate quantification and consistent sequencing performance.

1.8 Let samples rest overnight at Room Temperature (RT).

1.9 Following overnight rest, pipette mix 10X with a standard P200 pipette and analyze the recovery and purity as described in QC Procedure (Step 2).

1.10 After overnight rest at RT, DNA can be stored at 4 °C for up to four weeks, or -80 °C indefinitely.

2 Part 2: Pre-size selection DNA quantification (~30 minutes for 8 samples)

2.1 Hand-shear each sample ~ 20X with 1 mL Luer-Lock syringes and 1.5” needles (bringing sample up into needle and depressing plunger counts as 1 cycle).

Note: This step isn't to shear the DNA to a specific size, it is required to get an accurate QC reading for downstream processes.

2.2 Quantify by taking triplicate measurements (top, middle, bottom) on the Qubit.

2.3 If coefficient of variation (CV) is <10% for the three measurements, move on to the size selection.

2.4 If the CV is greater than 10%, further hand shearing is needed. Shear another 10x and take triplicate measurements on the Qubit. Repeat this until the CV is less than 10%. Once achieved, measure on the Nanodrop.

2.5 If the CV is less than 10% for the three Qubit measurements, measure on the Nanodrop and size on the Agilent Tapestation 4200 or femto pulse.

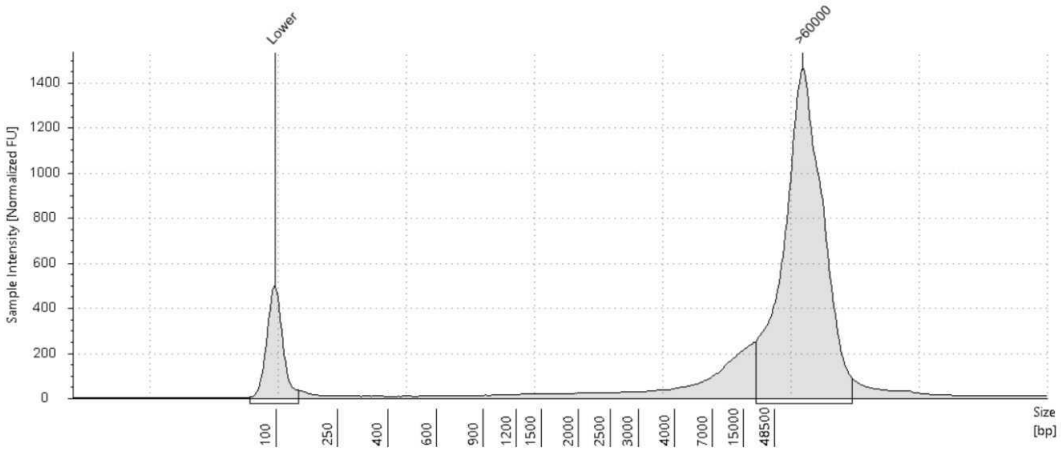

Figure 1. Example TapeStation trace pre-size selection

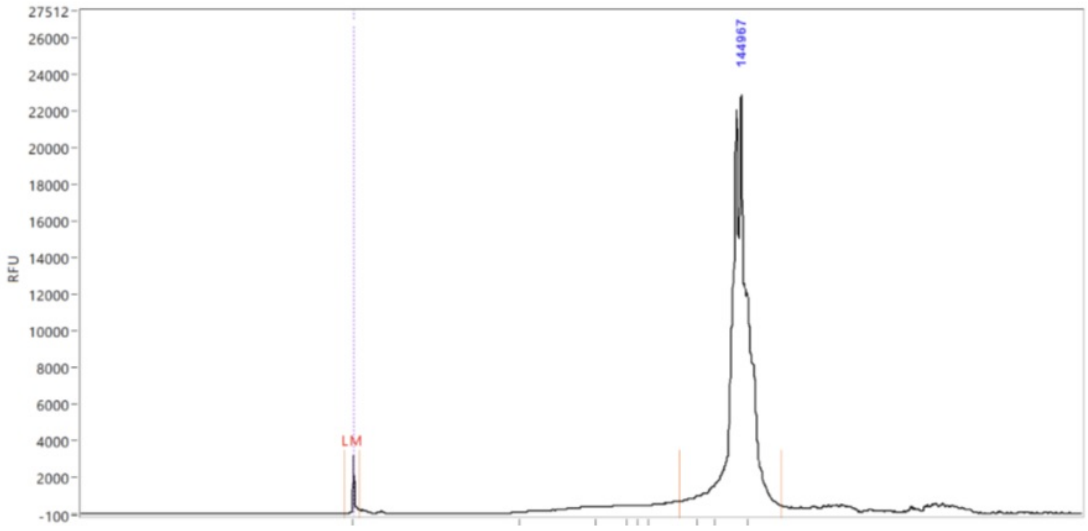

Figure 2. Example Femto Pulse trace pre-size selection

Note: It is recommended to use the Femto Pulse System to get a better representation of the size distribution of the DNA samples.

3

Part 3: Size selection (~1:15 hours for 8 samples).

Note: This process can be done before or after Megaruptor shearing depending on the nature of the cohort. The following protocol applies to the PacBio Short Read Eliminator Kit, Pacbio Short Read Eliminator-XS Kit, and PacBio Short Read Eliminator-XL Kit.

3.1 Ensure your sample is between 50-150 ng/uL. If it is > 150 ng/uL then it will need dilution. The

optimal range for the sample going into size selection is between 100-150ng/uL but protocol will work as long as it's above 50 ng/uL.

3.2 Start centrifuge at 10,000 rpm and 25 °C for 00:05:00 . 5m

3.3 In the meantime, once the sample is at the correct concentration, aliquot the desired volume (a minimum of 8ug is required or maximum 20 ug) into a 1.5ml DNA LoBind tube.

Note: Make sure that your volume is above 100 µL . If below, add TE buffer to reach 100 µL .

3.4 To the DNA LoBind tube with the sample, add equal volume of Buffer SRE from the PacBio Short Read Eliminator Kit.

3.5 Flick the tube thoroughly to mix.

3.6 Place tube in microcentrifuge at 10,000 rpm and Temperature25 °C
for Duration00:30:00
with hinge facing out as shown.

- Inserting tube with the hinge out is crucial to avoid aspirating the pellet if not visible.

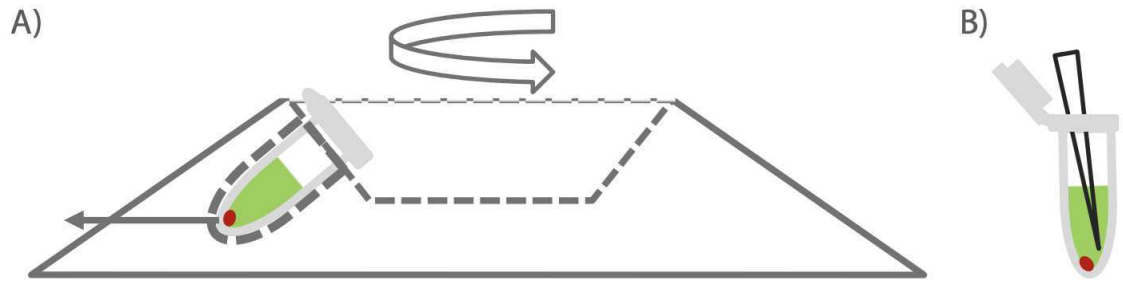

Figure 3. Taken from Circulomics Short Read Eliminator Kit Family Handbook v2.0 (07/2019)
A) Note orientation of tube in centrifuge. Pellet will form on the side of the tube facing

outwards at the bottom of the tube. B) Pipette from opposite side of tube on the thumb lip side to avoid disturbing pellet (pellet may not be visible).

3.7 Remove tube from centrifuge and remove supernatant from opposite wall of pellet formation.

Note: Do not remove all of the supernatant if close to aspirating pellet or if pellet has not properly formed (can happen with very viscous DNA). Pellet will form a tighter knot after the EtOH washes.

3.8 Carefully add 200 µL of fresh 70% EtOH on the opposite side of the hinge wall and centrifuge at 10,000 x g for 00:02:00 at RT. 2m

Note: Do not mix after adding EtOH.

3.9 Centrifuge at 10,000 rpm and 25 °C for 00:02:00 with hinge facing out. 2m

3.10 Carefully remove EtOH wash without disrupting the pellet.

3.11 Repeat steps 3.8 and 3.9

3.12 Remove as much EtOH as possible without disturbing the pellet.

3.13 Add 50-100 µL of Buffer EB from the Short Read Eliminator Kit.

3.14 Let sample rest at RT for 00:20:00 . 20m

3.15 After 00:20:00 , flick the tube to ensure proper mixing. 20m

4 Part 4: Post-size selection DNA quantification (~10 minutes for 8 samples)

4.1 Analyze size selection recoveries via Qubit.

Note: Quantification post size-selection can be difficult. If necessary, gently pipette mix the sample x5 before quantification to homogenize. The most accurate quantification will come after Megaruptor shearing, but an estimate of DNA concentration is important in this step to standardize the samples going into shearing.

5 Part 5: Shearing (~2.5 hours for 8 samples)

5.1 In a Diagenode DNA Fluid+ tube, make up the sample to 150 µL and 40-50ng/uL with TE buffer.

Note: It is ok if target concentration cannot be reached for all samples. If concentration differs from this range, make sure the Megaruptor 3 shearing settings are updated to reflect your sample concentration. If shearing more than one sample try to get all the concentrations to be as close as possible to this target range.

Note: The MR3 has a minimum volume of 100 ul and a maximum concentration of 150 ng/ul.

5.2 Attach the DNA Fluid+ needle onto the tube and push the entire item into the Megaruptor 3 slot until it fits snugly.

Note: If running fewer than 8 samples, put the tubes in the 1st and/or 8th slots, working your way in. Samples should always be balanced. If running an odd number of samples, samples can be balanced with an empty corresponding tube.

5.3 Shear at speed 45 for two rounds.

Note: From initial testing two cycles compared to one yields a more complete shear, which leads to higher data output when sequenced.

5.4 Once done, remove item from Megaruptor 3 and carefully remove syringe from tube. Make sure the plunger is fully depressed in order to avoid losing sample. Use a P200 pipette to aspirate any leftover sample on the syringe.

5.5 Avoid any vortexing of DNA from this point on to avoid any unnecessary further shearing, instead mix by gently flicking the tube and spin down.

6 Part 6: Post-shearing DNA Quantification (~30 minutes for 8 samples)

6.1 Quantify the samples with the Qubit and Nanodrop and size using the Agilent TapeStation 4200 or the Agilent Femto Pulse System.

Note: The desired post-shear peaks for the CARD long-read sequencing project should be between 25-35kb.

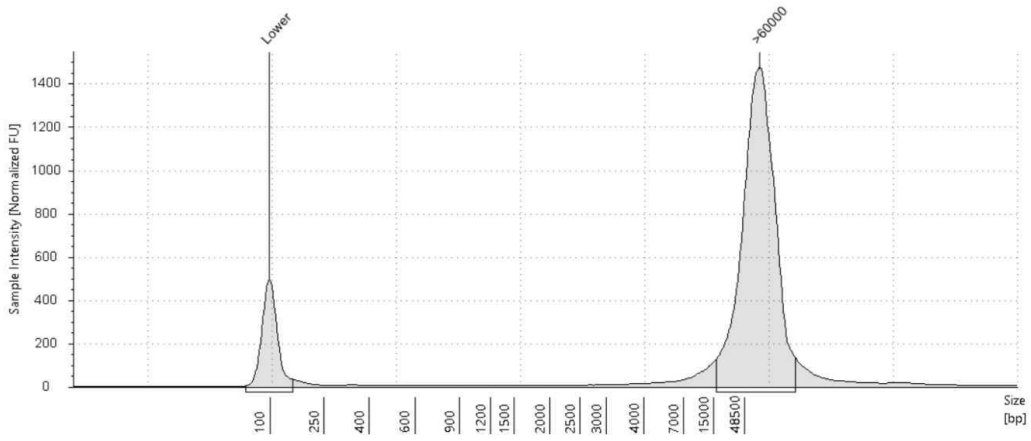

Figure 4. Example TapeStation trace post-size selection and shearing

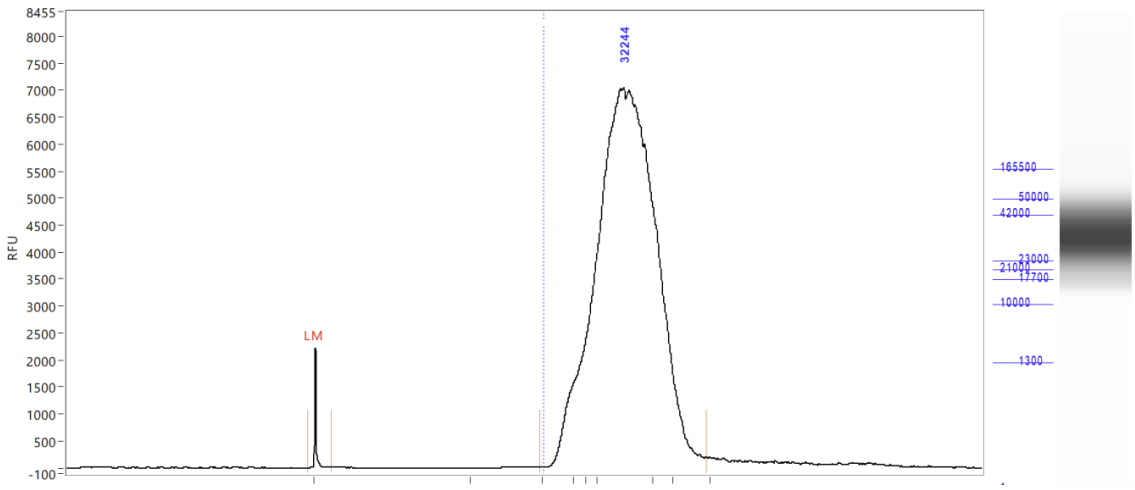

Figure 5. Example Femto Pulse trace post-size selection and shearing

6.2 Upload the TapeStation/Femto reports and quantifications to tracking google sheet.

6.3 At this point, at least 2.5 µg of DNA is necessary to move on to library prep.

6.4 DNA can be stored at 4 °C for up to four weeks, or -80 °C indefinitely.

7 Part 7: Optional-Size selection using High Pass Plus Cassettes for BluePippin

Note: Use if more aggressive size-selection is needed after SRE. Alternatively, the BluePippin can be used instead of PacBio SRE. High Pass Plus(Cat No. BPLUS10) cassettes are used to collect fragments greater than 15kb or 20kb.

7.1 Using TE, dilute up to 10ug of sheared DNA sample into a final volume of 30 µL or 60 µL.

7.2 Follow the Blue Pippin guide for entering the Sample IDs, running QC tests, and loading the

samples into the cassettes. The expected recovery on the region of interest can range between 40-75%.

7.3 Quantify using the Qubit Flex Fluorometer.

7.4 Prepare separate DNA dilutions(0.005 - 0.5ng/ul) and size using the Agilent Femto Pulse.

Note: The Blue Pippin instrument removes fragments less than 15kb or 20kb(depending on the software settings).

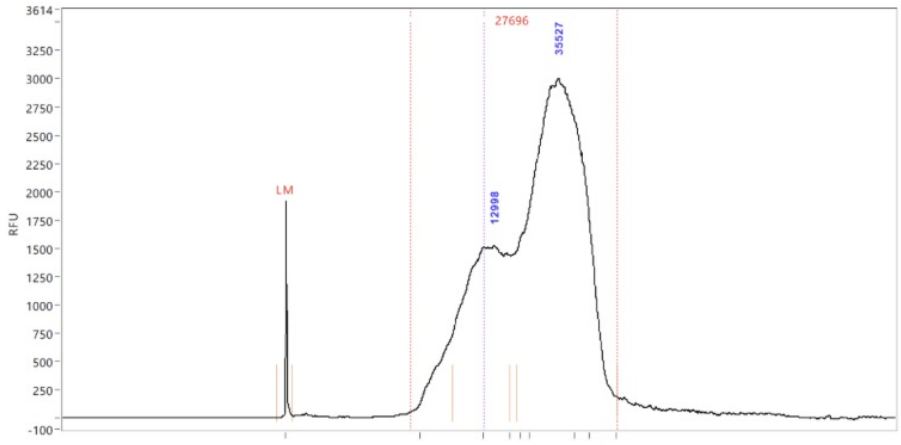

Figure 6: Example Femto Pulse Trace Pre-BluePippin(Post Shear). Short fragments under 10kb are apparent.

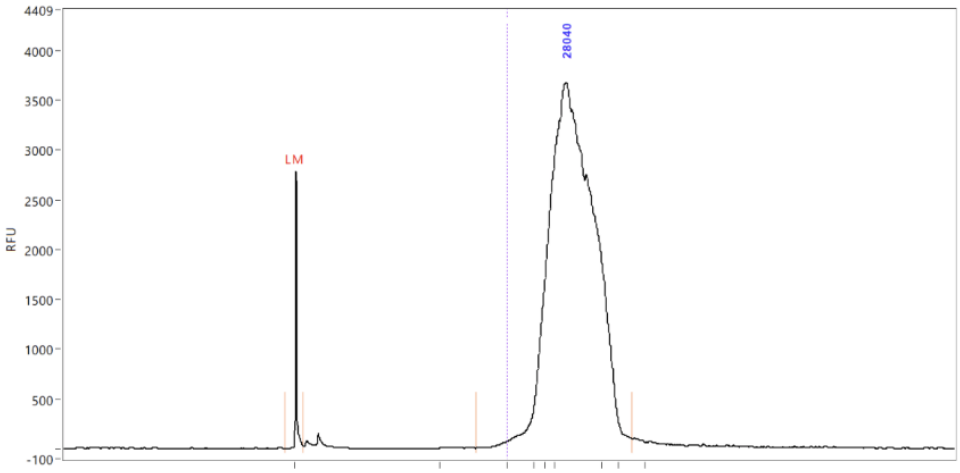

Figure 7: Example Femto Pulse Trace Post-BluePippin(Post Shear). Short fragments under 10kb no longer exist

7.5 Measure the volume of sample and calculate the amount. If the desired amount has not been reached, repeat extraction from Part 1.

8 Part 8: Manual SQK-LSK114 Library Prep and sequencing (~6 hours, including reloads but not including flushing and returning flow cells)

Note: Library prep is the same as the brain library prep with two exceptions. First, in step 7.1A we mix beads into the sample by flicking and not pipetting up and down. Second, the loading amount is 350ng/load for cells.

Note: Reagents are from Oxford Nanopore Ligation Sequencing Kit V14 (SQK-LSK114). Library prep is following the standard Oxford Nanopore Ligation Sequencing DNA V14 (SQK-LSK114) protocol with minor tweaks: https://community.nanoporetech.com/docs/prepare/library_prep_protocols/genomic-dna-by-ligation-sqk-lsk114/v/gde_9161_v114_revo_29jun2022

Note: Library prep can also be done on the Hamilton NGS star and can process 48 samples in ~4 hours. https://dx.doi.org/10.17504/protocols.io.n2bvj36mnlk5/v1

8.1 A. DNA Repair and End-Prep

19m 30s

1. Place all the necessary reagents on ice to thaw and the Agencourt AMPure XP beads out at room temperature.

2. Prepare the following in a 0.2 mL thin-walled PCR tube:

- 48 µL DNA (input 3ug, this might be over 48 µL but that is fine. Adjust the amount of beads to match the total volume of this mixture (sample + buffer + enzyme))
- 3.5 µL NEBNext FFPE DNA Repair Buffer (vortex and spin down)
- 3.5 µL Ultra II End-prep reaction buffer (vortex and spin down)
- 3 µL Ultra II End-prep enzyme mix (do not vortex, spin down)
- 2 µL NEBNext FFPE DNA Repair Mix (do not vortex, spin down)

Note: Do not exceed 168ul for total volume.

3. Mix thoroughly by gently flicking the tube or very gently pipetting up and down x10, and

then spin down.

4. Using a Thermocycler, incubate samples at 20 °C for 00:05:00 and 65 °C for 00:05:00 .

Note: Start and pause Thermocycler to allow lid to come to 85 °C before putting samples in.

5. Allow Thermocycler to cool to 4 °C and then remove your samples.

6. Re-suspend the AMPure XP beads by vortexing.

7. Transfer DNA samples to clean 1.5 mL Eppendorf DNA LoBind tube.

8. Add 60 µL (or equivalent volume, see step 2) of resuspended beads to the reaction and mix by flicking the tube x10. Do not pipette mix here as beads may clump around pipette tip.

9. Incubate for 00:05:00 at RT.

10. Prepare 500 µL per sample of fresh 80% ethanol in nuclease-free water.

11. Spin down and pellet sample on magnet until eluate is clear and colorless, about 00:02:00 .

12. Keep the tube on the magnet and pipette off the supernatant.

Note: Can retain the supernatant if needed just in case the following quant is low.

13. With the samples remaining on the magnet, wash the beads with 200 µL of 80% ethanol, pipetting on the opposite wall making sure not to disturb the pellet. Count to 3 and remove and discard ethanol.

Note: The goal here is to make sure the beads are fully covered, if initial volume of beads was a lot higher than 60uL, more ethanol may be used.

14. Repeat step 13.

15. Spin down and place the tube back on magnet, pipetting off any residual ethanol.

16. Allow to dry for ~ 00:00:30 but do not over-dry to the point of cracking.

17. Remove the tube from the magnetic rack and re-suspend the pellet in 60 µL nuclease-free water, incubate for 00:02:00 at RT gently flicking every so often.

18. Spin down and pellet the samples on a magnet until eluate is clear and colorless.

19. Remove and retain 60 µL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.

20. The sample concentration must be > 40ng/ul**.** If the sample does not reach this requirement restart from **Part 5.**

21. It is possible to store samples at 4 °C overnight at this step if needed

8.2 B. Adapter Ligation and Clean-Up

55m 30s

1. Spin down the AMX-F, Quick T4 ligase, and LNB, then return to ice.

Note: Do not allow AMX-F to remain at room temperature for too long.

2. Thaw LNB at RT and mix by pipetting up and down (vortexing is ineffective due to viscosity).

3. Thaw EB at RT, mix by vortexing, spin down, and place on ice.

4. Thaw SFB at RT, mix by vortexing, spin down, and keep at RT.

5. In a 1.5 mL Eppendorf DNA LoBind tube, mix the following in order:

- 60 µL DNA sample (if not 60uL, make up with water)
- 25 µL LNB
- 10 µL Quick T4
- 5 µL LA

6. Mix by gently flicking the tube and spin down.

7. Incubate the reaction for 00:10:00 at RT.

8. During this time, put flow cells out at RT.

9. Re-suspend beads by vortexing.

10. Add 40 µL of resuspended beads to the reaction and mix by flicking.

11. Incubate on a hula mixer for 00:05:00 at RT.

12. Spin down sample and pellet on magnet.

13. Keeping tube on magnet, pipette off the supernatant.

Note: Can retain if needed just in case the final elution quantification is uncharacteristically low.

14. Wash the beads with 250 µL SFB, remove from magnet and flick to re-suspend, spin down and re-pellet on magnet and then remove and discard supernatant.

15. Repeat step 14.

16. Spin down and place the tube back on magnet, pipetting off any residual SFB.

17. Allow to dry for ~00:00:30, but do not over-dry.

18. Remove the tube from magnet and re-suspend pellet in 26 µL EB, spin down, and incubate for 00:20:00 at 37 °C and 450 x g.

19. During this time, QC the flow cells (only use flow cells with >7000 pores if trying to reach 30 x coverage).

Note: Wait at least 00:20:00 after taking out flow cells to let them get to RT before loading onto PromethION to avoid condensation forming.

20. Pellet the beads on magnet until eluate is clear and colorless.

21. Remove and retain 26 µL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube (this is the DNA library).

22. Quantify 2ul of sample on Qubit.

23. Re-prep library from Part 6 if < 540ng (based on 10-20 fmol per load(which would be 30 to 60 fool overall) to avoid under or overloading the flow cell).

Note: We calculated this based on the DNA at a size of 30 kb and at a coverage of 230x over 3 loads. If you're sample is a differentiates from this size, recalculate to get 10-20 fmol per

load.

Note: 540ng will be enough for three loads of 180ng per load which is the amount that our testing has indicated is needed to hit 30x coverage.

24. Keep libraries on ice until ready to load on flow cell.

8.3 C. Priming and Loading Flow Cell

1h 15m

Note: This kit is only compatible with R10.4.1 flow cells (FLO-PRO114M).

1. Thaw Sequencing Buffer (SB), Library Solution (LIS) or Library Beads (LIB), Flow Cell Tether (FCT), and Flow Cell Flush (FCF) at RT, vortex, and spin down
2. Priming mix : in a new tube, add 30 µL of thawed and mixed FCT to 1170 µL of thawed and mixed FCF and vortex. l
3. Expose inlet port on flow cell, set P1000 pipette to 200 µL and draw back a small amount of volume to remove any air bubbles (usually about 20-30 µL, just until a small volume of buffer enters the pipette tip).
4. Flush 500 µL of Priming Mix into the inlet port of the flow cell, being extremely careful to avoid the introduction of air bubbles at the end.
5. Wait 00:05:00 .
6. During this time, make up your DNA library to 32 µL at 180ng using EB.
7. Prepare the library mix for loading:

- 100 µL SB
- 68 µL LIS or LIB
- 32 µL DNA library

8. Repeat steps 4 and 5.

10. Load all 200 µL of the library mix.

11. Close valve to seal inlet port and close PromethION lid.

12. Wait 00:10:00 and then initiate sequencing.

13. Ideally, the library quants yielded at least 540 ng to allow for 3 x 180ng loads, the latter 2 loaded approximately after 24 and 48 hours. However this will vary slightly depending on pore usage, data generated, as well as other factors i.e. if after 24 hours there are still +3000 pores then the sample does not need to be reloaded until 48 hours.

- To wash and reload a flow cell, begin by thawing Wash Mix (WMX) on ice and Wash Diluent (DIL) at RT

Note: DIL should be vortexed. WMX should NOT be vortexed, only spun

- In a new tube, add 2 µL WMX to 398 µL DIL and pipette mix. This is your flow cell wash mix.
- Pause the PromethION runs and export .pdf run reports
- With inlet port 1 closed, remove waste from port 2 or 3

- Rotate the inlet port 1 cover to reveal inlet port 1
- Using a P1000, insert tip into inlet port 1 and draw back a small volume using the wheel to remove any air bubbles (usually around 20-30 µL, just until a small volume enters the pipette tip).
- Load 400 µl flow cell wash mix into inlet port 1, avoiding any introduction of air.
- Wait 01:00:00
- Repeat priming steps and reload samples (steps 1 - 13)

8.4 D. Flushing and Recycling Flow Cells (~15 minutes per set of 4 flow cells)

1. Following the completion of the sequencing, flow cells may be removed from the sequencer.
2. Place enough absorbent material to take up approximately 4 mL of flush waste.
3. Rotate valve to reveal inlet port 1.
4. Place flow cell at a 45° angle on the absorbent material and, using a P1000, flush 1 mL of DI water into the inlet port.
5. Repeat 3 more times for a total of 4 mL.
6. Once complete, close the inlet port cover and remove all liquid from the waste port.
7. Dispose of absorbent material as local biological waste guidelines dictate.
8. Return flow cells to clear plastic tray in which it was shipped, making sure to record the flow cell IDs.
9. Seal the tray with the sticker provided in the packaging.
10. Put the clear plastic lid back on the tray.
11. Place the tray back in the packaging.
12. Place packaged cells in the returns box (large box can hold up to 80).
13. Once returns box is filled, follow the instructions here and follow the prompts to request the box to be sent back to Nanopore.

9 Part 9: Results

Following 72 hours of sequencing the sample should yield an N50 ~ 30kb with a data output ~ 100 GB.

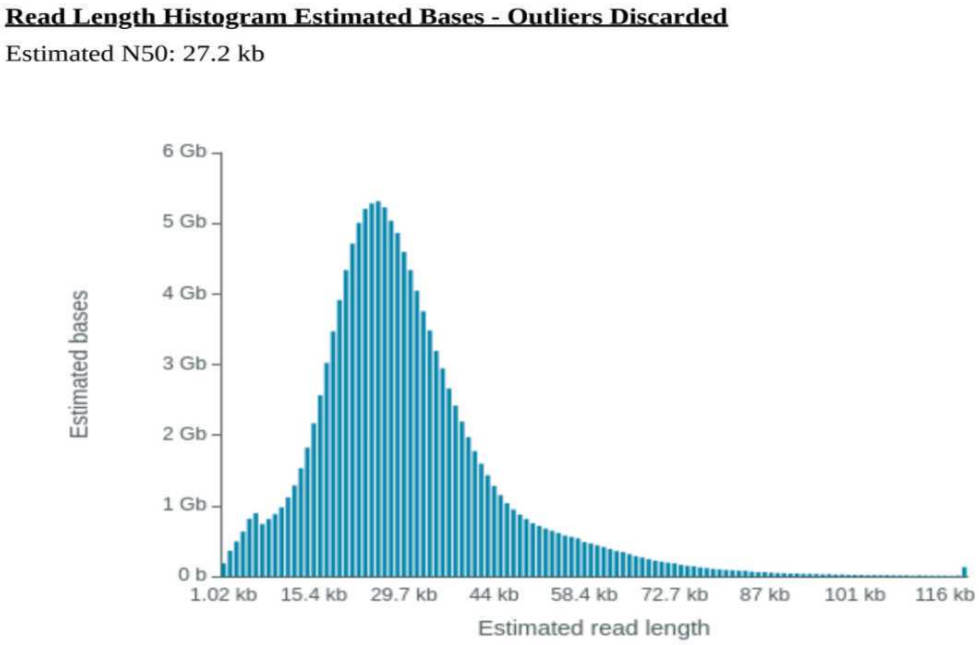

Figure 8. Expected read length histogram

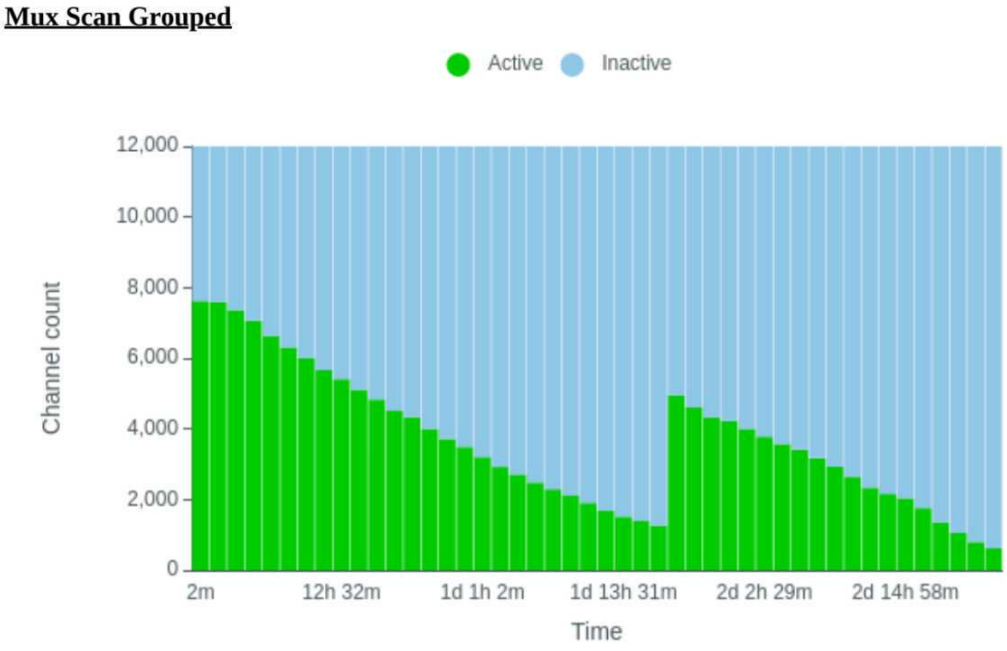

Figure 9. Expected Muxscan (two loads, three loads will have three humps)