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Modified Oxford Nanopore ligation-based whole-genome sequencing from human blood: simplified fragmentation and enhanced N50 and yield

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High molecular weight DNA extraction from all kingdoms
Tech. support email: See@each.protocol



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Protocol status: Working

We routinely apply this protocol in both diagnostic and research settings, with particular success in sequencing human genomes as part of the European "Genome of Europe" initiative. In our experience, the method has proven to be highly reliable, delivering consistent and high-quality results across multiple runs and sample types.

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Abstract

Problem identification: Modern analytical approaches, particularly those involving *de novo* human genome assemblies, demand the longest possible DNA fragments to ensure optimal resolution and accuracy. However, a fundamental trade-off exists with nanopore sequencing: sequencing yield tends to decrease as the average length of DNA fragments in the library increases. This inverse relationship poses a significant technical bottleneck. Compounding the issue, there is a lack of comprehensive data on the optimal sample preparation and processing parameters that maximise both fragment length and sequencing yield per Oxford Nanopore flowcell. This gap in knowledge hampers efforts to standardise high-efficiency protocols for long-read sequencing applications.

Presented solution: We developed a sequencing protocol that consistently produces high-yield genomic DNA datasets with read length N50 values exceeding 30 kb, approximately twice the N50 typically reported in standard datasets. Additionally, the protocol achieves total yields surpassing 100 Gb per Oxford Nanopore flow cell through 3–4 library reloads.

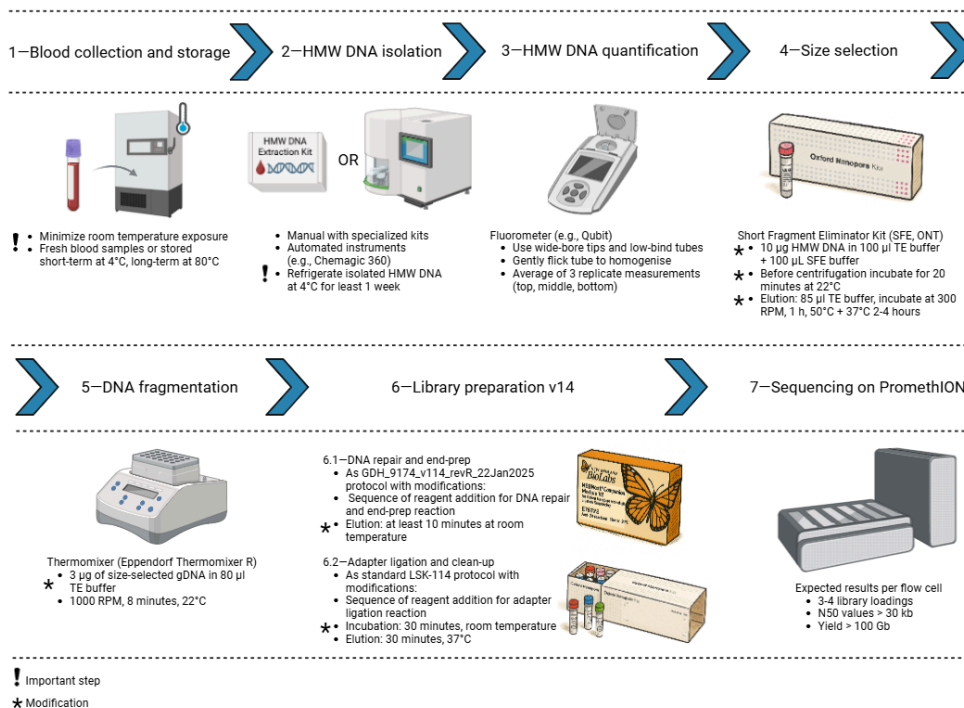


Figure 1: Visual overview of the protocol, emphasising important steps and modifications. Created with BioRender.



Materials

Kits

- Short Fragment Eliminator Kit (EXP-SFE001)
- Ligation Sequencing Kit V14 (SQK-LSK114)
- NEBNext Companion Module v2 for Oxford Nanopore Technologies Ligation Sequencing (NEB, E7672S or E7672L)
- Flow Cell Wash Kit (EXP-WSH004)

Consumables

- TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)
- Nuclease-free water
- Freshly prepared 80% ethanol in nuclease-free water
- Qubit 1x dsDNA HS Assay Kit (ThermoFisher Q33230) or equivalent QC check
- Qubit Assay Tubes (ThermoFisher Q32856)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- PromethION Flow Cells

Equipment

- PromethION 24 device
- Centrifuge
- Thermomixer (Eppendorf Thermomixer R)
- Vortex mixer
- Microcentrifuge (Eppendorf MiniSpin)
- Qubit fluorometer (or equivalent for QC check)
- Magnetic separation rack, suitable for 1.5 ml Eppendorf tubes
- Thermal cycler (Applied Biosystems SimpliAmp)
- Icebox, mini cooler or container with ice
- Timer
- P1000 pipette and tips
- P200 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- Wide-bore pipette tips

Safety warnings

 When pipetting HMW DNA always use wide-bore tips.



Before start

- 1.** Ensure your DNA is high-molecular weight (HMW); otherwise, the yield after the Short Fragment Eliminator (SFE) step will be low, as the SFE kit removes fragments up to 25 kb. Expected yield after SFE is 25–50% if HMW DNA was used, compared to 10% or less if fragmented.
- 2.** If HMW DNA was isolated recently, make sure it is completely resuspended, or stored at least a week at 4°C before use. To aid with resuspension, gently flick the tube or pipette mix a few times with a wide-bore tip. Do not vortex!
- 3.** HMW DNA is highly viscous and sticky, leading to thread formation during pipetting and resulting in imprecise concentration measurement. A commonly recommended method for UHMW DNA concentration measurement is taking 1 or 2 µl aliquots from the top, middle and bottom of the solution and using the average concentration value. If the measurements from three different points differ by more than 10%, prolong the rehydration time at 4°C.

1. Sample collection and HMW DNA isolation

1

Note

Initial sample collection, handling, and high-molecular weight (HMW) DNA isolation are crucial for successful Nanopore sequencing with N50 values >30 kb and yield per flow cell >100 Gb.

DNA fragmentation can occur during prolonged transport or storage at room temperature or higher. For HMW DNA extraction, the samples should be either fresh or stored short-term in cooled (4°C) or long-term in frozen conditions (-20°C to -80°C) without any freeze-thaw cycles.

The method used for DNA isolation also determines the molecular weight and integrity of the extracted DNA. Commercially available kits designed for HMW DNA extraction can be used, such as the Puregene or MagAttract HMW DNA Kit (Qiagen) and the Monarch HMW DNA Extraction Kit for Cell and Blood (New England Biolabs). Because HMW DNA is susceptible to mechanical shearing, extraction procedures should minimise vortexing, excessive pipetting, and rapid aspiration/dispensing. Alternatively, automated nucleic acid purification instruments, such as KingFisher Flex or Apex (ThermoFisher Scientific), Long String STAR V (Hamilton), QIA Symphony SP system (Qiagen), or Chemagic 360 (Revvity), can be used. Automated isolation helps minimise DNA fragmentation during isolation, increases sample throughput and enables standardisation.

Due to the high viscosity and tendency of HMW DNA to aggregate, sufficient time for rehydration and relaxation is required after elution. Ideally, HMW DNA should be extracted several days to a week before downstream analyses.

2 Human blood samples were collected in Vacutainer K2 EDTA Blood Collection Tubes and kept at -80°C until DNA extraction.

3 HMW DNA was extracted using the automated DNA purification instrument Chemagic 360-D (Revvity) and Chemagic BBS DNA Kit H24 (IVD-1074, Revvity), according to the manufacturer's protocol.

4 Isolated HMW DNA was stored at 4°C for at least a week before continuing on to library preparation.

5 Prior to quantification, HMW DNA should be fully resuspended by gently flicking the tube or pipetting up and down 5–10 times using a P1000 wide-bore tip.

Quantify your sample three times using the Qubit 1x dsDNA HS Assay Kit (or equivalent). Sample the DNA from three different points (e.g., top, middle, and





bottom). If the measurements are inconsistent, gently flick the tube a few times and repeat.

- 6 For short-term storage (up to several weeks), samples can be kept at 4°C, while long-term storage should be at -20°C to minimise degradation.

2. Size selection of gDNA using Short Fragment Eliminator Kit (SFE)

7

Note

The efficiency of adapter ligation in library prep and translocation through nanopores during sequencing is higher for smaller compared to longer DNA molecules, so size selection of gDNA improves data distribution (N50 value) by eliminating small DNA fragments.

Size selection can be done using various methods, from simple kits such as Short Fragment Eliminator (ONT), Short Read Eliminator kit (Circulomics), solid-phase reversible immobilisation (SPRI) beads, to automated size selection systems such as BluePippin (Sage Science).

In this protocol, the Short Fragment Eliminator Kit (SFE) by ONT was used. For this step, having HMW DNA is very important; otherwise, the yield after the SFE step will be very low (<1 µg), as the SFE buffer removes fragments up to 25 kb. If the input mass is >3 µg, the recovery after SFE will be too low, so this step can be omitted.

HMW DNA is harder to precipitate than short DNA at low concentrations. If it doesn't aggregate properly during SFE addition, it won't bind or pellet efficiently, especially without incubation or gentle mixing.

In this section, the protocol is modified in the following steps:

- substituting nuclease-free water with TE buffer for initial dilution of the sample (see step 8),
- incubation before centrifuging (see step 11)
- smaller elution volume (see step 17)
- prolonged elution time (see step 17)

- 8 In a 1.5 ml Eppendorf DNA LoBind tube, prepare 10 µg DNA in 100 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Note

Throughout this protocol, always use wide-bore tips when pipetting HMW DNA.

- 9 The SFE buffer should be kept at room temperature. Due to the high viscosity of the SFE buffer, pipette mix it at least 10–15 times with a wide-bore tip before use.
- 10 Add an equal volume (100 μ l) of SFE buffer to the DNA sample. It is very important that the mixture is homogenous; mix by pipetting with a wide-bore tip slowly 5 times, followed by flicking the tube gently and checking the consistency towards the light to ensure the mixture is homogenous.
- 11 Incubate the tube for 20 minutes at room temperature. Meanwhile, set the centrifuge to 22°C.
- 12 Centrifuge the sample at 10,000 g for 30 minutes at 22°C (fixed angle rotor), ensuring the tube is orientated with the hinge facing outward. Meanwhile, prepare 450 μ l of 80% ethanol (EtOH) in nuclease-free water per sample. For one sample, mix 360 μ l of 100% EtOH with 90 μ l of nuclease-free water.

Note

A larger volume of 80% EtOH can be prepared in advance if the DNA repair and end-prep step is performed on the same day (see step 34). For example, for one sample, mix 760 μ l of 100% EtOH with 190 μ l of nuclease-free water.

- 13 To prevent disruption of the DNA pellet, slowly remove the supernatant with a narrow-bore pipette tip, leaving about 15 to 20 μ l.
- 14 Wash the pellet by slowly adding 200 μ l of 80 % EtOH to the opposite side of the tube and centrifuging at 10,000g, 3 minutes. Ensure the tubes are orientated with the hinge facing outwards as before. Meanwhile, set the thermomixer to 50°C.
- 15 After washing with ethanol, the pellet might become visible as a white speck on the hinge side of the tube, towards the bottom on the side of the tube. If it is visible, remove as much ethanol as possible without disturbing the pellet. If it is not visible, remove most of the ethanol, leaving 10–15 μ l.
- 16 Repeat the washing step. This time, remove as much ethanol as possible (especially if the pellet is visible) using a 10 μ l pipette tip and allow it to air dry for a minute. If the DNA pellet is excessively dried, it will not easily dissolve in the next step, leading to low yield after the SFE step.
- 17 Resuspend the pellet in 85 μ l of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Gently flick the tube to aid resuspension and place it on a thermomixer for 1 hour at 50°C and 300 RPM. Then lower the temperature to 37°C and turn off mixing; let the



tube incubate for another 2-4 hours.

18 Gently flick the tube before taking out 1 µl for quantification using the 1x Qubit dsDNA HS Assay Kit (or equivalent). If the DNA mass is under 1.5 µg, let the pellet resuspend overnight at room temperature.

19 Quantify your sample from three different points using the 1x Qubit 1x dsDNA HS Assay Kit (or equivalent) (see step 5). If the measurements are inconsistent, gently flick the tube a few times and then let the pellet resuspend overnight at 4°C. Resuspension at 4°C can be prolonged to a few days if needed; however, if Qubit readings are consistent, the yield is unlikely to improve.

Note

Expected yield after SFE is 25–50% if HMW DNA was used.

3. gDNA fragmentation with shaking

20



Note

Similar to size selection, controlled HMW DNA fragmentation can also improve sequencing outcomes. Light shearing breaks up the longest DNA molecules, which can then be more readily sequenced, leading to an increased lengths of sequenced reads. Additionally, it also reduces pore blocking, leading to higher yield per flow cell.

To achieve the optimal DNA fragmentation, different methods can be used, such as Megaruptor 3 (Diagenode), g-TUBE (Covaris), needle shearing, or borosilicate or soda lime glass beads. Alternatively, DNA can be fragmented with shaking on a thermomixer, which eliminates the use of specialised equipment or kits, as described below.

When using mechanical shaking to fragment DNA, several key parameters must be carefully optimized to achieve consistent and predictable results. These include shaking time and speed, as well as the concentration and volume of the DNA sample. Because different thermoshakers or thermomixers can vary significantly in their motion profiles and energy transfer efficiency, it's essential to fine-tune these variables specifically for the instrument being used. Additionally, the choice of tube type plays a critical role: round-bottom tubes can influence fluid dynamics differently than conical ones, and regular plastic tubes may introduce more surface interaction than low-bind variants. These factors can affect the distribution of shear forces within the sample, ultimately altering DNA fragmentation patterns and fragment length distribution. Systematic testing and empirical calibration are often necessary to achieve optimal fragmentation profiles.

To simplify the optimization process, we fixed the reaction volume at 80 μ L and the DNA input at 3 μ g, while systematically varying the shaking speed (RPM) and duration. Speed trials revealed that shaking at 1600 RPM led to over-fragmentation, yielding a relatively low N50 of approximately 6.0 kb. In contrast, 800 RPM produced minimal fragmentation, with an N50 of 33.7 kb but a notably lower sequencing yield. We next evaluated shaking duration at an intermediate speed of 1000 RPM. Fragmentation efficiency improved between 5 and 8 minutes of shaking, with N50 values consistently exceeding 30 kb and showing a gradual increase toward the upper end of this time range. This suggests that 5–8 minutes at 1000 RPM strikes a practical balance between maintaining long DNA fragments and achieving sufficient fragmentation for downstream applications and sequencing yield.

Based on our results, we concluded that shaking at 1000 RPM for 8 minutes provides an effective and reproducible method for shearing high molecular weight (HMW) DNA isolated using the Chemagic 360-D*. Under these conditions, we consistently achieved N50 values exceeding 30 kb, indicating sufficient fragmentation while preserving long DNA molecules. Furthermore, sequencing runs conducted with DNA prepared using this protocol routinely yielded over 100 Gb of data per flow cell, demonstrating its suitability for high-throughput long-read sequencing applications.

*We hypothesize that the key determinants for achieving reproducible results with this mechanical shearing method are the consistency of sample collection, handling, and the integrity of the isolated high molecular weight (HMW) DNA. Given that DNA quality can vary significantly between preparations, we recommend that shaking parameters—namely speed and duration—be carefully optimized based on the fragmentation state of the input DNA. If the starting material is already partially fragmented, it is advisable to begin with lower RPM settings and shorter shaking durations to avoid over-shearing. Tailoring these parameters to the specific characteristics of your DNA sample will help ensure consistent fragment size distribution and maximize sequencing performance.

- 21 Using a wide-bore tip, transfer 3 µg of size-selected gDNA into a clean 1.5 ml Eppendorf DNA LoBind tube. Adjust the volume to 80 µl using TE buffer. Spin down briefly (1-2 seconds).
- 22 Place the tube on a thermomixer (Eppendorf Thermomixer R) set to 1000 RPM for 8 minutes at 22°C or room temperature.
- 23 Continue with DNA repair and end-prep, or store the sample at 4°C overnight.

4. DNA repair and end-prep

24

Note

In this section, the protocol is modified in the following steps:

- the sequence of reagent addition (see step 28)
- prolonged elution time (see step 37)

- 25 For DNA repair and end-prep use the NEBNext Companion Module v2 for Oxford Nanopore Technologies Ligation Sequencing (E7672S/E7672L, New England Biolabs), which contains all the NEB reagents required for the updated SQK-LSK114 singleplex ligation sequencing library prep protocol.
- 26 Thaw all reagents on ice. Flick and/or invert the NEBNext FFPE DNA Repair Mix and Ultra II End-prep Enzyme Mix, then spin down and place on ice. Vortex the NEBNext FFPE DNA Repair Buffer v2 and place on ice.
- 27 Turn on the thermal cycler and set the program for incubation at 20°C for 5 minutes, then 65°C for 5 minutes, and hold at 4°C. You can add a 20°C infinity step before the incubation to ensure the thermal cycler reaches the required temperature before placing in the samples.
- 28 In a 0.2 ml thin-walled PCR tube prepare the reaction as per Table 1. Before adding DNA from the previous step, pipette mix the prepared solution. Then use a wide-bore tip to add DNA and with the same tip slowly pipette mix 5 times to ensure the reaction is well mixed and homogenous.

Table 1: DNA repair and end-prep reaction

Reagent	Volume
NEBNext FFPE DNA Repair Buffer v2	11.7 µl
NEBNext FFPE DNA Repair Mix	3.3 µl
Ultra II End-prep Enzyme Mix	5 µl
DNA from the previous step	80 µl
Total	100 µl

- 29 Shortly spin down the PCR tubes and place them in a thermal cycler. Incubate at 20°C for 5 minutes, then 65°C for 5 minutes and hold at 4°C.
- 30 Meanwhile, take the AMPure XP beads (AXP) from the freezer and bring to room temperature. Vortex the beads to resuspend before using. Prepare 500 µl of fresh 80% ethanol in nuclease-free water per sample. Keep the AXP beads thawed for use in the subsequent adapter ligation and clean-up step (see step 45).
- 31 Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube. Add 100 µl of the resuspended AXP Beads to the end-prep reaction and mix by gently flicking the tube.
- 32 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
- 33 Spin down the sample and pellet on a magnet for 10 minutes until the supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant very slowly to ensure long DNA molecules are not pulled off the beads.
- 34 Keep the tube on the magnet and wash the beads with 250 µl of freshly prepared 80% ethanol without disturbing them. Incubate for 30 seconds and remove the ethanol.
- 35 Repeat the washing step for a total of two washes.
- 36 To remove as much of the ethanol as possible, briefly spin down and place the tube back on the magnet. Pipette off any residual ethanol with a 10 µl tip. Allow to air dry for 30 seconds, inspecting the beads to ensure they do not dry to the point of cracking.
- 37 Remove the tube from the magnetic rack and add 61 µl of nuclease-free water. Gently flick the tube to resuspend the beads. Incubate for at least 10 minutes at room temperature.

- 38 Place the tube on the magnet and allow the beads to pellet until the eluate is clear and colourless, for at least 1 minute. Using a wide-bore tip, transfer 60 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube, making sure not to transfer any beads.

Note

If possible, quantify the leftover 1 µl using the Qubit 1x dsDNA HS Assay Kit (or equivalent). However, regardless of the measured concentration, use the entire volume in the adapter ligation step (see step 42).

- 39 Continue with the adapter ligation and clean-up step or store the sample at 4°C overnight.

5. Adapter ligation and clean-up

40

Note

In this section, the protocol is modified in the following steps:

- the sequence of reagent addition (see step 42)
- prolonged incubation time (see step 43)
- prolonged elution time (see step 49)

- 41 Thaw Ligation Buffer (LNB) at room temperature, mix well by pipetting, spin down and place on ice.

Take the Ligation Adapter (LA) and Salt-T4 DNA Ligase out of the freezer, mix by flicking or inverting the tubes, spin down and place on ice. Thaw the Elution Buffer (EB) and Long Fragment Buffer (LFB) at room temperature, mix by vortexing and place on ice.

- 42 In a 1.5 ml Eppendorf DNA LoBind tube prepare the reaction as per Table 2. Add LNB to DNA and gently flick to ensure the LNB is well mixed with the sample (check towards the light). Then add LA and Salt-T4 DNA Ligase and gently flick the tube to mix.

Table 2: Adapter ligation reaction

Reagent	Volume
DNA sample from the previous step	60 µl



Reagent	Volume
Ligation Buffer (LNB)	25 µl
Ligation Adapter (LA)	5 µl
Salt-T4 DNA Ligase	10 µl
Total	100 µl

- 43 Spin down the tube and incubate at room temperature for 30 minutes. Meanwhile, bring the AXP Beads to room temperature and resuspend by vortexing.
- 44 Add 40 µl of the resuspended AXP Beads to the sample and mix by flicking the tube.
- 45 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
- 46 Spin down the sample and pellet on a magnet until the supernatant is clear and colourless. Slowly pipette off the supernatant while the tube is on the magnet.
- 47 Remove the tube from the magnet and wash the beads by adding 250 µl LFB. Gently flick the tube to resuspend the beads, then spin down and place the tube back on the magnet. Allow the beads to pellet for at least 5 minutes, making sure the supernatant is clear. Slowly pipette off the supernatant and discard.
- 48 Repeat the washing step for a total of two washes. Spin down and place back on the magnet. Pipette off as much residual supernatant as possible. Air dry the beads on the magnet for ~30 seconds, but ensure they do not dry to the point of cracking.
- 49 Add 97 µl of EB to the beads and resuspend by gently flicking the tube. Spin down and incubate for 30 minutes at 37°C.

Note

Volume of the final library per sequencing reaction is 32 µl with a mass of 300 ng. If DNA mass after end-prep was low (>1000 ng), elute with a smaller volume of EB. For example, 65 µl for two or 33 µl for one sequencing load.

- 50 Gently flick the tube, spin down and place on the magnet until the supernatant is clear and colourless.

- 51 While keeping the tube on the magnet, transfer 96 μ l of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube. Use wide-bore tips and pipette very slowly to avoid transferring any beads.
- 52 Gently flick the tube and quantify 1 μ l using a Qubit fluorometer.

Note

The expected yield should be at least 1200 ng of DNA library in a volume of 96 μ l, allowing for four 300 ng loads into the flow cell per sample.

- 53 The finished library can now be used for loading into the flow cell or stored short-term (for reloading flow cells between washes) at 4°C. For long-term storage (more than 3 months), store the final library at -80°C.

6. Priming and loading the PromethION Flow Cell

54

Note

Perform all the steps according to Nanopore protocols and recommendations for priming and loading the PromethION flow cell.

- 55 Take the flow cell out of the fridge and wait at least 20 minutes to reach room temperature before inserting the flow cell into the PromethION.
- 56 Thaw the Sequencing Buffer (SB), Library Beads (LIB), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.
- 57 To prepare the flow cell priming mix, add 30 μ l of FCT to 1,170 μ l of FCF. Mix by vortexing at room temperature.
- 58 Remove any air bubbles from the flow cell in the following way. Set a P1000 pipette tip to 200 μ l. Insert the tip into the inlet port and turn the wheel until the dial shows 220-230 μ l or until you see a small volume of liquid entering the pipette tip.
- 59 Load 500 μ l of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait 5 minutes. During this time, prepare the library for loading.

- 60 The LIB tube contains a suspension of beads that settles very quickly. Before use, thoroughly mix the LIB.
- 61 In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the sequencing reaction as per Table 3.

Table 3: Sequencing reaction.

Reagent	Volume
Sequencing Buffer (SB)	100 µl
Library Beads (LIB) thoroughly mixed before use	68 µl
DNA library (300 ng)	32 µl
Total	200 µl

Store the library on ice or at 4°C until ready to load.

- 62 Complete the second flow cell priming by slowly loading 500 µl of the priming mix into the inlet port.
- 63 Mix the prepared library gently by flicking the tube just prior to loading.
- 64 Load 200 µl of library into the inlet port using a P1000 pipette. Pipette very slowly or by turning the wheel.
- 65 Close the valve to seal the inlet port. Make sure the light shield is placed on the flow cell.
- 66 Close the PromethION lid. Wait at least 20 minutes after loading the flow cells before initiating sequencing.
- 67 After 1 hour into the sequencing run, pay attention to the pore occupancy and pore scan results in MinKNOW. See section 8 for expected results for pore activity during sequencing.

7. Washing and reloading a PromethION Flow Cell

68

Note

Perform all the steps according to the Nanopore Flow Cell Wash Kit protocol and recommendations for washing and reloading a PromethION flow cell. Keep the light shield on the flow cell during washing and reloading.

69

Note

Sequencing performance can be monitored by tracking the percentage of active pores over time. When pore activity drops to around 30–25%, we typically pause the run and perform a flow cell wash to enhance pore recovery—particularly during the first two library loads, where recovery tends to be more effective. This drop in active pores usually occurs between 20 to 24 hours into the sequencing run, depending on library quality and loading efficiency. It's important to note that the efficiency of pore recovery diminishes with each successive load. As a result, sequencing duration tends to decrease with each reloading cycle. In practice, a single flow cell can be washed and reloaded 3 to 4 times, and occasionally up to 5 times, depending on the total amount of DNA library available. However, the fifth load typically yields a relatively low amount of data and often contributes minimally to the overall sequencing output.

70 Take the Wash Mix (WMX) tube from the freezer and place it on ice. Mix by flicking and inverting, then spin down and place on ice. Thaw Wash Diluent (DIL) at room temperature.

71 In a new 1.5 ml Eppendorf tube, prepare the Flow Cell Wash Mix by adding 398 μ l DIL and 2 μ l WMX. Mix by pipetting, and place on ice. Do not vortex.

72 Pause the sequencing experiment in MinKNOW.

73 Make sure the inlet port is closed, insert a P1000 pipette into a waste port and remove the waste buffer.

74 Open the inlet port and remove the air bubble as before by drawing back a small volume.

75 Perform the flow cell wash by slowly loading 200 μ l of the flow cell wash mix into the inlet port. Wait 5 minutes and carefully load the remaining 200 μ l. Close the inlet port and wait for 1 hour.



- 76 In the meantime, prepare the flow cell priming mix as before (step 57). After the wash incubation is over, remove the waste buffer and perform the first flow cell prime as before (steps 58-59).
- 77 In a new 1.5 ml Eppendorf DNA LoBind tube prepare the sequencing reaction as before (steps 60-61).
- 78 Perform the second flow cell prime and load the prepared library as before (steps 62-67).

8. Troubleshooting and expected results

- 79 PromethION sequencing run results of the same HMW DNA human blood sample before (Fig. 1) and after (Fig. 2) gDNA fragmentation by shaking.



Note

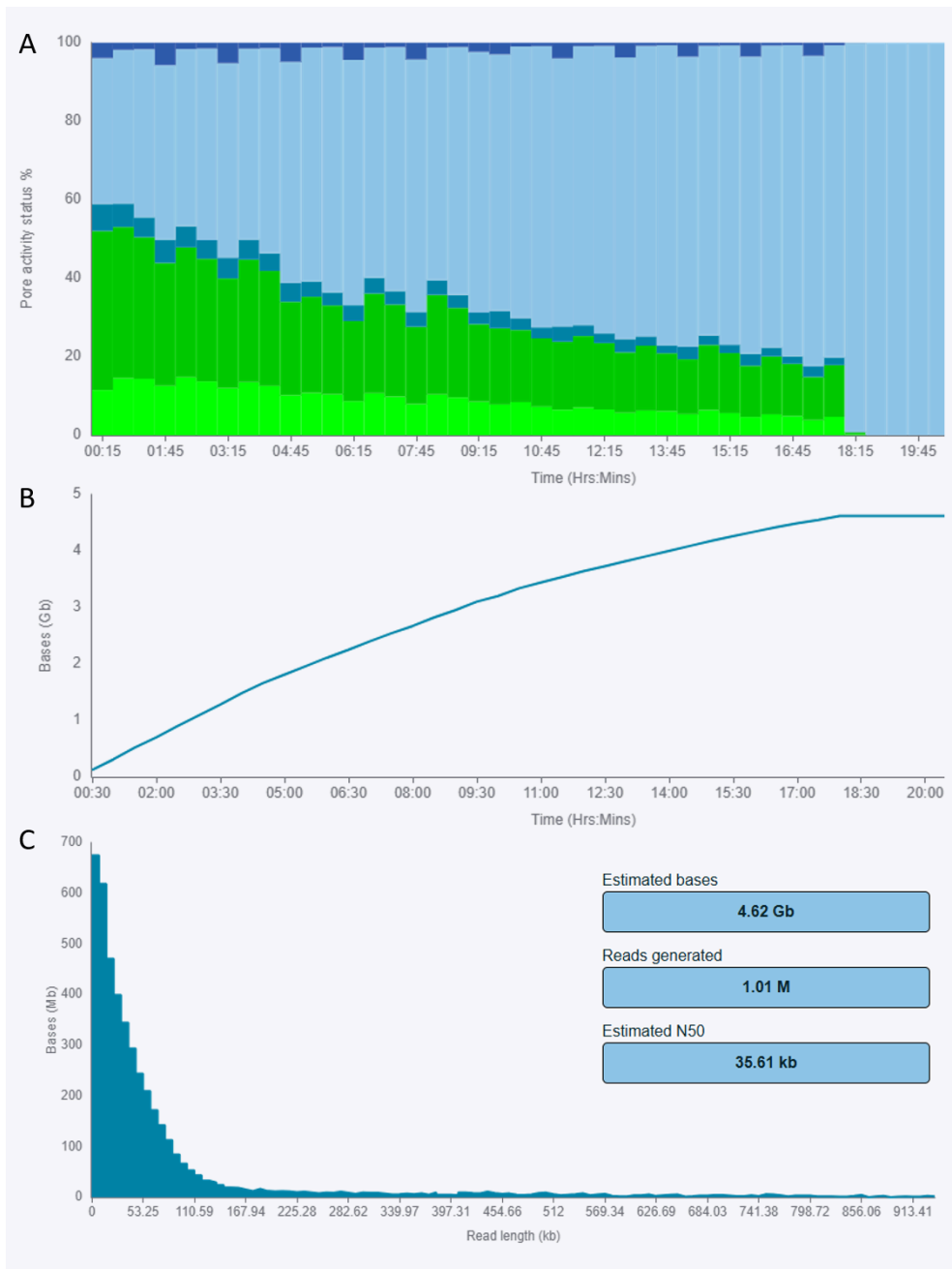


Figure 2: Sequencing run results of HMW DNA human blood sample before gDNA fragmentation.

(A) Pore activity graph showing a high percentage of pores available for sequencing (dark green) and a low percentage of pores actively sequencing (bright green).

(B) Cumulative yield plot showing data generation over time.

(C) Read length distributions with estimated total bases (Gb), reads generated (M), and N50 values (kb).

Such results can be observed when sequencing HMW DNA samples with very long DNA molecules that fail to enter the pores or get stuck and block the pores for new DNA molecules. This sample was loaded only once due to poor sequencing performance.

Expected result

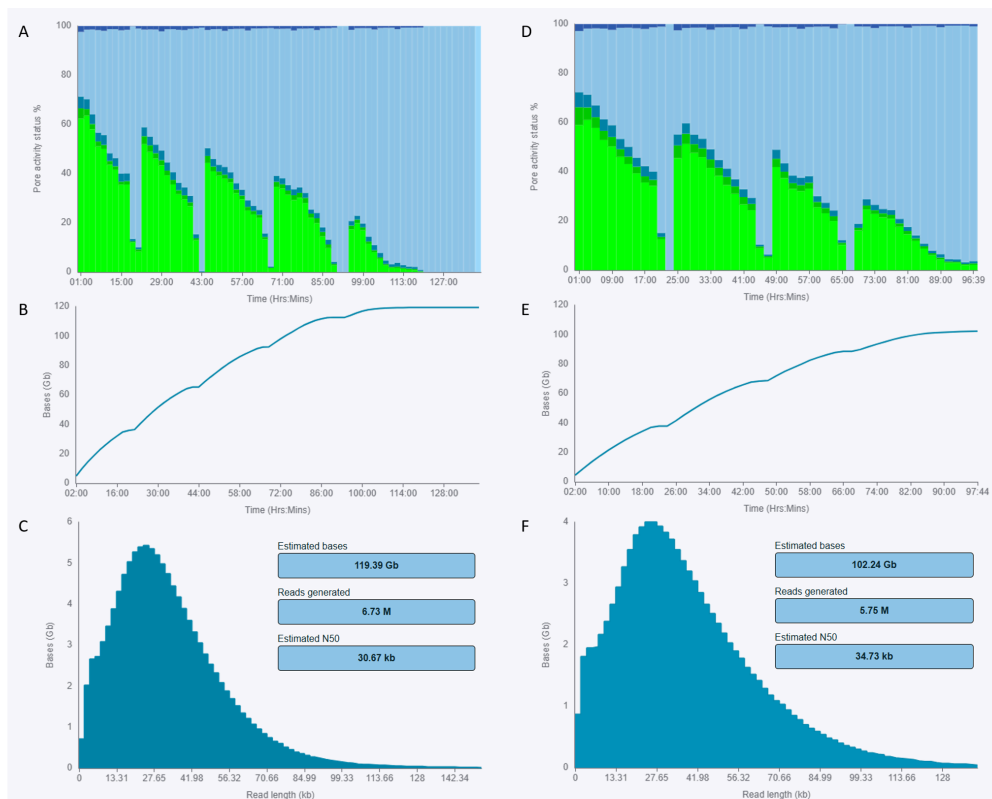


Figure 3: Sequencing run results from two PromethION runs of the same HMW DNA human blood sample, where the gDNA was fragmented by shaking at 1000 RPM for either (A–C) 5 minutes or (D–F) 8 minutes at 22°C.

(A, D) Pore activity graph showing a high percentage of pores actively sequencing (bright green) and a low percentage of pores available (dark green). Progressive pore depletion can be seen over time, with five (A) and four (D) loadings per flow cell.

(B, E) Cumulative yield plot showing data generation over time.

(C, F) Read length distributions with estimated total bases (Gb), reads generated (M), and N50 values (kb).

These results show that gDNA fragmentation greatly increased the percentage of pores actively sequencing and improved the overall yield compared to the unfragmented sample (Fig. 2). Extending the shaking time by 3 minutes resulted in greater fragmentation of long DNA molecules, which in turn increased the N50 value. Based on pore activity and cumulative yield, performing four loadings of a single sample per flow cell appears optimal to maximise flow cell output. The yield also depends on the initial pore count; in this case, the starting pore counts were 5,836 and 5,733, respectively.

These results illustrate the trade-off between achieving longer read lengths and maximising total output when optimising fragmentation conditions for long-read sequencing.



Protocol references

Removal of short fragments with the Short Fragment Eliminator Kit (EXP-SFE001)

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Human variation sequencing from 30kb extracted cell line samples using SQK-LSK114

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Ligation sequencing DNA V14 (SQK-LSK114)

<https://nanoporetech.com/document/genomic-dna-by-ligation-sqk-lsk114?device=PromethION>