

Jun 03, 2024

Processing frozen archival human DNA samples for large-scale SQK-LSK114 Oxford Nanopore long-read DNA sequencing SOP v1

 Forked from [Processing frozen human blood samples for population-scale SQK-LSK114 Oxford Nanopore long-read DNA sequencing SOP](#)



DOI

<https://dx.doi.org/10.17504/protocols.io.5jyl82morl2w/v1>

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Protocol Citation: Alicia Wenghöfer, Kimberly Paquette, Laksh Malik, Breeana Baker, Cedric Kouam, Kimberley J Billingsley, on behalf of the CARD Long-read Team 2024. Processing frozen archival human DNA samples for large-scale SQK-LSK114 Oxford Nanopore long-read DNA sequencing SOP v1. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.5jyl82morl2w/v1>

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Protocol status: In development

We are still developing and optimizing this protocol

Created: April 17, 2024

Last Modified: June 03, 2024

Protocol Integer ID: 98352

Keywords: Long-read sequencing, Oxford Nanopore sequencing, High molecular weight DNA extraction, DNA extraction, Human blood extraction, Whole blood extraction, DNA size selection, DNA shearing, frozen archival human dna sample, sequencing frozen human dna sample, frozen human dna sample, archival human dna sample, human dna sample, drop in dna quality, scale nanopore data, read dna, oxford nanopore long, sequencing data, dna quality, lsk114 oxford nanopore, read sequencing, dna length, genetic architecture of parkinson, terms of dna length, sequencing, processing frozen archival, parkinson, sequencing sop v1 abstract

Disclaimer

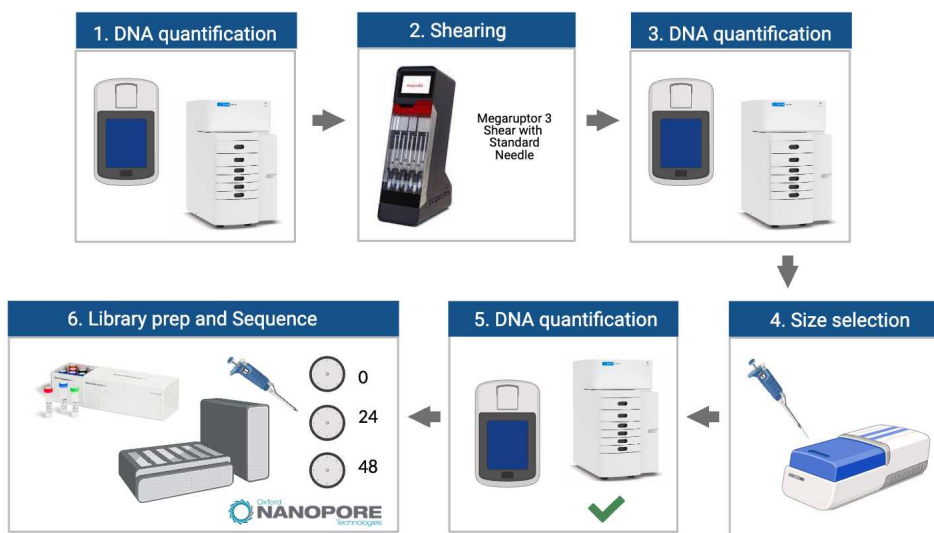
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Abstract

Abstract:

As part of the GP2 monogenic network we will generate long-read sequencing data 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 DNA samples, ideally targeting an N50 of ~30kb and ~30X coverage. However, with archival human DNA samples, we usually see a drop in DNA quality in terms of DNA length. Therefore, this protocol is focused on attempting to achieve the highest N50 and coverage possible from the input available.



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Figure 1. Overview of DNA processing steps.

Materials

	A	B
	Description	Supplier
	1.5mL DNA LoBind Tubes	Eppendorf
	Genomic DNA ScreenTape	Agilent
	5.0mL DNA LoBind Tubes	Eppendorf
	Megaruptor 3 Shearing Kit	Diagenode
	0.75% High Pass Plus Cassettes kit	Sage Science
	0.2mL Thin-Walled PCR Tubes	Thermo Fisher
	PromethION Flow Cells (FLO-PRO114M)	Oxford Nanopore Technologies

Table 1. Consumables

	A	B
	Description	Supplier
	1x dsDNA BR Working Solution	Thermo Fisher
	Genomic DNA 165 kb Kit	Agilent
	Genomic DNA Reagents	Agilent

	A	B
	TE Buffer	Thermo Fisher
	Ultra Pure Water	KD Medical
	Absolute Ethanol	Thermo Fisher
	Ligation Sequencing Kit (SQK-LSK114)	Oxford Nanopore Technologies
	AMPure XP Beads	Agencourt
	NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing	New England BioLabs
	Flow Cell Wash Kit (EXP-WSH004)	Oxford Nanopore Technologies

Table 2. Reagents

	A	B
	Description	Supplier
	Qubit Flex Fluorometer	Thermo Fisher
	Femto Pulse System	Agilent
	TapeStation 4200	Agilent
	Centrifuge 5425 R	Eppendorf
	Megaruptor 3	Diagenode
	Blue Pippin Size	Sage Science

	A	B
	Selection System	
	C1000 Touch Thermal Cycler with 96-Well Fast Reaction Module	Bio-Rad
	MyFuge Mini Centrifuge	Benchmark Scientific
	Vortex-Genie 2T	Scientific Industries
	Rocking Laboratory Shaker	Reliable Scientific
	DynaMag-2 Magnet	Thermo Fisher
	ThermoMixer C with 1.5mL SmartBlock	Eppendorf
	PromethION 24 or 48	Oxford Nanopore Technologies

Table 3. Equipment



1 Part 1: Thaw DNA samples On ice (~ 00:05:00)

5m

Note: Ideally for best results, DNA needs to be extracted with high-molecular weight methods straight from tissue. See dx.doi.org/10.17504/protocols.io.5qpvo3639v4o/v1 or dx.doi.org/10.17504/protocols.io.6qpvr347bvmk/v1 or dx.doi.org/10.17504/protocols.io.kxygx3zzog8j/v1 based on tissue type. However, if only archival DNA samples are available, continue with this protocol.

- 1.1 Remove the old DNA samples stored at  -80 °C and thaw them  On ice .

2 Part 2: DNA Quantification (measuring DNA concentration and size) (~ 00:30:00 for 16 samples)

4h 45m

Note: This step is to determine if shearing and/or size selection are needed. This is based off fragment length distribution and DNA mass available per sample.

Note: This duration does not include the time it takes to size the samples. The total time it takes to size 16 samples on the TapeStation or the Femto Pulse is ~  00:45:00 or ~  03:30:00 , respectively.

- 2.1 Pipette mix samples 10x.

- 2.2 Quantify by taking a single measurement on the **Qubit Flex Fluorometer**. Use  1 µL or  2 µL of the sample out of the middle of the pipette-mixed aliquot.

- 2.3 Quantify the purity of the samples (absorbance ratios (260/280 and 260/230)) by taking a single measurement on the **NanoDrop**. Use  1 µL of the sample out of the middle of the pipette-mixed aliquot.



- 2.4 Size on the Agilent TapeStation 4200 or **Agilent Femto Pulse System**. The expected size range for samples post-extraction is  >40 kb .

Note for the use of Agilent Femto Pulse System: use DNA sample dilutions between  0.005-0.5 ng/ul in 1x TE or EB-Buffer.

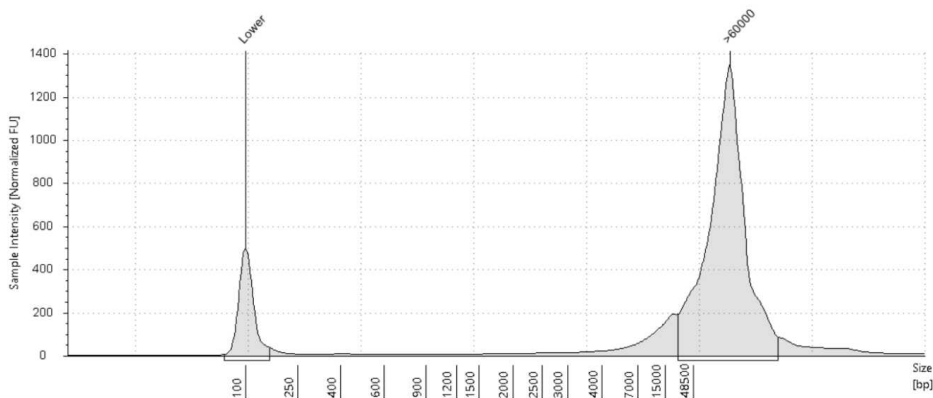


Figure 2. Example TapeStation trace pre-size selection.

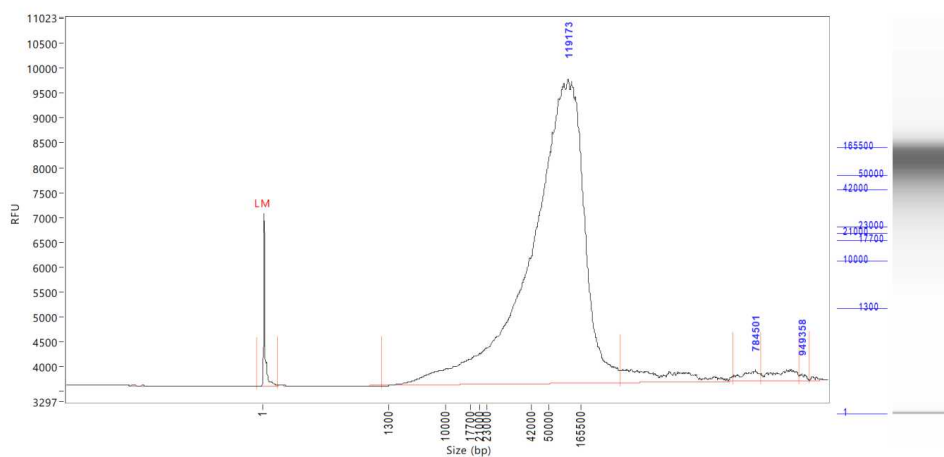


Figure 3. Example Femto Pulse trace pre-size selection.

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

2.5 Once all the samples are properly quantified, move on to size selection.

3 Part 3: Shearing (~ 03:00:00 for 8 samples)

3h

3.1 We will be using the Diagenode Megaruptor 3 Shearing Kit (E07010003).

In a Megaruptor 3 shearing tube, make up the size selected sample to  100-150 μ L and  100-150 ng/ μ L with TE Buffer or water. Do not go over maximum volume of  180 μ L and maximum concentration of  150 ng/ μ L .



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 and volume. If shearing more than one sample try to get all the concentrations to be as close as possible to this target range and/or to each other. Use the Megaruptor 3 for the different samples in the same range of concentration and volume:

- range of the concentration: +/- 10 ng/ul
- range of volume: +/- 15 µL

Note: We recommend taking in a larger concentration into shearing since you will lose some sample when removing the syringe post-shear. If the sample concentration is higher than 70 ng/ul diluting it with TE buffer or water to 150 µL would make it less than 70 ng/ul, it is suggested to dilute to 100 µL rather than 150 µL. If the sample concentration does not reach 70 ng/ul, it is suggested to take all sample into shearing and do not dilute with TE buffer or water. Make sure Megaruptor 3 shearing settings reflect your exact sample volume.

Note: If the sample size is >30 kb shear it 1x. If the sample size >100 kb shear it 2x. If the sample size is <30 kb the shearing step can be skipped. If DNA is viscous and high molecular weight (>100 kb), use fluid + needles (gray). Otherwise the standard needles (black) can be used.

- 3.2 Attach the Megaruptor 3 shearing syringe onto the tube and push the entire item into the MR3 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.

Note: Samples should always be balanced. If running an odd number of samples, samples can be balanced with an empty corresponding tube and syringe.

- 3.3 Shear once or twice at speed 20 (takes ~ 03:00:00 if the MR3 is set to 150 µL volume and sheared twice; takes 01:00:00 if the MR3 is set to 100 µL volume and sheared once).

Note: There is no setting for Megaruptor to shear twice. It requires the cycle to be manually restarted for shearing a second time.

4h



- 3.4 Once finished, remove the sample from the Megaruptor 3 and carefully remove the syringe from the tube. Make sure the plunger is fully depressed in order to avoid losing sample. Use a P20 pipette to aspirate any leftover sample on the syringe.
- 3.5 Avoid any vortexing of the DNA from this point on to avoid any unnecessary further shearing. Instead mix by gently flicking the tube and spin down.

4 Part 4: Post-Shear DNA Quantification (~ 00:10:00 for 8 samples)

10m

- 4.1 Quantify the samples on the **Qubit** and size using the Agilent TapeStation 4200 or the **Agilent Femto Pulse System**. Although the targeted size is  30 kb , the observed size range for samples post-shear is between  30-70 kb . The samples usually still sequence around  30 kb .

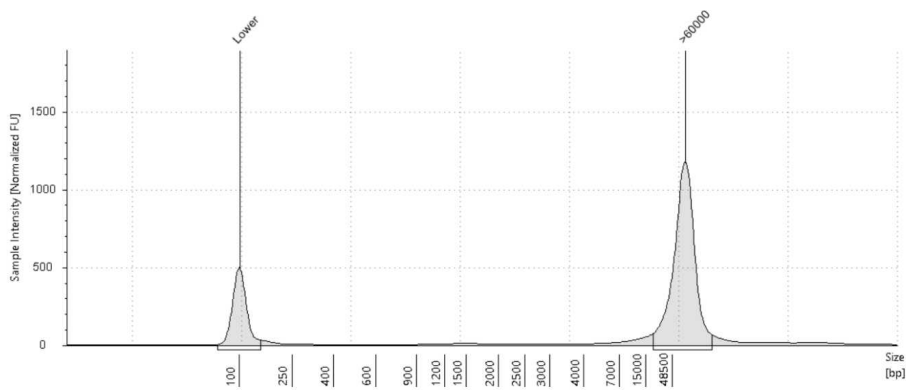


Figure 4. Example TapeStation trace post-size selection and shearing.

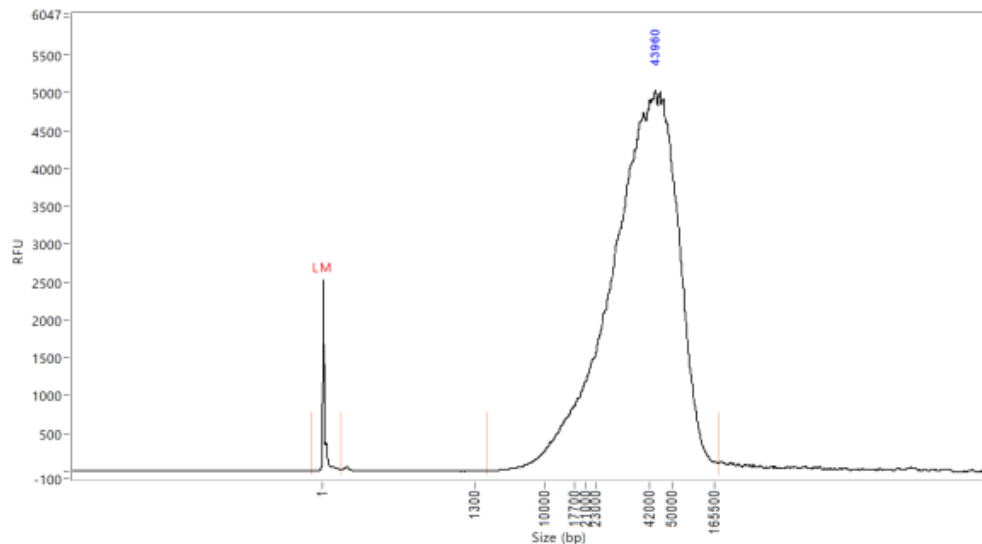


Figure 5. Example Femto Pulse trace post-size selection and shearing.

5 Part 5: Size selecting using Blue Pippin (~ 04:00:00 for 4 samples)

4h

- 5.1 Using TE, dilute up to  10 μg of sheared DNA sample into a final volume of  60 μL .

Note: The standard protocol to start with is 60/20 ( 60 μL +  20 μL standard loading solution). The sample dilution can also be made up to  30 μL for the 30/10 standard loading protocol or  70 μL for the 70/10 (use 2x loading solution) loading protocol. See **Table 4** for more information.

- 5.2 In the Blue Pippin software, click the "Protocol Editor" Tab and click the folder icon to add a new protocol.
- 5.3 Click "Casette Type" folder icon and select the greyed out option 0.75 Agarose Dye-free. Then choose "10 Kb High Pass Plus Marker U1" or "15 Kb High Pass Plus Marker U1" based on the desired protocol.
- 5.4 Enter the Sample ID into the "Sample ID" fields. Make sure that the check box for "End Run when Elution is Completed" is selected. Enter Marker for the Marker Well.

Note: Lane 4 is the preferred lane for the Marker.

- 5.5 In the "Range" column, make sure that all of the rectangle blocks are selected (light grey color) except of the Marker well (dark grey).

Note: The Marker lane requires a manual deselection of the row (changes color of row to dark grey).

- 5.6 In the Reference Lane field, choose the Marker well number (lane 4) and press the "APPLY REFERENCE TO ALL LANES" button.
- 5.7 Enter the number "10000" (10 kb = 10000) or "15000" (15 kb = 15000) or in the BP "Start" field. The BP "End" and "Target" values are cassettes will still collect anything past the start field.
- 5.8 Click "Save As" to name and save the run.
- 5.9 In the software, click the "Main" Tab.
- 5.10 Place the calibration fixture onto the optical nest. The dark side of the fixture must be down, and completely cover all the LED detectors.
- 5.11 Close the lid and press "Calibrate". The calibration status will read "Calibration Underway" and the LED photocurrents will automatically adjust to a factory setting. Once calibration is successful the "Calibration Status" field will contain the message "Calibration OK". In the resulting sub-window, check that the calibration target box ("Target I ph, mA") at the top of the window reads "0.60". If not, manually change the target value to "0.60". Press "EXIT".
- 5.12 Remove the cassette from the packaging. Inspect the buffer chambers to make sure there are no chambers that are too low. If so, make a mental note of it and add more buffer when you load the cassette in step 5.16.

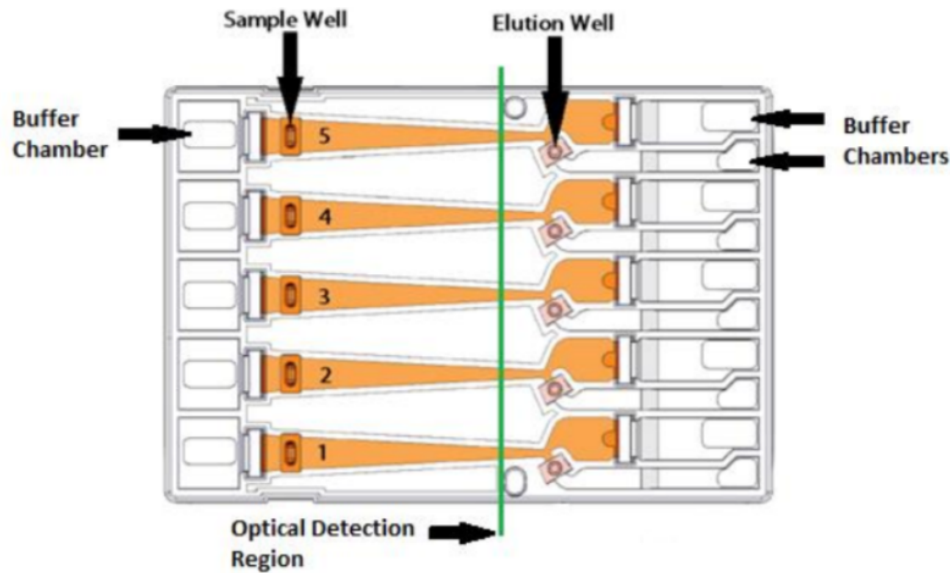


Figure 6. Picture of cassette with the orientation from left to right (Buffer chamber, Sample Well, Elution Well, Buffer Chamber). The region of optical detection is marked.

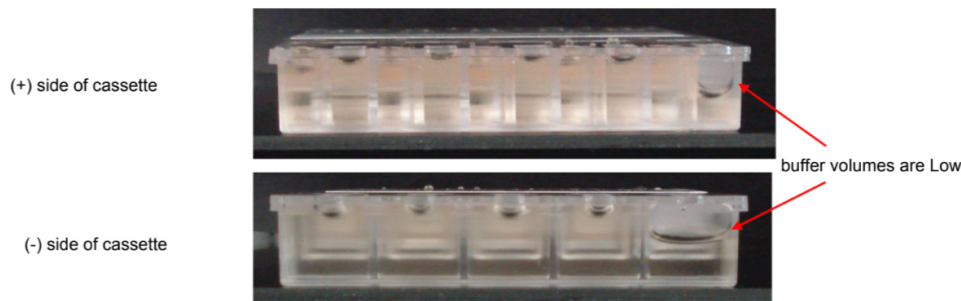


Figure 10.1. Low buffer levels in cassette buffer chambers.

Figure 7. Low buffer levels in cassette buffer chambers.

- 5.13 Place the cassette under a light source and inspect for bubbles due to delimitation of agarose from the bottom of the cassette in the region used for optical detection of DNA. If a bubble is detected, do not use the affected lane to run the DNA reference marker. Affected lanes can be used for size selection of sample DNA.

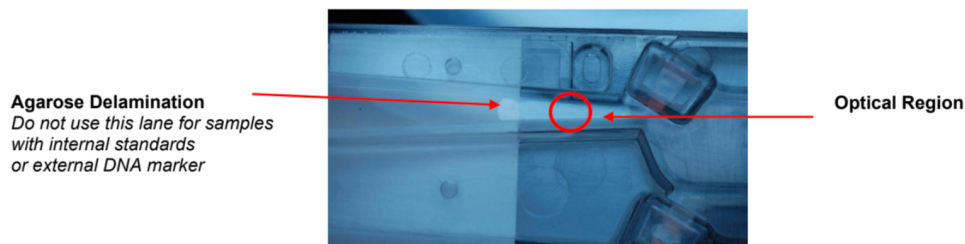


Figure 8. Bubble in optical path. Do not use the lane for the DNA reference marker.

- 5.14 If there are any trapped air bubbles behind the elution wells, tilt the cassette, sample well side down, and gently tap the sides to release the bubbles to the top in direction to the Buffer chambers.
- 5.15 Place the cassette into the optical nest, but keep the cassette slightly tilted down so that the air bubbles do not return to the area behind the elution modules.

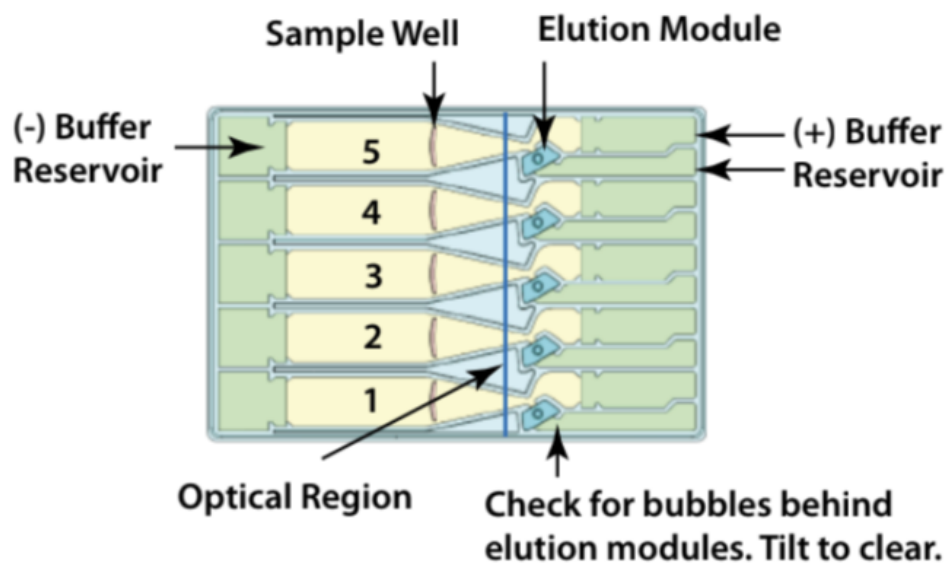


Figure 9. Cassette orientation in the optical nest.

- 5.16 Remove  450 μL of buffer from the large buffer reservoirs to reduce the amount of leakage after the run; however, if any of the left or right buffer reservoirs appear to be extremely low from step 5.12, do not remove buffer. Instead, add extra buffer to match to the level of other ones.



- 5.17 Remove both buffer chamber adhesive strips by using the white tabs and pulling the strips slowly towards the front of the instrument until they are removed. To prevent any issues, such as contamination, it may be a good idea to leave the adhesive strips on the sample wells until you are ready to use them. Wipe excess liquid from around the elution modules (not in the well).
- 5.18 Completely remove all buffer from each elution module (around  90 µL). Move the pipette tip slowly as you aspirate liquid from the corners of the elution modules to avoid piercing them. You can use the same pipette tip to remove the buffer from each elution module but remember to go back with a new pipette tip to remove any leftovers and reintroduced bubbles. You can also change tips between wells.
- 5.19 At this time, remove the loading solution from the  4 °C to allow it to reach  Room temperature .
- 5.20 Add  80 µL of the provided electrophoresis buffer to each well. Change tips between wells. To avoid trapping more air bubbles, it is good to go slowly raise the pipette tip as the liquid is being dispensed.
- 5.21 Seal the elution modules with the provided adhesive tape strip and seal. Use a edge to tightly seal and remove any wrinkles.
- 5.22 Close the lid and person Continuity Test by clicking the "Test" button in the "Main" tab. The test sub-window will open and the current in each channel will automatically be measured. If the test returns a "PASS" message, click "RETURN" to exit. If there is a "FAILED" message, make sure there is buffer in every chamber and elution module.
- 5.23 Remove adhesive strips of sample wells by using the white tabs and pulling the strips slowly towards the front of the instrument until they are removed.
- 5.24 Remove  40 µL of buffer from the marker well. Remove  80 µL (if sample volume is =  60 µL +  20 µL LS) from the sample wells (see **Table 4** for additional protocols). Take care not to pierce the agarose with the pipette tip. When removing buffer, it may be good to immerse the tip just below the buffer surface and follow the liquid down with the tip as the buffer is being removed.
- 5.25 At this time, remove the DNA Marker from the  4 °C . Do not leave out for any longer than  00:05:00 . If you will not be able to use it by then, remove the DNA marker

5m

from the  4 °C at a later step.

5.26 Add  40 µL of marker to the marker well and return the marker to the  4 °C .

Add loading solution (LS) to the sample tube based on the sample volume, pipette mix, and pulse-centrifuge:

	Sample Volume	Type of LS	Vol of LS to add	Vol to remove from sample well
	30 uL	Standard	10 uL	40 uL
	60 uL	Standard	20 uL	80 uL
	70 uL	2x	10 uL	80 uL

Table 4. Sample volume and loading solution (LS) volume.

Note: For the 70/10uL sample to LS mix, the 2x LS is needed.

- 5.27 When adding samples to the respective wells, place the tip of the pipette just below the surface of the buffer and slowly dispense. Do not be concerned if the sample well slightly overfills. The density of the sample will allow it to sink during the run. Sample wells should be completely filled to the top with buffer. If any wells are under-filled, top them off with additional electrophoresis buffer.
- 5.28 Close the lid and press "Start" on the "Main" tab. A checklist screen will appear reminding the user to do the LED calibration, which should have been done earlier. Press "Start".
- 5.29 For best recovery, wait at least  00:45:00 (better overnight) after the end of the run before removing samples from the elution modules. Slowly, remove samples. The volumes should be close to  80 µL 45m
- 5.30 Additional 10-30% yield can often be achieved by adding  80 µL of the provided Tween solution to each empty elution module. Wait  00:01:00 , then remove the solution and place in separate tubes. 1m
- 5.31 **Preventative Maintenance** - Electrodes should be rinsed after 5 runs or once per week. Fill the provided rinse cassette with deionized water. Load the rinse cassette onto the

sample tray and close the lid. Allow the electrodes to rinse for several seconds. Open the lid and remove the cassette.

6 Part 6: Post-BluePippin DNA Quantification (~ 00:10:00 for 8 samples)

10m

- 6.1 Quantify the samples on the Qubit and NanoDrop and size using the Agilent TapeStation 4200 or the Agilent Femto Pulse System. Although the targeted size is 30 kb , the observed size range for samples post-shear is between 30-70 kb . The samples usually still sequence around 30 kb .

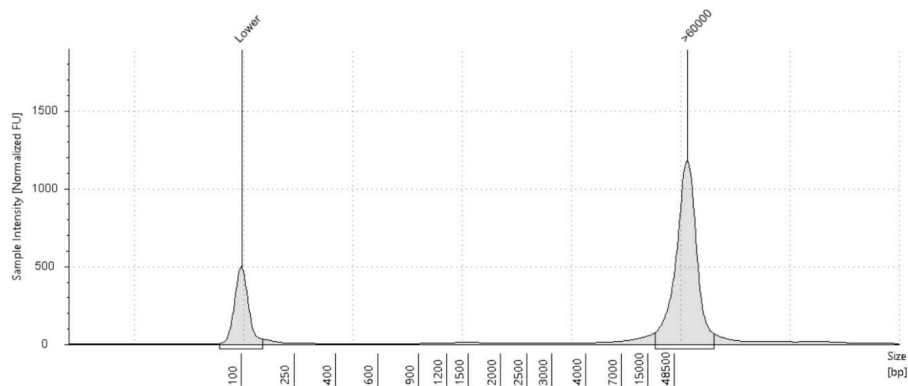


Figure 10. Example TapeStation trace post-size selection and shearing.

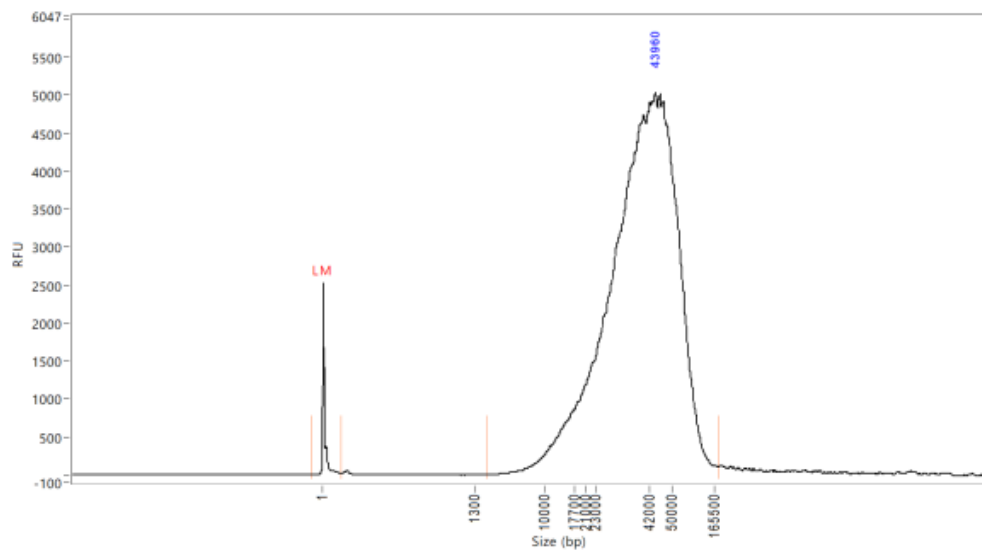


Figure 11. Example Femto Pulse trace post-size selection and shearing.

- 6.2 At this point, ideally 2.5 μ g of DNA is needed to move on to library prep.



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

7 Part 6: Manual SQK-LSK114 Library Prep and Sequencing (06:00:00 , including reloads)

6h 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 SQK-LSK114 Library preparation SOP: <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?devices=promethion

Minor tweaks:

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

7.1 A. DNA Repair and End-Prep

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 0.2mL thin-walled PCR tube:

-  48 µL DNA (input  2.5 µg , 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))

Note: The DNA mass should be at least  1.15 µg . However, this protocol is optimized for  2.5 µg of starting input. Do not exceed  168 µL of sample +  12 µL of the buffers/enzymes added (max. total volume of  180 µL).

-  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: In our experience, AMPure XP beads and Short Fragment Buffer perform best when thawed and used at  Room temperature rather than  On ice .

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  00:30:00 and then  65 °C for  00:05:00 and then  4 °C for infinity.

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

5. Resuspend the AMPure XP beads by vortexing.

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

7. Add  60 μL (or equivalent volume to the previous sample-end-prep-mix, 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.

8. Incubate on a rocking laboratory shaker or hula mixer for  00:05:00 at  Room temperature .

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

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

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

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



12. With the samples remaining on the magnet, wash the beads with  200 μL of 80% EtOH, pipetting on the opposite wall making sure not to disturb the pellet. Once EtOH has been added to all samples, immediately remove and discard the EtOH.

Note: The goal here is to make sure the beads are fully covered. If initial volume of beads was significantly higher than  60 μL , more EtOH may be required.

13. Repeat step 12.

14. Spin down and place the tube back on the magnet, pipetting off any residual EtOH.

15. Allow to dry for ~  00:00:30 but do not overdry to the point of cracking.

16. Remove the tube from the magnetic rack and resuspend the pellet in  62 μL nuclease-free water. Incubate on a rocking laboratory shaker or hula mixer for  00:03:00 at  Room temperature. If not quantifying the sample post-DNA repair and end-prep, user can resuspend the pellet in  60 μL water.

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

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

19. Optional: Quantify  1 μL of sample on the Qubit.

Note: It is possible to store samples at  4 $^{\circ}\text{C}$  Overnight at this step if necessary.

7.2 B. Adapter Ligation and Clean-Up

1h 15m
30s

1. Spin down the Ligation Adapter (LA) and NEBNext Quick T4 DNA Ligase, then return to  On 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 at  -20 $^{\circ}\text{C}$, it is possible to leave them in the



freezer until needed.

2. Thaw Ligation Buffer (LNB) at Room temperature , 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 Room temperature , mix by vortexing, spin down, and place On ice .

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

- 60 μ L DNA sample
- 25 μ L LNB
- 10 μ L Quick T4
- 5 μ L LA

6. Mix by gently pipetting and spin down.

7. Incubate the reaction for 00:30:00 at Room temperature .

8. During this time, put flow cells out at Room temperature .

9. Resuspend AMPure beads by vortexing.

10. Add 45 μ L of resuspended beads to the reaction and mix by flicking.

11. Incubate on a rocking laboratory shaker or hula mixer for 00:05:00 at Room temperature .

12. Spin down sample and pellet on magnet.

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

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

14. Wash the beads with 250 μ L SFB, remove from magnet and flick to resuspend, 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 supernatant.

17. Allow to dry for ~  00:00:30 , but do not overdry 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 allow them to reach  Room temperature before loading onto the PromethION to avoid condensation formation.

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

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

21. Remove and retain  26 μL of eluate into a clean 1.5mL Eppendorf DNA LoBind tube, being careful not to aspirate any beads (this is the DNA library).

22. Quantify  1 μL of sample on the Qubit.

23. Re-prep library from **Part 6** if  [M] <20 fmol . Calculate the mass (ng) of DNA need to achieve this based on averaging the size of DNA from the **Femto Pulse** traces using <https://nebiocalculator.neb.com/#!/dsdnaamt>. This should allow a coverage of 30x over three loads. If your sample differs from this size, recalculate based on 10-20 fmol per load.

Note: For example,  740 ng would be enough for three loads of  370 ng each, which is calculated for 20 fmol of DNA averaging at 30 kb. If necessary, user can load a higher fmol on the first load and lower fmol on the next two loads.

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



7.3 C. Priming and Loading R10 Flow Cell

2h 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 Room temperature , 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.

Note: You can make a Master-Priming mix as well: add 25 μL of thawed and mixed FCT to 975 μL of thawed and mixed FCT and vortex. Multiply by the number of samples and adjust for a little more volume than needed.

3. Rotate valve to reveal 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 20 fmol using EB.

7. Prepare the library mix for loading:

- 100 μL SB
- 68 μL LIS or LIB
- 32 μL DNA library (20 fmol)

8. Repeat steps 3 and 4.

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 directly after the 2nd load of priming mix.

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

12. Wait at least  00:10:00 (better up to  01:00:00) then initiate sequencing (see **step 7.4**).

13. Ideally, the library quant's yielded allow for 3  20 fmol 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).

Note: See step 7.5 Recovery of Library if you don't have 3 times  20 fmol loads in total.

14. To wash and reload a flow cell, begin by thawing Wash Mix (WMX)  On ice and Wash Diluent (DIL) at  Room temperature

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

15. In a new tube, add  2 μL WMX to  398 μL DIL and pipette mix. This is your flow cell wash mix.

16. Pause the PromethION runs and export .pdf run reports.

17. Rotate the inlet port 1 cover to reveal inlet port 1.

18. 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).

19. Load  400 μL flow cell wash mix into inlet port 1, avoiding any introduction of air.

20. Wait  01:00:00

21. With inlet port 1 closed, remove waste from port 2 or 3 ( 920-950 μL are expected)



22. Repeat priming steps and reload samples (steps 1 - 13).

7.4 D. Starting a Run on PromethION

10m

1. Once library is loaded and the time has passed in step 12, navigate to 'Start sequencing'

Note: be sure that there is at least 2TB of data storage capacity per sample which can be found under 'Host settings'

2. Select proper flow cell position and enter experiment name and sample ID name

Note: Type of flow cell should be automatically detected. Do not change this setting.

3. Select kit "Ligation Sequencing Kit SQK-LSK114"

4. Chose Run limit: "75 hours duration" (72 hours of running time plus additional time for paused wash and reloading steps) with a minimum read length of "200 bp" and Adaptive Sampling "off"

5. Chose basecalling options: Basecalling off is standard.

Note: We typically basecall after the run with Dorado (the advantage being with storage capacity and accuracy of the data). If selecting basecalling during the run, be sure to check if PromethION can handle the capacity (i.e. Fast basecalling can keep up with the speed of data acquisition on most Nanopore platforms. High Accuracy basecalling cannot.)

6. Chose data output files: ".POD5" for raw reads

7. After final review, start the sequencing process

Note: Check after  00:10:00 if the sequencing run looks good and there are not any air bubbles etc. If there is an issue with the flow cell, recover the library immediately with **Step E**.

7.5 E. Recovery of Library (optional)

*

1. If there is not enough library for 3 complete 20 fmol loads, recovery of the previous library is possible. **Before** you doing the steps in **7.3.13**: rotate the inlet port 1 and remove  220 µL volume (last library load; a little bit yellow) and put that immediately on ice.

2. Add  20 µL of SB and pipette mix.



3. Add any additional amount or rest of your library.
4. Continue with steps **7.3.13** and mix instead of your library at step 7.3.10 with the total volume ( >200 μL).

Note: You can not overload the flow cell if it will be more than  20 fmol in the end.

7.6 F. Removing Flow Cells

1. After runs are complete, if basecalling was selected, make sure the basecalling shows 100% before removing flow cells.
2. Remove flow cells from PromethION and place aside to send back to Nanopore for recycling.

Sequencing results:

3d

- 8 Following  72:00:00 of sequencing the sample should yield an N50 ~  30 kb with a data output ~  80 Gb

3d

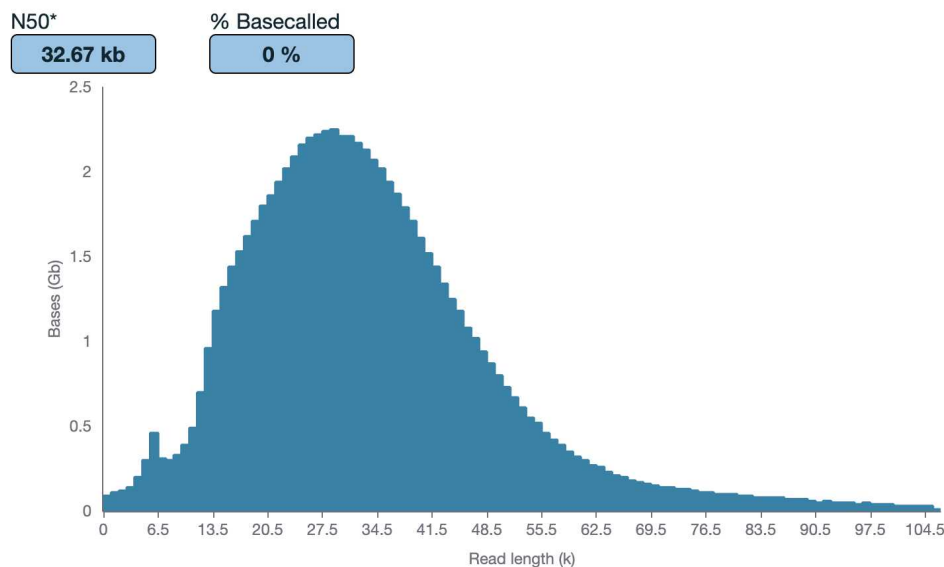


Figure 12. Expected Read Length Histogram

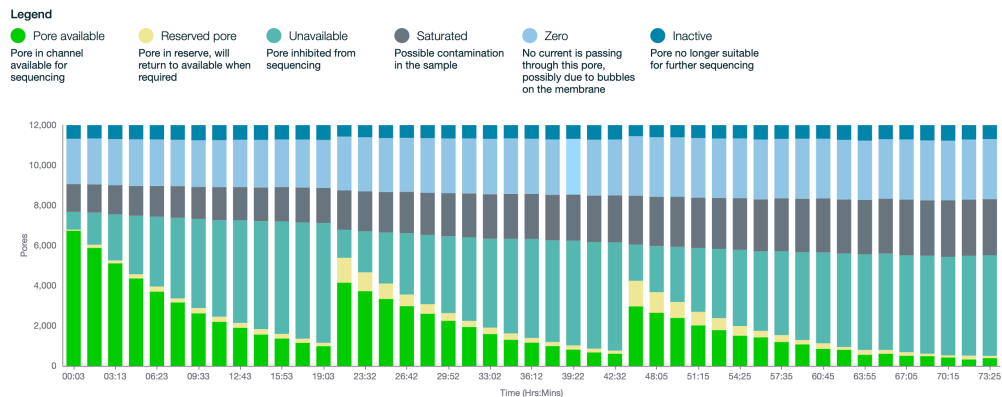


Figure 13. Expected Pore Scan (3 loads)

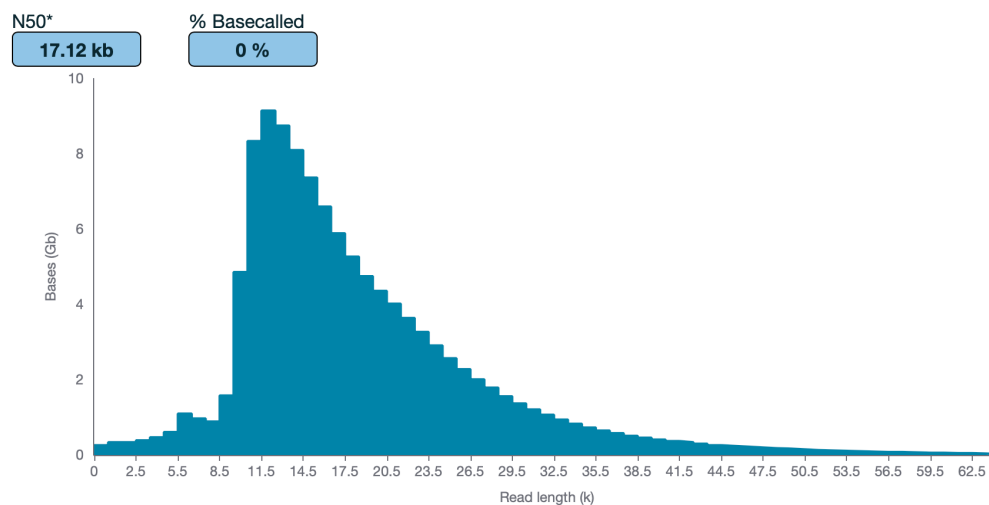


Figure 14. Not optimal Expected Read Length Histogram