Processing human frontal cortex brain tissue for population-scale SQK-LSK114 Oxford Nanopore long-read DNA sequencing SOP

Forked from Processing human frontal cortex brain tissue for population-scale Oxford Nanopore long-read DNA sequencing SOP

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NIH_CARD

Breeana Baker

DISCLAIMER

In development
We are still developing and optimizing this protocol.

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ABSTRACT

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LIbrary preparation for population-scale Oxford Nanopore long-read DNA sequencing SOP

At the NIH's Center for Alzheimer's and Related Dementias (CARD) https://card.nih.gov/research-programs/long-read-sequencing we will generate long-read sequencing data from thousands of patients with Alzheimer's disease, frontotemporal dementia, Lewy body dementia, and healthy subjects. With this research, we will build a public resource consisting of long-read genome sequencing data from a large number of confirmed people with Alzheimer's disease and related dementias and healthy individuals. To generate this large-scale nanopore sequencing data we have developed a protocol for processing and long-read sequencing human frontal cortex brain tissue, targeting an N50 of ~30kb and ~30X coverage.

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ATTACHMENTS

Untitled-2.pdf image.png

MATERIALS

List of reagents/equipment needed:

Consumables:

	A	B

	A	B
	Description	Supplier
	1.5mL DNA LoBind tubes	Eppendorf
	1.5mL Protein LoBind tubes	Eppendorf
	Sterile Weigh Boat	
	Razor Blade	VWR
	Cooling Block	
	15mL round-bottom tube	Perkin Elmer
	TissueRuptor Disposable Tips	Qiagen
	2mL Protein LoBind tubes	Eppendorf
	3mm Nanobind Disks	Circulomics/PacBio -Tissue big DNA kit
	1mL Luer-lock Syringes	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 Tissue Big DNA Kit	Circulomics/ PacBio
	TE pH 8	Millipore Sigma
	Isopropyl Alcohol	
	Agencourt AMPure XP beads	Agencourt

	A	B
	NEBNext® Companion Module for Oxford Nanopore Technologies ® Ligation Sequencing	New England BioLabs
	Ligation Sequencing Kit (SQK-LSK114)	Oxford Nanopore Technologies
	Flow Cell Wash Kit (EXP-WSH004)	Oxford Nanopore Technologies
	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
	Chemical Fume Hood	
	Weigh Scale	
	Centrifuge 5425R	Eppendorf
	ThermoMixer C	Eppendorf

	A	B
	TissueRuptor II	Qiagen
	KingFisher Apex/96 Deepwell plates for DW Magnets	Thermo Fisher
	Megaruptor	Diagenode
	C1000 Touch Thermocycler	BioRad
	NanoDrop 8000	Thermo Fisher
	Qubit 4	Thermo Fisher
	Vortex Genie-T	Scientific Industries
	Microfuge	
	Magnetic separator (suitable for 1.5mL Eppendorf tubes)	
	PromethION 24/48	Oxford Nanopore Technologies

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Part 1: Brain Tissue Cutting (~2 hours for 8 samples)

1.1 Add dry ice to an ice bucket.

1.2 Place supplies (sterile weigh boats, razor blades, spatulas, tweezers, labeled empty 2ml Eppendorf Protein LoBind tubes and cooling block) on dry ice and allow to chill for ~ 00:10:00 . 10m

Note: For any metal tools, clean with 70% ethanol, RNase Zap, and distilled water. Then, place

them on plastic wrap before placing them into the ice bucket.

1.3 Obtain tissue samples from the -80°C freezer and place them into the ice bucket.

1.4 Wear all necessary protective equipment (lab coat, face shield and/or mask, gloves) and complete the following steps in a chemical fume hood.

1.5 Wipe down hood with 70% ethanol and RNase Zap.

1.6 Put on a 2^{nd} pair of gloves.

1.7 Weigh the labeled empty 2ml tube to tare the scale, ensuring that the tube is centered. Then, place the chilled weigh boat on top of the cooling block.

1.8 Using the right hand to grip the blade and left hand to shield any flying pieces, cut the brain tissue.

1.9 Lift the cut tissue piece, transfer to the chilled labeled empty tube, and weigh immediately. Place it back on the dry ice immediately to avoid tissue thawing.

1.10 Add or remove tissue using the method outlined above as required by input specifications. Each brain region yields slightly different extraction results so input amounts may need to be optimized per region. In our experience frontal cortex, parietal cortex, and visual cortex require ~35mg-45mg however for cerebellum less input is required (8-10mg).

Note: DNA recovery varies based on amount of gray matter vs. white matter.

1.11 Dispose of used weighing boat in a burn box and razor blade in the sharps waste container between each sample to prevent inter-sample contamination. Keep all tissue samples on dry ice when not in use.

1.12 Discard the top layer of gloves and place in a burn box. Repeat Steps 1.4 to 1.11 for subsequent brain samples.

2 Part 2: TissueRuptor Brain Tissue Disruption and Digestion (~3.5 hours)

2.1 Clean the surfaces and pipettes with 70% ethanol.

2.2 Place 15mL round-bottom tubes and cold Buffer CT on wet ice, chill centrifuge to 4 °C, and warm ThermoMixer to 55 °C. Keep the brain samples on dry ice. Turn the Kingfisher UV light on for an 01:00:00 . 1h

2.3 Add 750 µL of cold Buffer CT to the 15ml tubes and transfer the cut brain samples from Part 1 to the 15ml tubes(keep on ice during the entire disruption process).

Note: If desired, the 2ml tube can be placed on dry ice and be reused during the rest of the extraction process.

Note: If the brain samples are going to be difficult(i.e. shavings) to remove from the 2ml tubes, add ~ 400 µL of Buffer CT to the 15ml tubes instead of 750 µL. Then, use a wide bore P1000 pipette to add 350 µL of Buffer CT to the 2ml tubes, getting as much brain as possible into the Buffer CT. Do a quick spin down if necessary. Then, use a wide bore P1000 pipette to transfer or manually pour everything into the 15ml tube. If leftover brain pieces still remain in the 2ml tube, use a wide bore tip to add ~ 100 µL of Buffer CT and transfer again to the 15ml tube.

2.4 In a chemical fume hood, submerge the TissueRuptor probe tip into the buffer and blend at a max speed for ~10s (place probe tip off to side to be cleaned later if desired).

2.5 Transfer the homogenate to a 2 mL Protein LoBind tube(or same original tube from before) including all undisrupted tissue chunks and any foam that forms.

Note: It may be better to quickly pour the liquid portion of the homogenate into the 2ml tube and use a wide-bore P1000 pipette to transfer the foam. Since it takes a while for the foam to dispense out of the tip, it may be better to set the pipette to ~ 400 µL and quickly remove smaller portions so that the 2ml tube does not warm up too much. If the 2ml tube starts to warm up, place it on the wet ice and continue removing.

2.6 Pellet homogenate by centrifuging at 6,000 x g and 4 °C for 00:05:00. Discard supernatant (pellet may not be visible, so pipette carefully and avoid pipetting from the bottom of the tube). 5m

2.7 Add 1 mL of cold Buffer CT and pipette mix ~10X with a wide bore P1000 pipette to resuspend tissue.

2.8 Pellet homogenate by centrifuging at 6,000 x g and 4 °C for 00:04:00. Discard supernatant (pellet may not be visible, so pipette carefully and avoid pipetting from the bottom of the tube). 4m

2.9 Add 20 µL of Proteinase K to the previous pellet.

2.10 Add 50 µL 1X TE pH 8.

2.11 Add 60 µL Buffer CS.

2.12 Add 100 µL Buffer CLE3 and pipette mix ~15X with a wide bore P200 pipette.

2.13 Incubate for 01:00:00 on a ThermoMixer at 55 °C and 900 rpm. 1h

2.14 Spin on a mini-centrifuge for 00:00:02 to remove liquid from the cap. 2s

2.15 Add 20 µL of RNaseA and pipette mix 3X with a wide bore P200 pipette.

2.16 Incubate for 00:30:00 on a ThermoMixer at 55 °C and 900 rpm. If desired, the Kingfisher plates in Part 3 can be prepared. 30m

2.17 Spin the tube on a mini-centrifuge for 00:00:02 to remove liquid from the cap. 2s

2.18 Add 50 µL Buffer SB and vortex for 00:00:10 at maximum speed. 10s

2.19 Spin the tube on a mini-centrifuge for 00:00:02 to remove liquid from the cap. Transfer everything(including the foam) to the prepared Kingfisher lysis plate in Part 3. 2s

3 Part 3: KingFisher Apex Nanobind Tissue Big DNA protocol (~2 hours)

3.1 Prepare KingFisher Apex plates as follows:

Plate 1 - Lysis Binding: Sample + 50 µL BL3
Plate 2 - Nanobind Storage: one 3mm Nanobind disk
Plate 3 - CW1 Wash 1: 600 µL Buffer CW1
Plate 4 - CW1 Wash 2: 600 µL Buffer CW1
Plate 5 - CW2 Wash 1: 600 µL Buffer CW2
Plate 6 - CW2 Wash 2: 600 µL Buffer CW2
Plate 7 - Elution: 100 µL Buffer EB
Plate 8 - Tip: KingFisher Flex 96-Tip Combo

3.2 Run KingFisher Apex program “210804_nanobind_tissue_kf_apex_v2.kfx” (KF script available by request from PacBio Inc).

3.3 After 00:12:00 when the program pauses, add 300 µL IPA. 12m

3.4 Collect DNA by transferring eluate to a new 1.5 mL microcentrifuge tube OR if sample is to be sheared, the sample can be transferred to a DNA Fluid+ tube.

3.5 Let the sample rest at room temperature overnight or weekend to allow DNA to solubilize.

4 Part 4. Pre-Shear DNA Quantification(~3 hours for 8 samples)

4.1 Hand-shear 10X with 1mL 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.

4.2 Quantify using the Qubit Flex Fluorometer.

Note: Taking top-middle-bottom Qubit measurements may be useful for new cohorts. If the measurements vary greatly, extra hand-shearing may be necessary.

Note: If the Qubit concentrations are lower than expected, repeat the extraction from Part 3.

4.3 Size on the Agilent Tapestation 4200 or prepare separate DNA dilutions(0.005 - 0.5ng/ul) and size using the Agilent Femto Pulse.

Note: Samples can be sized at any stage during the QC process(i.e. post-extraction, post-size selection, post-BluePippin, and post-Megaruptor shearing).

Note: Depending on the nature of the cohort, post-extraction peaks can range between 45-120kb and be heterogeneous. If the Femto does not yield desired peak sizes, repeat the extraction from Part 3.

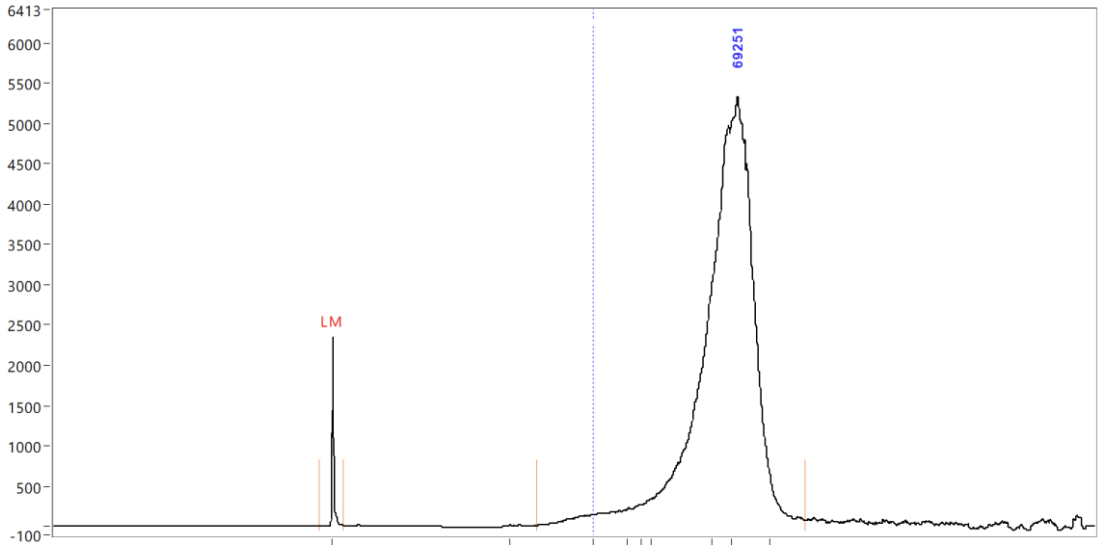

Figure 1: Example Femto Pulse Trace Post-Extraction

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

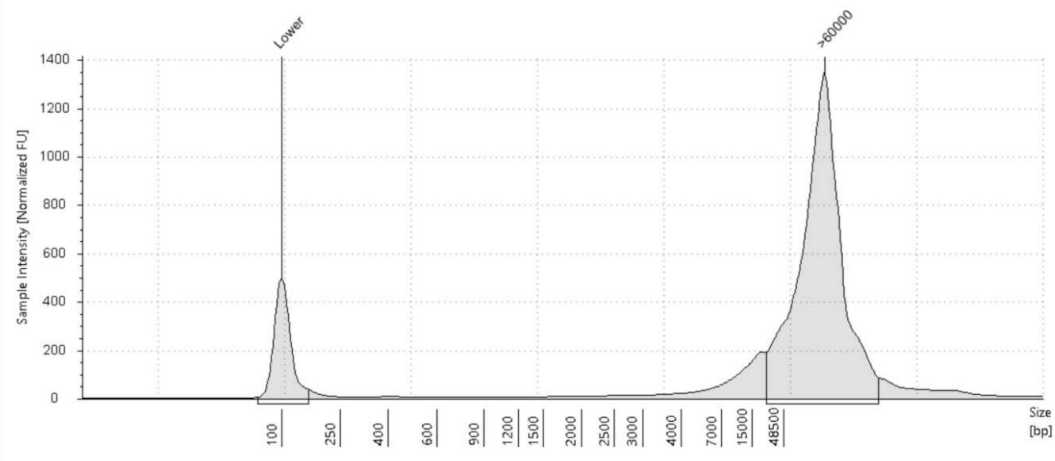

Figure 2: **Example TapeStation trace Post-Extraction**

4.4 Measure the volume of sample and calculate the amount. If the desired amount has not been reached, repeat extraction from Part 3.

5 Part 5: DNA Size Selection (1.5 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.

5.1 Set a temperature-controlled centrifuge between 26-29 °C.

5.2 Adjust the DNA sample to a Qubit concentration of 50-150 ng/ul and place the sample into a 1.5ml Eppendorf DNA LoBind tube if not already. If the SRE-XS kit is being used, adjust the DNA sample to a Qubit concentration of 25-150ng/ul.

5.3 Add an equal volume of Buffer SRE to the sample. Mix thoroughly by tapping the tube or pipette mixing.

5.4 Load the tubes into the centrifuge with the hinge facing out and make sure that the samples are balanced.

Note: Inserting tube with the hinge out is crucial to avoid aspirating the pellet if not visible in later steps.

5.5 Centrifuge at 10,000 x g for 00:30:00 . If the total volume in the tube is high(i.e. 200 µL), spin for an additional 00:05:00 . 35m

5.6 Remove tubes from centrifuge and carefully remove the supernatant without disturbing the DNA pellet. Pellet may not always be visible but will have formed on the bottom of the tube under the hinge region. Pipette from the opposite wall. See **Figure 3** below.

Note: For very viscous DNA, the pellet may not initially form tightly and can lead to easy aspiration of DNA. If this is the case, remove as much supernatant as possible but leave behind what you cannot pipette without aspirating DNA.

Note: Pellet will be tighter after the EtOH wash.

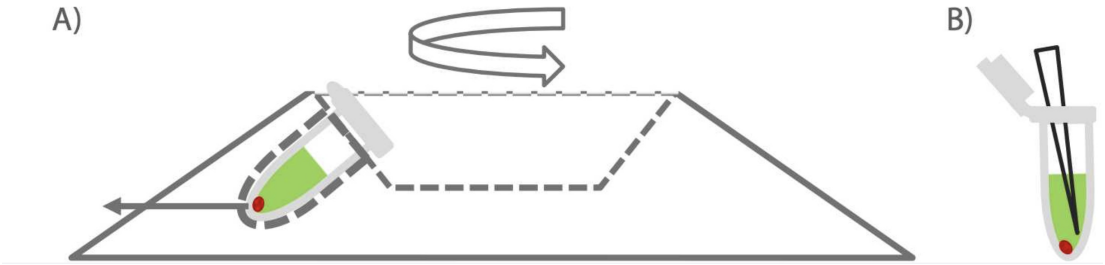

Figure 3: Taken from Circulomics Short Read Eliminator Kit Family Handbook v2.0 (07/2019) a) note orientation of the tube in centrifuge. Pellet will form on the side of the tube facing outwards at the bottom of the tube. b) Pipette from opposite side of tube on the thumb lip side to avoid disturbing pellet (pellet may not be visible).

5.7 Carefully add 200 µL of fresh 70% EtOH on the opposite side of the hinge wall and centrifuge at 10,000 x g for 00:03:00 . 3m

Note: Do not mix after adding EtOH.

5.8 Carefully remove EtOH wash without disrupting the pellet.

5.9 Repeat Steps 5.7 and 5.8.

5.10 Add 50-100 µL of Buffer EB to the tube and pipette mix ~10-15 times along the hinge side to thoroughly mix the pellet. Incubate at RT for 00:45:00 . 45m

Note: Letting the sample incubate for longer or in the 4 °C may better homogenize the sample and may give better quantification readings.

5.11 Quantify using the Qubit Flex Fluorometer.

Note: Taking top-middle-bottom Qubit measurements may be useful for new cohorts. If the measurements vary greatly, extra hand-shearing may be necessary if samples are still heterogeneous.

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

Note: It may be useful to size the sample at the post-size selection stage if a new cohort is being processed or if further analyses needs to be done on a heterogeneous cohort. If the Femto does not yield desired peak sizes, repeat extraction from Part 3.

5.13 Measure the volume of sample and calculate the amount. If the amount is lower than expected, repeat extraction from Part 3.

6 Part 6: Megaruptor 3 Shear with DNA Fluid+ Kit (2.5 hours per 8 samples)

Note: Samples can be sheared before or after size-selection.

6.1 If possible, normalize the samples between ~40 - 60 ng/uL in 140 µL total volume (to be made in TE or Buffer EB and equates to 5600-8200ng per sample) in DNA Fluid+ tubes. This ensures that there will be enough for future processes; however, the final concentrations can be adjusted if recoveries are low or if future sequencing requires any changes.

Note: The Megaruptor 3(MR3) has a minimum volume of 100ul and a maximum concentration of 150ng/ul when using the DNA Fluid+ tubes.

Note: If shearing is done before size-selection, normalize to at least 80ng/ul in 120ul total volume. Since volume is lost after the MR3 process, this ensures that there will be an adequate volume and concentration for size-selection. However, normalization can be

adjusted if recoveries are low or if future sequencing requires any changes.

6.2 Attach the DNA Fluid+ needle onto the tube and push the entire item into the Megaruptor 3 slots until it fits snugly. If running fewer than 8 samples, put the tubes in the 1st and/or 8th slots, working your way inward. Use a balance if there is an odd number of samples. Shear at speed 45. Some cohorts may require slower speeds(i.e. 43) to obtain 25-30kb fragment sizes. Shearing takes around ~ 01:00:00). 1h

Note: The Megaruptor can be set to the average concentration if the sample Qubit readings and volumes do not vary greatly from each other(+/- 10ng/ul to +/- 20ul). Make sure that the volume is set to the sample with the highest volume.

6.3 Once the MR3 shearing is finished, repeat the run by navigating back to the main menu and running another cycle(takes around ~ 01:00:00). 1h

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

6.4 When disassembling the MR3 needle use a P20 pipette to remove remaining liquid from inside of the needle and syringe.

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

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

7 Part 7: Post-shear DNA Quantification (~2.5 hours for 8 samples)

7.1 Quantify using the Qubit Flex Fluorometer and Nanodrop.

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

Note: The desired post-shear peaks for the CARD long-read sequencing project should be between 25-35kb. If the Femto does not yield desired peak sizes, repeat extraction from Part 3.

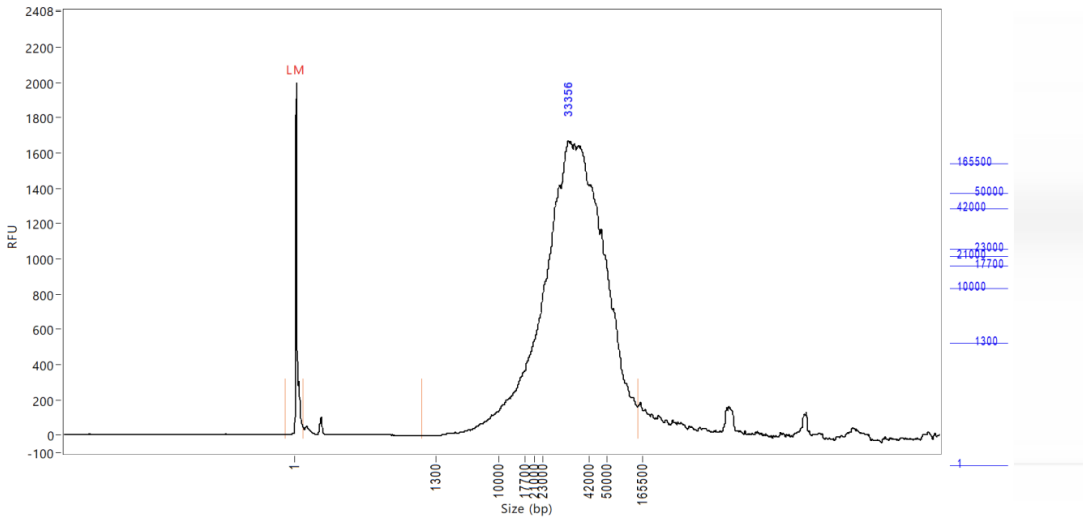

Figure 4: Example Femto Pulse trace post-sheared

7.3 Measure the volume of sample and calculate the amount. If the desired amount has not been reached, repeat extraction from Part 3.

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

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

8 Part 8: 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.

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

8.2 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%.

8.3 Quantify using the Qubit Flex Fluorometer.

8.4 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).

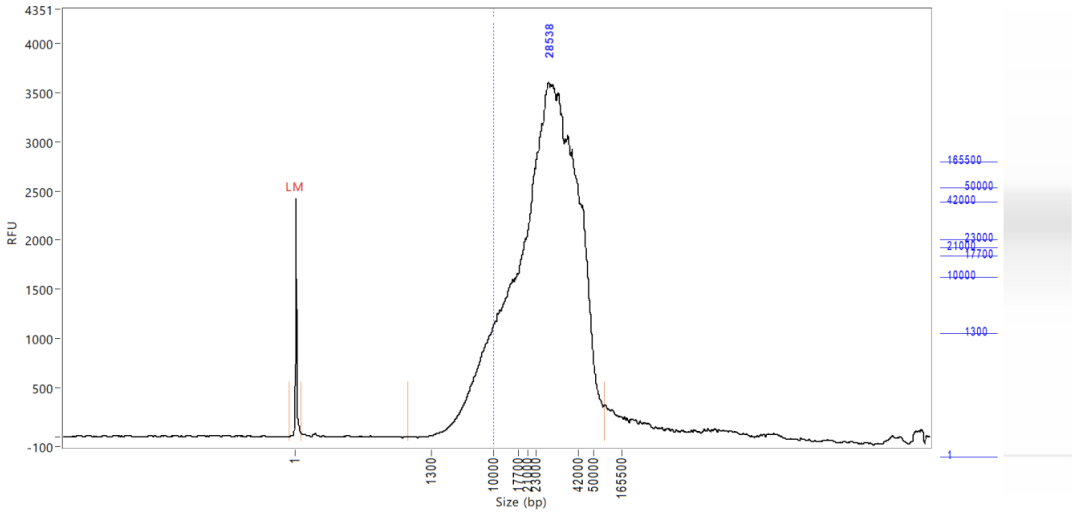

Figure 5: Example Femto Pulse Trace Pre-BluePippin(Post-SRE/Post Shear). Short fragments under 10kb are apparent.

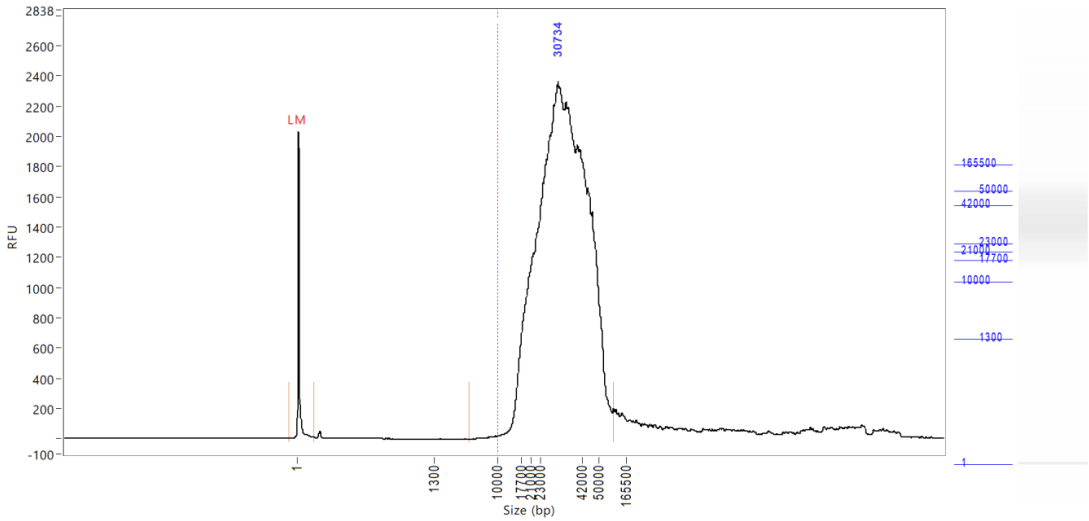

Figure 6: Example Femto Pulse Trace Post-BluePippin(Post-SRE/Post Shear). Short fragments under 10kb no longer exist.

8.5 Measure the volume of sample and calculate the amount. If the desired amount has not been reached, repeat extraction from Part 3.

9 Part 9: Manual SQK-LSK114 Library Prep and Sequencing (~6 hours, including reloads but not including flushing and returning flow cells)

20m

Note: Library prep can also be done on the Hamilton NGS Star and can process 48 samples in ~4 hours using the [HAMILTON NGST STAR Oxford Nanopore LSK114 Library preparation] protocol: https://dx.doi.org/10.17504/protocols.io.n2bvj36mnlk5/v1

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

Minor tweaks:

- 2.5 µg DNA library in 48 µL (Part A Step 2)
- Add 45 µL of beads (Part B Step 10)
- Use short fragment buffer (Part B Step 14)
- Incubate for 00:20:00 at 37 °C at 300-450 x g (Part B Step 18)

9.1 A. DNA Repair and End-Prep

20m 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 2.5ug, this might be over 48 µL but that is fine. Adjust the amount of beads to match the total volume of this mixture (sample + buffers/enzymes)
- 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 10x, 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. Resuspend 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 10x. Do not pipette mix here as beads may clump around the pipette tip.

9. Incubate on a hula mixer 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 uncharacteristically 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 significantly higher than 60uL, more ethanol may be used.

14. Repeat step 13.

15. Spin down and place the tube back on the 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 resuspend the pellet in 62 µL nuclease-free water. Incubate for 00:03:00 at RT gently flicking every so often. If not quantifying the sample post-DNA repair and end-prep, can resuspend the pellet in 60uL water.

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

19. Remove and retain 62 µL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.

20. Quantify 2uL of sample on the Qubit. If not quantifying sample post-DNA repair and end-prep, can resuspend the pellet in 60uL water in step 17.

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

9.2 B. Adapter Ligation and Clean-Up

55m 30s

1. Spin down the Ligation Adapter (LA) and NEBNext Quick T4 DNA Ligase, then return to ice.

Note: Do not allow LA or Quick T4 to remain at room temperature for too long. Since LA and Quick T4 do not freeze, it is possible to leave them in the freezer until needed.

2. Thaw Ligation Buffer (LNB) at RT, mix by pipetting up and down (vortexing is ineffective due to viscosity), and place on ice.

3. Thaw Elution Buffer (EB) and Short Fragment Buffer (SFB) at RT, mix by vortexing, spin down, and place on ice.

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

- 60 µL DNA sample
- 25 µL LNB
- 10 µL Quick T4
- 5 µL LA

6. Mix by gently pipetting and spin down.

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

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

9. Resuspend AMPure beads by vortexing.

10. Add 45 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 resuspend, spin down and repellet 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 supernatant.

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

18. Remove the tube from magnet and resuspend pellet in 26 µL EB, spin down, and incubate for 00:20:00 at 37 °C and 300-450 x g.

19. During this time, QC the flow cells.

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

Note: If trying to reach 30x coverage, it is recommended to only use flow cells with >7000 pores.

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, being careful not to aspirate any beads (this is the DNA library).

22. Quantify 2uL of sample on the Qubit.

23. Reprep library from **Part 6** if < 540ng. We have calculated this based on a DNA size of 30kb and at a coverage of 30x over three loads. If your sample differs from this size, recalculate based on 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.

9.3 C. Priming and Loading R10 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. Add 30 µL of thawed and mixed FCT directly to tube of thawed and mixed FCF and vortex. Alternatively, in a new tube, add 30 µL of thawed and mixed FCT to 1170 µL of thawed and mixed FCF and vortex. This is your priming mix
3. Expose inlet port 1 on the flow cell, set P1000 pipette to 200 µL and use the dial to 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 inlet port 1 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.
9. Gently pipette mix the prepared library mix right before loading.
10. Load 200 µL of the library mix into inlet port 1 on the flow cell.
11. Close valve to seal inlet port and close PromethION door.
12. Wait 00:10:00 and then initiate sequencing.
13. Ideally, the library quants yielded at least 540ng 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).

9.4 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. Put the clear plastic lid back on the tray.
10. Place the tray back in the packaging.
11. Place packaged cells in the returns box (large box can hold up to 80).
12. Once returns box is filled, follow the instructions here and follow the prompts to request the box to be sent back to Nanopore.

Sequencing results:

10 Following 72 hours of sequencing the sample should yield an N50 ≥ 30kb with a data output ~ 100-160GB.

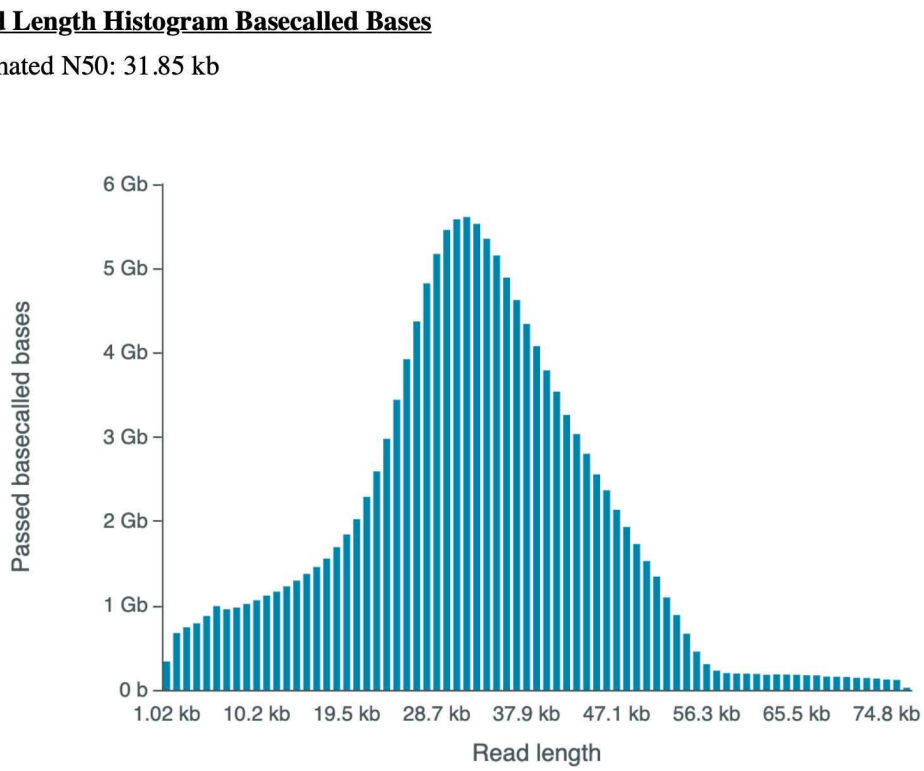

Figure 7. Expected Read Length Histogram:

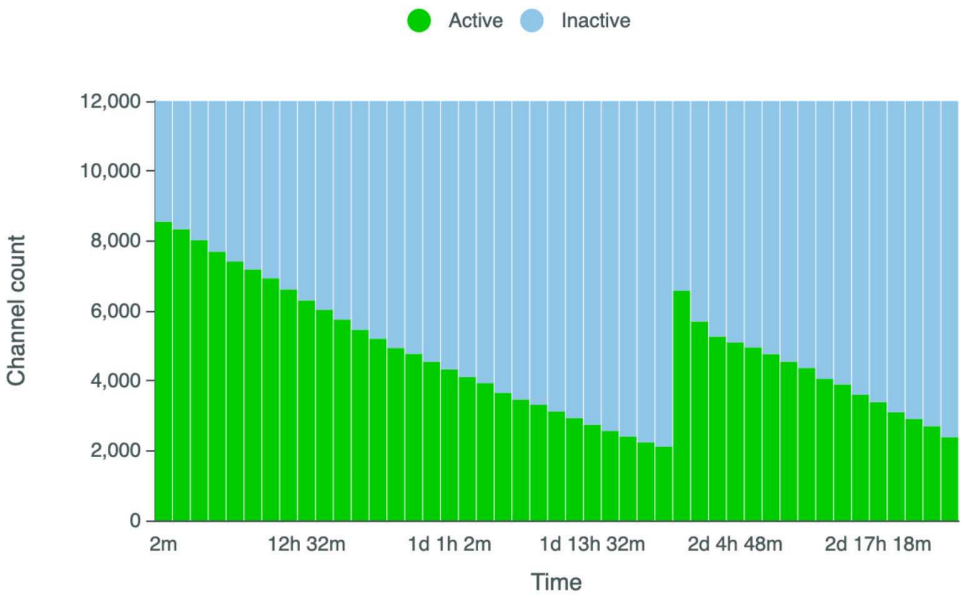

Figure 8. Expected Muxscan (from 2 loads):