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

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Kimberley J Billingsley<sup>1,2</sup>, Ramita Dewan<sup>2</sup>, Laksh Malik<sup>1</sup>, Pilar Alvarez Jerez<sup>1,2</sup>, Stith Kiley<sup>1</sup>, Cornelis Blauwendraat<sup>1,2</sup>, on behalf of the CARD Long-read Team<sup>1</sup>

<sup>1</sup>Center for Alzheimer's and Related Dementias, National Institute on Aging, Bethesda, Maryland, USA;

<sup>2</sup>Laboratory of Neurogenetics, National Institute on Aging, Bethesda, Maryland, USA

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Kimberley J Billingsley

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**Protocol status:** Working

**We use this protocol and it's working**

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**Keywords:** sequencing human frontal cortex brain tissue, sequencing data, scale oxford nanopore, sequencing sop processing, oxford nanopore, human frontal cortex brain tissue for population, nanopore team, sequencing, sequencing sop, read dna, processing human frontal cortex brain tissue, human frontal cortex brain tissue, brain tissue, people with alzheimer, patients with alzheimer, dna, lewy body dementia, related dementia, alzheimer, center for alzheimer, frontotemporal dementia

## Disclaimer

### In development

We are still developing and optimizing this protocol.



## Abstract

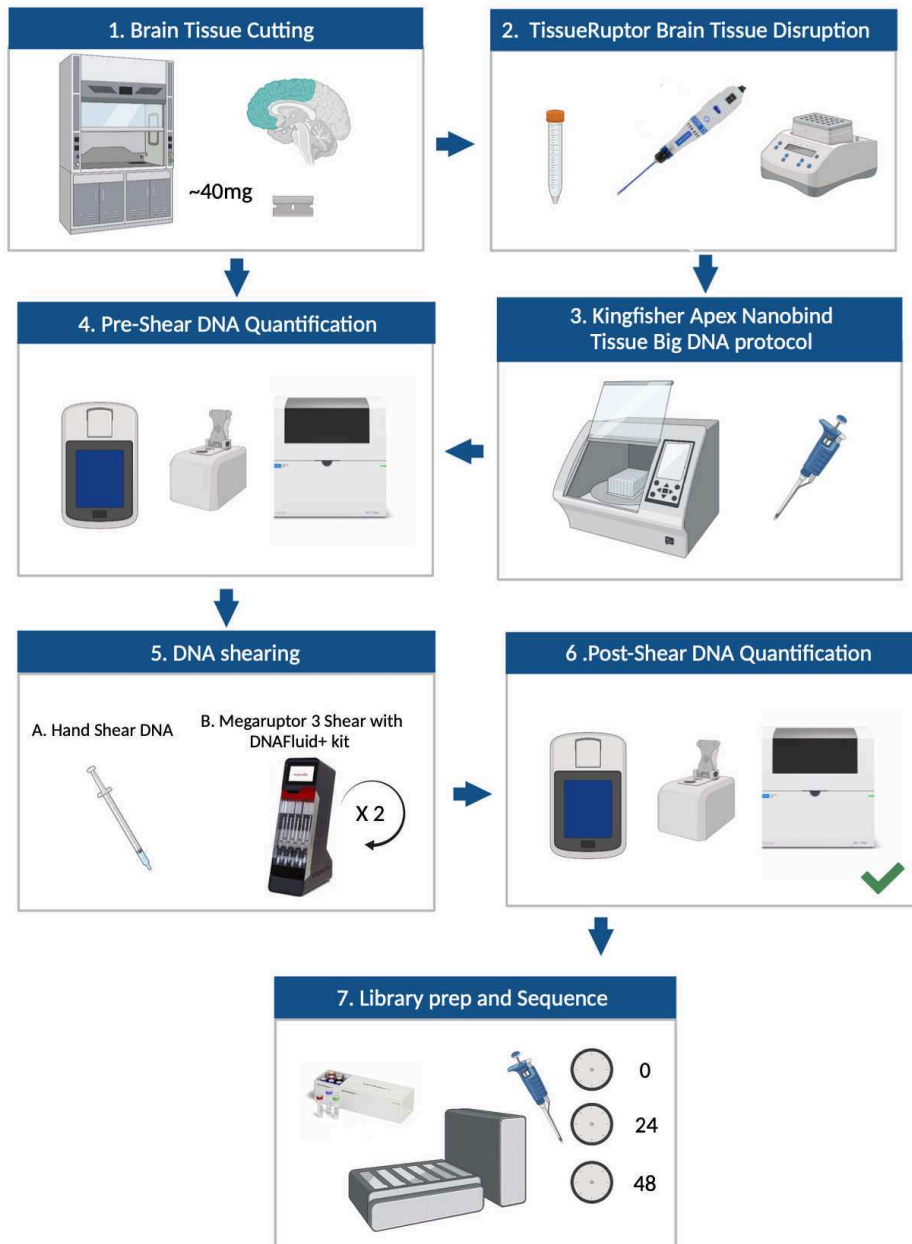
### Processing human frontal cortex brain tissue for population-scale Oxford Nanopore long-read DNA sequencing SOP

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.

†Correspondence to: Kimberley Billingsley [billingsleykj@nih.gov](mailto:billingsleykj@nih.gov) and Cornelis Blauwendraat [cornelis.blauwendraat@nih.gov](mailto:cornelis.blauwendraat@nih.gov)

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**Figure 1. Overview of HMW Brain DNA extraction and ONT sequencing protocol**

## Attachments



Untitled-2.pdf

746KB



image.png

1KB

## 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
- 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

5m

**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  $\mu\text{L}$  Buffer CLE3 and pipette mix 15X with a wide bore P200 pipette
- 2.14 Incubate for  01:00:00 on a ThermoMixer at  55  $^{\circ}\text{C}$  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  $\mu\text{L}$  of RNaseA and pipette mix 3X with a wide bore P200 pipette
- 2.17 Incubate for  00:30:00 on a ThermoMixer at  55  $^{\circ}\text{C}$  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  $\mu\text{L}$  Buffer SB and vortex for  00:00:10 at maximum speed (transfer any foam that appears) 10s

### 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  $\mu\text{L}$  BL3

Plate 2 - Nanobind Storage: one 3mm Nanobind disk

Plate 3 - CW1 Wash 1:  600  $\mu\text{L}$  Buffer CW1

Plate 4 - CW1 Wash 2:  600  $\mu\text{L}$  Buffer CW1

Plate 5 - CW2 Wash 1:  600  $\mu\text{L}$  Buffer CW2

Plate 6 - CW2 Wash 2:  600  $\mu\text{L}$  Buffer CW2

Plate 7 - Elution:  100  $\mu\text{L}$  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  $\mu$ L 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  $\mu$ L 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 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)
  -  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  20 °C for  00:05:00 and  65 °C for  00:05:00
  - Start and pause thermal cycler 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 pipetting up and down 10X
9. Incubate for 00:05:00 at room temperature
10. Prepare 500 µL per sample of fresh 75% ethanol in Nuclease-free water
11. Pellet sample on magnet until eluate is clear and colorless, about 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 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 ~ 00:00:30 but do not over-dry
17. Remove the tube from the magnetic rack and resuspend the pellet in 61 µL Nuclease-free water, incubate for 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 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 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  $\mu\text{L}$  LNB
  -  10  $\mu\text{L}$  Quick T4
  -  5  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{L}$  EB, spin down, and incubate for  00:20:00 at  37  $^{\circ}\text{C}$
  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  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{L}$ , just until a small volume of buffer enters the pipette tip)
5. Flush  500  $\mu\text{L}$  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  $\mu\text{L}$  with EB. (i.e, if your final elution

is exactly 1200 ng in 24 ul, move  8  $\mu\text{L}$  to three separate tubes and add  16  $\mu\text{L}$

of EB to each.)

8. Prepare the first library mix for loading:

-  75  $\mu\text{L}$  SBII (vortex)
-  51  $\mu\text{L}$  LB (pipette up and down immediately before use)
-  24  $\mu\text{L}$  DNA library in EB (400 ng)

9. Immediately load all  150  $\mu\text{L}$  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 quant yields 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  $\mu\text{L}$  WMX into  398  $\mu\text{L}$  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  $\mu\text{L}$ )
- Load  400  $\mu\text{L}$  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:

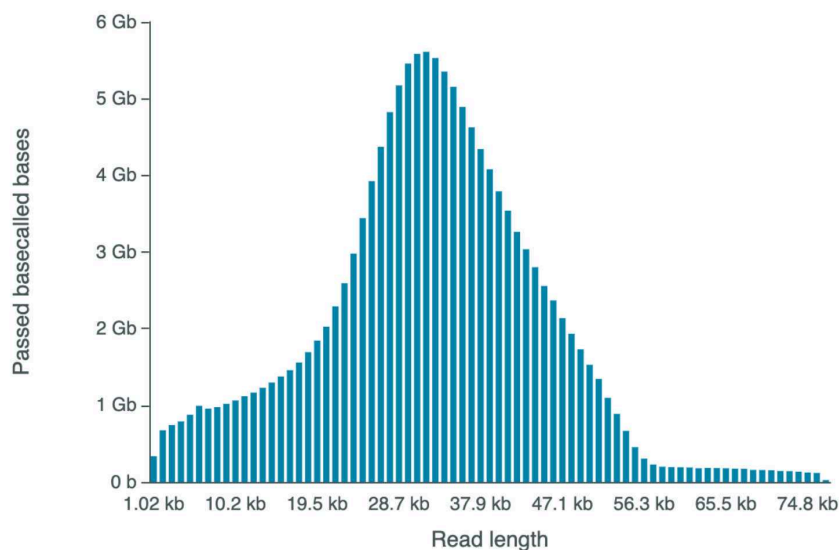
8

1. Following 72 hours of sequencing the sample should yield an N50  $\geq$  30kb with a data output  $\sim$  100-160GB.

9

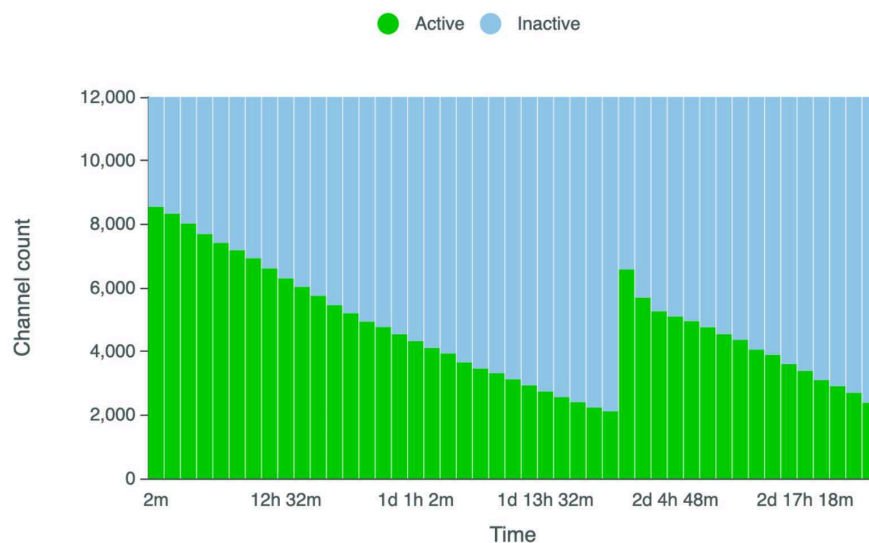
### **Read Length Histogram Basecalled Bases**

Estimated N50: 31.85 kb



**Figure 2. Expected Read Length Histogram:**

### Mux Scan Grouped



**Figure 3. Expected Muxscan (from 2 loads):**