NIH CARD

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Protocol status: Working
We use this protocol and it's working

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

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NIH-CARD

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DISCLAIMER

In development
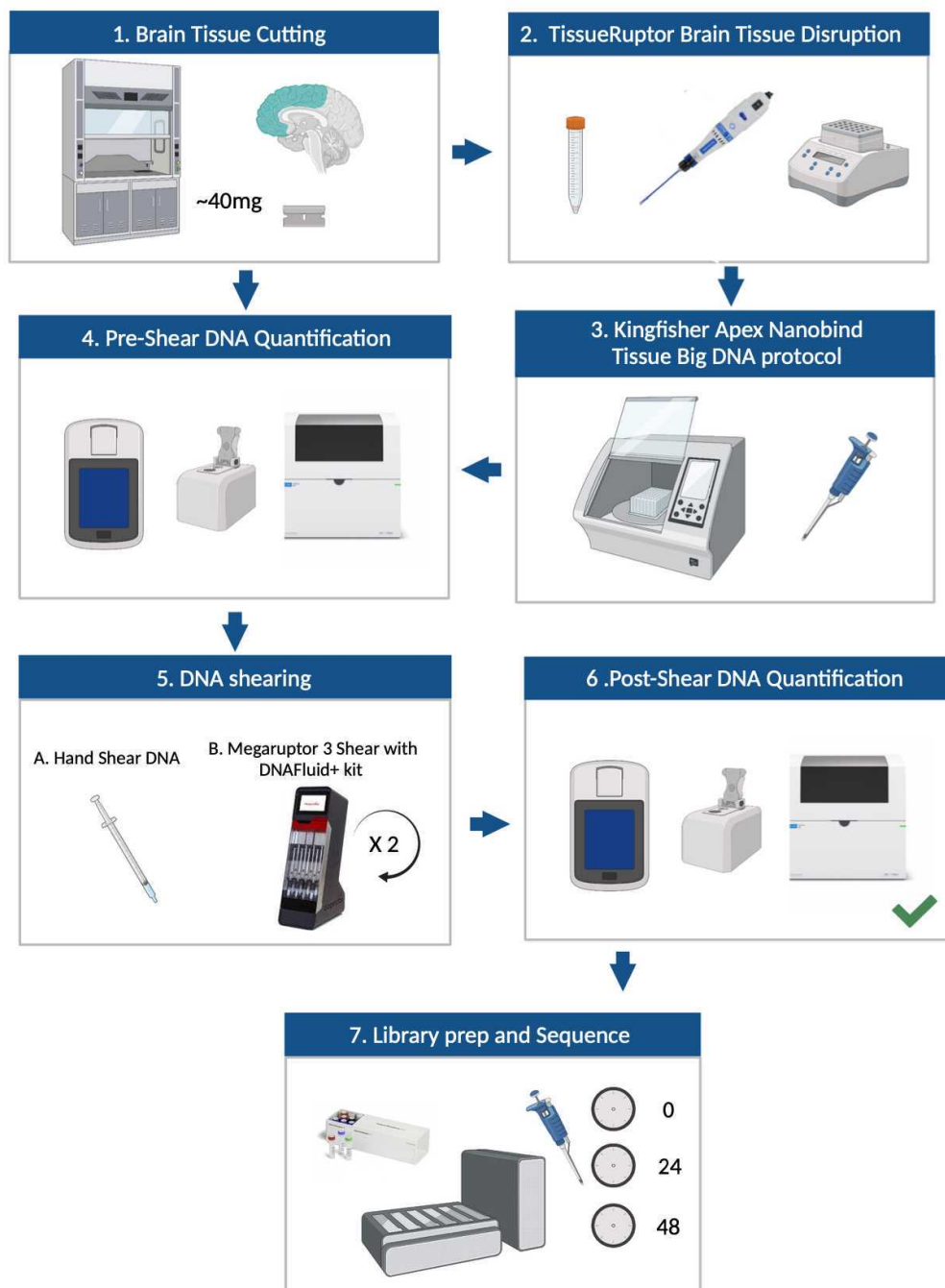We are still developing and optimizing this protocol.

ABSTRACT

Processing human frontal cortex brain tissue 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 roughly 4000 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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Figure 1. Overview of HMW Brain DNA extraction and ONT sequencing protocol

MATERIALS

List of reagents/equipment needed:

Consumables:

Description	Supplier
1.5mL DNA LoBind tubes	Qiagen
1.5mL Protein LoBind tubes	Qiagen
Sterile Weigh Boat	
Razor Blade	
Cooling Block	
15mL conical-bottom tube	
TissueRuptor Disposable Tips	
2mL Protein LoBind tubes	
3mm Nanobind Disks	Circulomics -Tissue big DNA kit
1mL Luer-lock Syringes	
1.5" Needles	
DNA Fluid+ Kit	Diagenode
0.2mL thin-wall PCR tubes	
PromethION Flow Cells	Oxford Nanopore Technologies

Reagents:

Description	Supplier
Nanobind Tissue Big DNA Kit	Circulomics
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-LSK110)	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	
Genomic DNA Screen Tape	Agilent

Equipment:

Description	Supplier
Chemical Fume Hood	
Weigh Scale	
Centrifuge 5425R	Eppendorf
ThermoMixer C	Eppendorf
TissueRuptor II	Qiagen
KingFisher Apex	Thermo Fisher
Megaruptor	Diagenode
C1000 Touch Thermocycler	BioRad
NanoDrop 8000	Thermo Fisher
Qubit 4	Thermo Fisher
Tapestation 4200	Agilent
Vortex Genie-T	Scientific Industries
Microfuge	
Magnetic separator (suitable for 1.5mL Eppendorf tubes)	
PromethION 48	Oxford Nanopore Technologies

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Part 1: Brain Tissue Cutting (~2.5 hours for 16 samples)

1.1 Add dry ice to a ice bucket

1.2 Place supplies (sterile weigh boat, razor blade, labeled empty 2mL protein LoBind microcentrifuge tubes and cooling block) in dry ice and allow to chill for ~ ⏱ 00:05:00   `5m`

1.3 Obtain tissue samples from 🌡 -80 °C freezer and place in dry ice

1.4   Wear all necessary protective equipment (lab coat, face shield, double gloved) and complete the following steps within a chemical fume hood

1.5   Weigh labeled empty tube to tare scale, ensuring tube is centered

1.6   Working in the chemical fume hood, place the chilled weighing boat on top of the cooling tissue (use right hand to grip blade and cut tissue), use left hand just above, in between tissue and in front of hood, to shield any flying pieces)

1.7   Using the chilled razor blade, gently lift the cut tissue piece and transfer to the chilled labeled empty tube and weigh immediately, place back in dry ice immediately to avoid tissue thawing

1.8   Add or remove tissue using the method outlined above as required by input specifications ~40mg for frontal cortex, DNA recovery varies based on amount of gray matter vs. white matter)

1.9   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 and keep all tissue samples on dry ice when not in use

2   **Part 2: TissueRuptor Brain Tissue Disruption (~3 hours)**

2.1   Place 15mL round tubes and cold Buffer CT on ice, chill centrifuge to 🌡 4 °C , and warm ThermoMixer to 🌡 55 °C

2.2   Transfer brain tissue from previous steps to 15mL round tubes (keep on ice during the entire disruption process)

2.3   Add [🧪 750 µL] of cold Buffer CT

2.4   Submerge TissueRuptor probe tip in buffer and blend at max speed for [🕐 00:00:10] (place probe tip off to side to be cleaned later)   `10s`

2.5   Transfer homogenate to a 2 mL Protein LoBind microcentrifuge tube including all undisrupted tissue chunks and any foam that forms

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 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 [🕐 00:02:00]. Discard supernatant (pellet may not be visible, so pipette carefully and avoid pipetting from the bottom of tube)   `2m`

2.9   Pulse vortex for [🕐 00:00:01] x 5 times (max setting) to dislodge pellet   `1s`

2.10　Add 🧪 20 μL of Proteinase K to the previous pellet

2.11　Add 🧪 50 μL 1X TE pH 8

2.12　Add 🧪 60 μL Buffer CS

2.13　Add 🧪 100 μL Buffer CLE3 and pipette mix 15X with a wide bore P200 pipette

2.14　Incubate for ⏱ 01:00:00 on a ThermoMixer at 🌡 55 °C and 900 rpm　`1h`

2.15　Spin on a mini-centrifuge for ⏱ 00:00:02 to remove liquid from the cap　`2s`

2.16　Add 🧪 20 μL of RNaseA and pipette mix 3X with a wide bore P200 pipette

2.17　Incubate for ⏱ 00:30:00 on a ThermoMixer at 🌡 55 °C and 900 rpm　`30m`

2.18   Spin the tube on a mini-centrifuge for ⏱ 00:00:02 to remove liquid from the cap   `2s`

2.19   Add ⚗ 50 µL Buffer SB and vortex for ⏱ 00:00:10 at maximum speed (transfer any foam   `10s`
that appears)

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 Circulomics 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 to allow DNA to solubilize

4   Part 4. Pre-Shear DNA Quantification

4.1   **Hand shear DNA**

Hand-shear 10X with 1mL Luer-Lock syringes and 1.5" needles (bringing sample up into needle and depressing plunger counts as 1 cycle)

4.2   Quantify

1. Quantify the samples with the nanodrop and Qubit and size using the Agilent Tapestation 4200
2. Upload the tapestation reports
3. Add the quantifications
4. Only take forward samples that are > 3ug. If a sample does not reach this requirement then repeat DNA extraction from Part 1.

5   Part 5: DNA Shearing (8 hours per 48 samples)

5.1   **Megaruptor 3 Shear with DNAFluid+ kit:**   `2h`

1. Normalize samples to 40-60 ng/uL in ⚗ 150 µL total volume (to be made up with nuclease-free water and equates to 4000 - 6000ng per sample) in DNA Fluid+ tubes.
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 in. Samples should always be balanced, if running an odd number of samples, samples can be balanced with an empty corresponding tube. Shear at speed 45 (takes around ~ 🕑 01:00:00)
3. Once the MR3 shearing is finished, repeat the run by navigating back to the main menu and select speed 45 again (takes around ~ 🕑 01:00:00)
4. 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-shear DNA Quantification

6.1   Quantify the samples with the nanodrop and Qubit and size using the Agilent Tapestation 4200

6.2   Upload the tapestation reports and and quantifications

6.3   For Step 6 the starting material must be > 3ug. If a sample does not reach this requirement then repeat DNA extraction from Part 1.

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

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Part 7: Library Prep (~6 hours, not including flushing and returning cells)

7.1   **A. DNA Repair and End-Prep**   `20m 30s`

1. Put 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 (load 3 ug, this will likely be over 48 µL but that is fine, just adjust the amount of beads to match the total volume of this mixture)
   - 🧪 3.5 µL NEBNext FFPE DNA Repair Buffer (vortex)
   - 🧪 3.5 µL Ultra II End-prep reaction buffer (vortex)
   - 🧪 3 µL Ultra II End-prep enzyme mix (do not vortex)

- $\boxed{\text{2 µL}}$ NEBNext FFPE DNA Repair Mix (do not vortex)

3. Mix thoroughly by gently flicking tube or pipetting up and down 10X, and then spin down

4. Using a thermal cycler, incubate samples at $\boxed{\text{20 °C}}$ for $\boxed{\text{00:05:00}}$ and $\boxed{\text{65 °C}}$ for $\boxed{\text{00:05:00}}$

- Start and pause thermal cycler to allow lid to come to $\boxed{\text{85 °C}}$ before putting samples in

5. Allow Thermocycler to cool to $\boxed{\text{4 °C}}$ 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 $\boxed{\text{60 µL}}$ (or equivalent volume, see step 2) of resuspended beads to the reaction and mix by pipetting up and down 10X

9. Incubate for $\boxed{\text{00:05:00}}$ at room temperature

10. Prepare $\boxed{\text{500 µL}}$ per sample of fresh 75% ethanol in Nuclease-free water

11. Pellet sample on magnet until eluate is clear and colorless, about $\boxed{\text{00:02:00}}$

12. Pipette off the supernatant and retain, just in case the following quant is uncharacteristically or surprisingly low

13. With the samples remaining on the magnet, wash the beads with $\boxed{\text{200 µL}}$ of the ethanol, pipetting on the opposite wall (the goal here is to make sure the beads are fully covered) and making sure not to disturb the pellet, count to 3 and remove and discard ethanol

14. Repeat previous step

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

16. Allow to dry for ~ $\boxed{\text{00:00:30}}$ but do not over-dry

17. Remove the tube from the magnetic rack and resuspend the pellet in $\boxed{\text{61 µL}}$ Nuclease-free water, incubate for $\boxed{\text{00:03:00}}$ at room temperature (hold in hands and gently flick every so often)

18. Pellet the samples on a magnet until eluate is clear and colorless

19. Remove and retain $\boxed{\text{61 µL}}$ 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 6.

21. It is possible to store samples at $\boxed{\text{4 °C}}$ overnight at this step if needed

7.2   B. Adapter Ligation and Clean-Up   `35m 30s`

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

- 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 60, add water until it is)
- ⚗ 25 µL LNB
- ⚗ 10 µL Quick T4
- ⚗ 5 µL AMX-F

6. Mix gently by 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. Resuspend beads by vortexing

10. Add ⚗ 45 µL of resuspended beads to the reaction and mix by flicking

11. Incubate for ⏱ 00:05:00 at RT

12. Spin down sample and pellet on magnet

13. Pipette off the supernatant and retain, just in case the final elution quant is uncharacteristically or surprisingly low

14. Wash the beads with ⚗ 250 µL SFB, flick to resuspend and repellent, remove and discard supernatant

15. Repeat previous step

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 resuspend pellet in 25 µL EB, spin down, and incubate for ⏱ 00:20:00 at 🌡 37 °C

19. During this time, QC the flow cells (only use flow cells with >6000 pores)

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

21. Remove and retain ⚗ 25 µL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube (this is the DNA library)

22. Quantify samples on Qubit

23. Reprep library from Part 6 if < 1200 ng.

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

7.3   C. Priming and Loading Flow Cell   `1h 15m`

1. Thaw SBII, FLT, and FB, vortex, and spin down

2. Thaw LBII

3. Add ⚗ 30 µL of thawed and mixed FLT directly to tube of FB and vortex

4. Expose inlet port on flow cell and draw back a small volume to remove any air bubbles (usually about 20-30 µL, just until a small volume of buffer enters the pipette tip)

5. 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

6. Wait ⏱ 00:05:00

7. During this time, separate the DNA library into three equal aliquots (ideally with 400 ng of DNA each). Bring each aliquot up to ⚗ 24 µL with EB. (i.e, if your final elution is exactly

1200 ng in 24 ul, move `🧪 8 µL` to three separate tubes and add `🧪 16 µL` of EB to each.)

8. Prepare the first library mix for loading:
   - `🧪 75 µL` SBII (vortex)
   - `🧪 51 µL` LB (pipette up and down immediately before use)
   - `🧪 24 µL` DNA library in EB (400 ng)

9. Immediately load all `🧪 150 µL` of the library mix

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

11. Wait `🕑 00:10:00` and then initiate sequencing

12. Ideally, the library quants yielded at least 1200 ng to allow for 3X 400 ng 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 (DIL should be vortexed, WMX should NOT be vortexed, only spun)

   - Add `🧪 2 µL` WMX into `🧪 398 µL` DIL and pipette mix

   -Pause the PromethION runs and export .pdf reports

   -Rotate the inlet port cover to reveal inlet port 1

   -Using a P1000, insert tip into inlet port and draw back a small volume using the wheel to remove any air (usually around 20-30 µL)

   -Load `🧪 400 µL` Flow Cell Wash Mix into the inlet port, avoiding any introduction of air

   -Wait `🕑 01:00:00`

   -Repeat priming steps and reload samples (steps 1 - 12)

7.4 **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 input 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 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
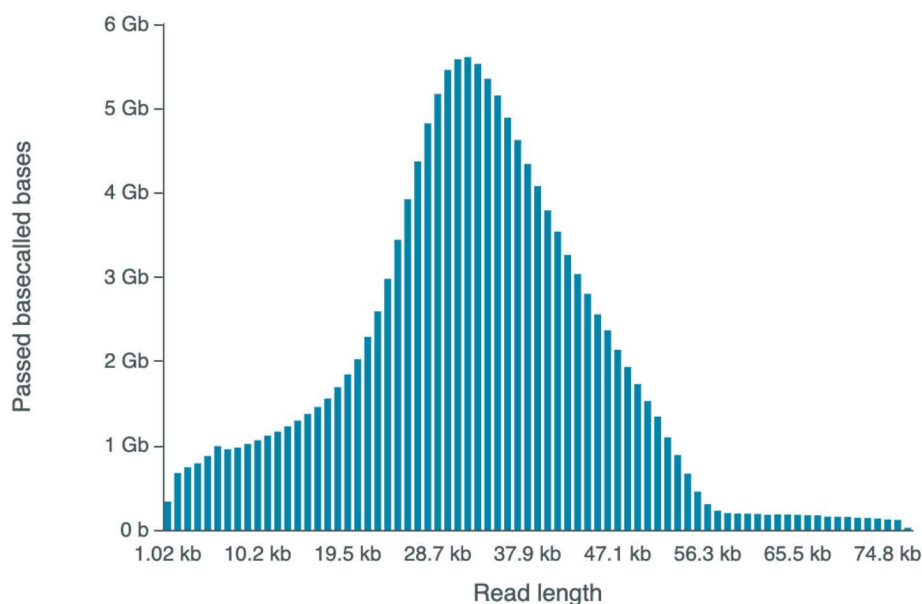
Sequencing results:

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1. Following 72 hours of sequencing the sample should yield an N50 ≥ 30kb with a data output ~ 100-160GB.
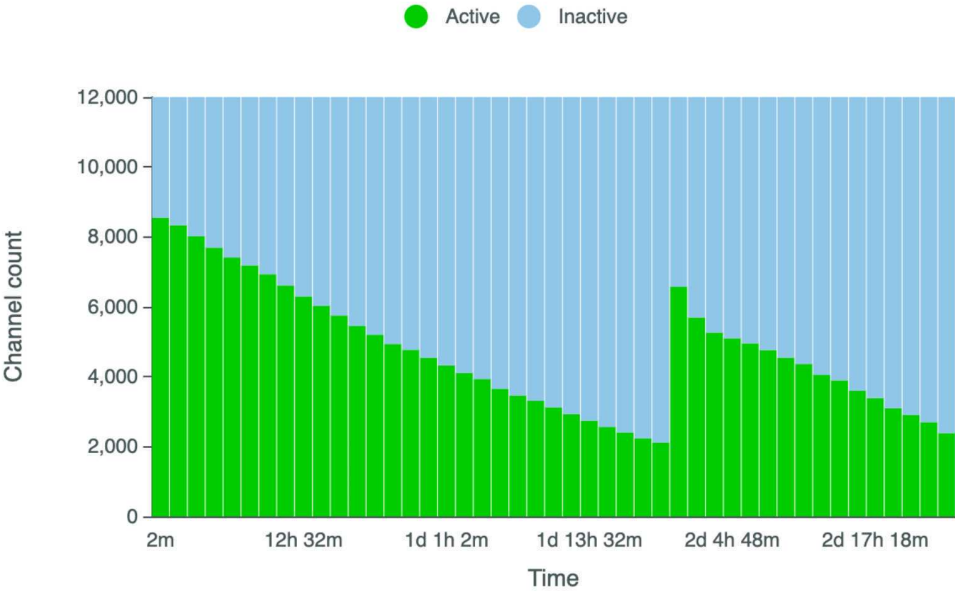
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Read Length Histogram Basecalled Bases

Estimated N50: 31.85 kb

Figure 2. Expected Read Length Histogram:

Mux Scan Grouped

Figure 3. Expected Muxscan (from 2 loads):