1D Genomic DNA by ligation (SQK-LSK108)

Overview

- This protocol uses genomic DNA

- High yield

- Library preparation time ~70 minutes

- Fragmentation optional

- No PCR

For Research Use Only

Overview

Overview of the 1D Sequencing by Ligation protocol

Introduction of the Ligation Sequencing Kit 1D

Checklist protocol

Lab Checklist Protocol - 1D Sequencing by Ligation

Prepare for your experiment

Preparing input DNA

Check the quality, quantity and formulation of the input DNA

Equipment and consumables

Computer requirements and software downloads

Check your flow cell

Prepare your library ~100 minutes

DNA fragmentation (optional)

Optional for when experiment requires specific fragment sizes

DNA repair (optional)

To repair nicks in the DNA to maximise read lengths

End-prep

End-repair and dA-tail of double-stranded DNA fragments

Adapter ligation

Addition of Adapters

AMPure XP bead binding

Priming and loading the SpotON flow cell

Priming and loading of the sensory array in the flow cell

Start sequencing and data analysis ~10 minutes

Starting a sequencing run

Progression of MinKNOW protocol script

Further analysis with EPI2ME (optional)

Setting up and starting the upload and download of reads

Complete the experiment ~2 minutes

Close down MinKNOW and the Desktop Agent

Prepare the flow cell for re-use or return to Oxford Nanopore.

1D Genomic DNA by ligation (SQK-LSK108)

Overview of the 1D Sequencing by Ligation protocol

Print step

Introduction of the Ligation Sequencing Kit 1D

1. Ligation Sequencing Kit features

   This kit is recommended for users who:

   - want to optimise their sequencing experiment for throughput
   - require control over read length
   - would like to utilise upstream processes such as size selection or whole genome amplification

2. Introduction to the Ligation Sequencing 1D protocol

   This protocol describes how to carry out sequencing of genomic DNA using the Ligation Sequencing Kit 1D (SQK-LSK108). It is highly recommended that a Lambda control experiment is completed first to become familiar with the technology.

   Steps in the sequencing workflow:

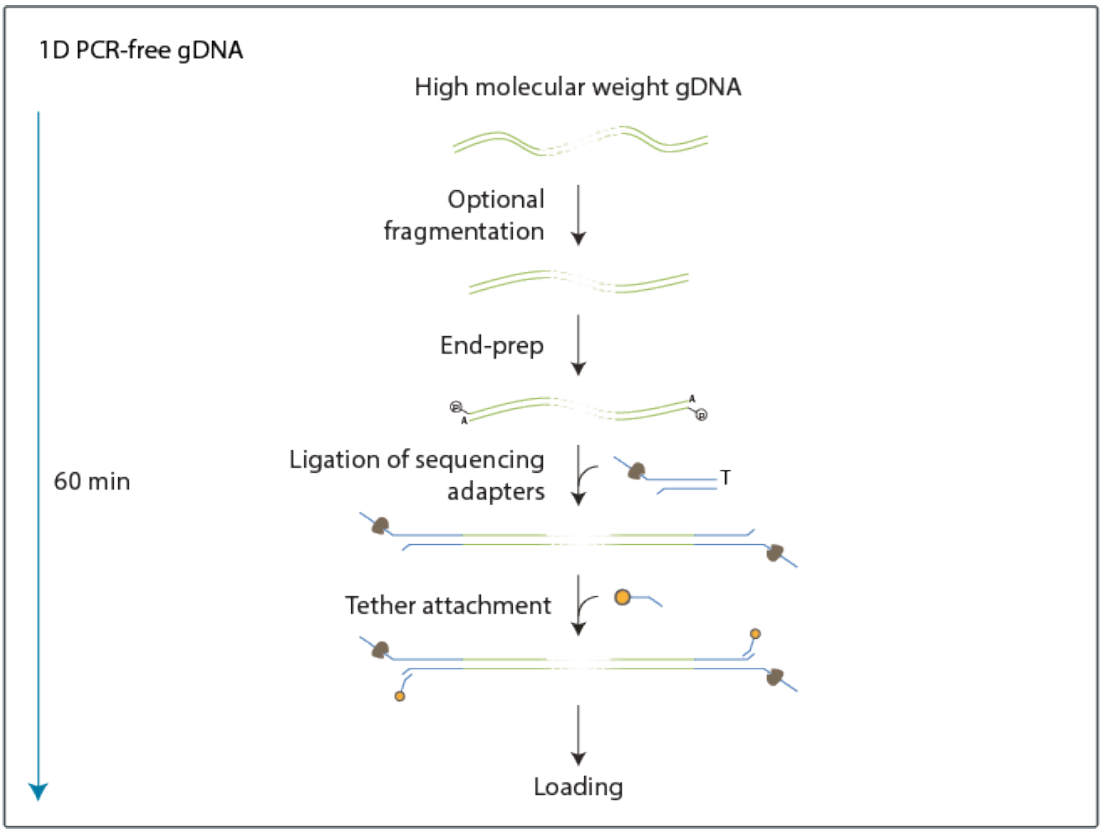
   Prepare for your experiment

   You will need to:
   - Ensure you have your sequencing kit, the correct equipment and third-party reagents

- Download the software for acquiring and analysing your data
- Check your flow cell to ensure it has enough pores for a good sequencing run

Library preparation

You will need to:
- Fragment your DNA (this step is optional)
- Prepare the DNA ends for adapter attachment
- Attach sequencing adapters supplied in the kit to the DNA ends
- Prime the flow cell, and load your DNA library into the flow cell



Sequencing and analysis

You will need to:
- Start a sequencing run using the MinKNOW software, which will collect raw data from the device and convert it into basecalled reads
- Start the EPI2ME software and select a workflow for further analysis (this step is optional)

3. Important Compatibility of this protocol

This protocol should only be used in combination with:

- Ligation Sequencing Kit 1D (SQK-LSK108)
- Flow Cells R9.5 version (FLO-MIN107) or R9.4 version (FLO-MIN106)
- MinKNOW scripts ending in ...FLO_MIN107 or FLO_MIN106

- MinKNOW script for local basecalling ending in *_plus_1D_Basecaller.py*
- Wash Kit (EXP-WSH002)
- Barcoding Kits (EXP-PBC001 and EXP-PBC096)

1D Genomic DNA by ligation (SQK-LSK108)

Checklist protocol

Print step

Lab Checklist Protocol - 1D Sequencing by Ligation

1. Lab checklist protocol - 1D Genomic DNA Sequencing SQK-LSK108

   Please read through the full protocol prior to using this abbreviated lab and printer-friendly version*.

   pdf

   Checklist protocol

   1D Genomic DNA by ligation (SQK-LSK108).pdf

   Massflow coming soon

1D Genomic DNA by ligation (SQK-LSK108)

Preparing input DNA

Print step

Check the quality, quantity and formulation of the input DNA

Materials

- 1-1.5 µg high molecular weight genomic DNA

Consumables

- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- Microfuge

1. Important Importance of DNA QC

   It is important that you check your input DNA for quality before beginning library preparation. Low molecular weight, incorrectly quantified and/or contaminated DNA (e.g. salt, EDTA, protein,

organic solvents) can have a significant impact on downstream processes and ultimately, your sequencing runs.
Below are some guidelines for how to check the DNA quality to ensure the highest possible throughput.

Access to laboratory equipment is not always possible in field conditions, however the recommendations should be used to optimise extraction and purification in the laboratory before doing fieldwork.

2. Important Input DNA mass and molarity

Oxford Nanopore's protocols recommend an input quantity in mass (e.g. 1 µg), as it is relatively easy to measure. The library preparation kit components are prepared with these input amounts in mind, but are robust to deviations from the input amount.

We expect many users to try and optimise protocols for maximum performance, and input amounts can mean different things in moles depending on the average length of the DNA fragments.

If you are unble to quantify your input DNA mass, please use the table below as a guide. Then, take forward the appropriate amount of DNA based on the average fragment length and known concentration.

Mass	Molarity if fragment length = 2 kb	Molarity if fragment length = 8 kb	Molarity if fragment length = 50 kb
10 µg	7.7 pmol	1.9 pmol	308 fmol
5 µg	3.9 pmol	963 fmol	154 fmol
3.5 µg	2.7 pmol	674 fmol	108 fmol
2 µg	1.5 pmol	385 fmol	62 fmol
1.5 µg	1.2 pmol	289 fmol	46 fmol
1 µg	770 fmol	193 fmol	31 fmol
500 ng	385 fmol	96 fmol	15 fmol
400 ng	308 fmol	77 fmol	12 fmol
200 ng	154 fmol	39 fmol	6.2 fmol
100 ng	77 fmol	19 fmol	3.1 fmol
30 ng	23 fmol	5.8 fmol	0.9 fmol
10 ng	7.7 fmol	1.9 fmol	0.3 fmol
10 pg	0.0077 fmol	0.009 fmol	0.0003 fmol

3. Prepare the DNA in nuclease-free water.
    - Transfer 1-1.5 µg genomic DNA into a DNA LoBind tube
    - Adjust the volume to 46 µl with nuclease-free water
    - Mix thoroughly by inversion avoiding unwanted shearing
    - Spin down briefly in a microfuge
4. Record the quality, quantity and size of the DNA.
5. Important Criteria for input DNA

    Before beginning library preparation, please ensure that the high molecular weight genomic DNA sample meets the following criteria:

    - Purity as measured using Nanodrop - OD 260/280 of 1.8 and OD 260/230 of 2.0-2.2
    - Average fragment size, as measured by pulse-field, or low percentage agarose gel analysis >30 kb
    - Input mass, as measured by Qubit - 1 µg (~ 1.5 µg if carrying out a DNA repair step)
    - No detergents or surfactants in the buffer

    For long-term storage of high molecular weight gDNA, we recommend the use of TE buffer.

6. Correct quantification

    The majority of RNA should be removed by RNase digestion. We have found RiboShredder (Epicentre RS 12500) to be particularly effective. However, since Riboshredder is being discontinued, you can use the RNase Cocktail Enzyme Mix (ThermoFisher, AM2286) instead.

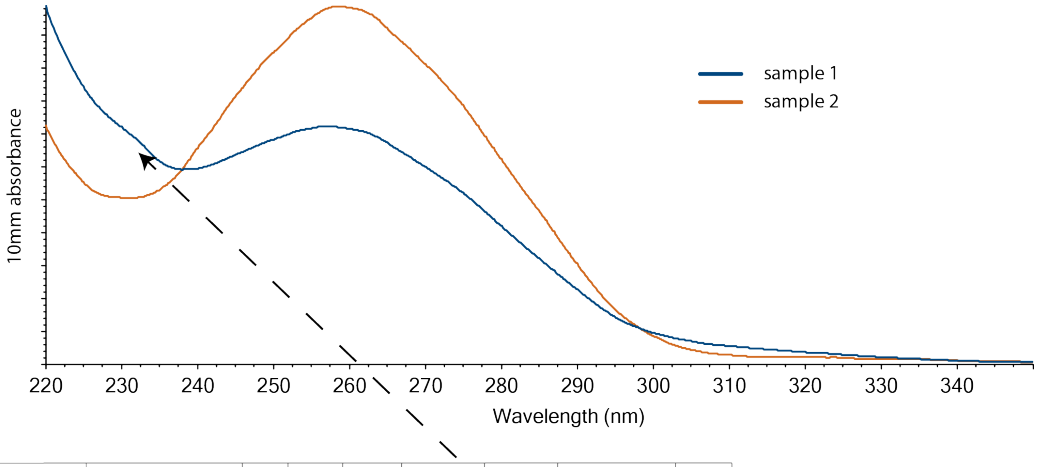
    We recommend that the DNA stock is quantified using Qubit analysis. A Qubit measures DNA specifically. Even after RNAse digestion, residual RNA is a common contaminant in gDNA preparations and is not well identified by Nanodrop measurements. Incorrect quantification could mean that you will proceed with less DNA than intended, resulting in poor performance. Also, contamination from bases (dNTPs and NTPs) will interfere with Nanodrop measurements. Therefore, we recommend that Qubit is used for all quantification measurements (i.e. after all clean-up steps).

    Additionally, high concentration, high molecular weight DNA preparations (and those with heavy RNA contamination) can lack homogeneity, which will give rise to inaccurate quantification. If you encounter this with your RNase-treated DNA sample, we recommend that you dilute the DNA further with TE, and that you rotate the tube gently until the suspension is homogeneous. Vortexing the DNA or pipetting up and down will cause shearing, which will limit the fragment sizes available to the nanopore.

7. Assessment of DNA quality
    - Chemical impurities such as detergents, denaturants, chelating agents and high concentrations of salts should be avoided as these may affect the efficiency of enzymatic steps.
    - Other contaminants such as single stranded DNA, RNA, proteins and dyes may also reduce the efficiency of steps in the library preparation.

- Prior to fragmentation, the quality of DNA may be assessed by Nanodrop (for samples with concentration >20 ng/µl).
- We recommend that sample DNA has a 260/280 ~ 1.80 and a 260/230 ~2.0-2.2.
- A 260/280 which is higher than ~1.8 indicates the presence of RNA.
- A 260/280 which is lower than ~1.8 can indicate the presence of protein or phenol
- If the 260/230 is significantly lower than 2.0-2.2 indicates the presence of contaminants, and the DNA may need additional purification.

In the NanoDrop trace shown below (figure DQND), Sample 1 had a 260/230 of ~1.0 and the resulting library performed badly in a sequencing run. If additional purification is not possible, amplification of the library by PCR (following the low input protocol) can be performed to improve library cleanliness.



Sample ID	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
sample 1	207.5	ng/µl	4.150	2.410	1.72	0.99	DNA	50.00
sample 2	1080.1	ng/µl	21.601	12.369	1.75	2.17	DNA	50.00

Figure DQND. NanoDrop trace of absorbance.

8. Assessing molecular weight

Nanopore sequencing devices reads that reflect the lengths of the fragments loaded into the flow cell. To have control over the size of the fragments generated in the library prep it is important to begin with high molecular weight DNA.

The shearing of HMW DNA can be minimised by:

- Using wide-bore pipette tips to handle the gDNA
- Mixing gently but thoroughly by inversion, as opposed to vortexing or pipetting
- Avoiding unnecessary freeze-thaw cycles
- Avoiding pH <6 and >9
- Avoiding high temperatures, which can lead to degradation

Conventional agarose gels cannot resolve DNA fragments greater than 15-20kb, but the molecular weight of starting material can be measured by pulsed-field gel analysis (figure AGLD).

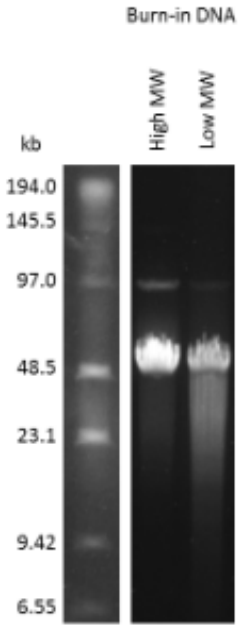


Figure AGLD: Two samples of Lambda DNA: one of intact high molecular weight fragments and one containing a significant proportion of low molecular weight fragments

Low % agarose gel analysis can be used to detect substantial degradation / shearing (figure AGDS):

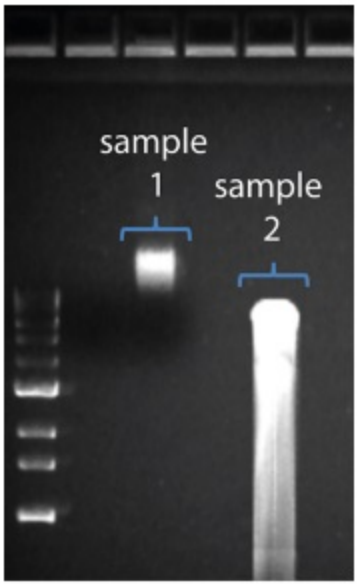


Figure AGDS: The figure shows two samples of input DNA: Sample #1 is of high molecular weight and sample #2 is of lower molecular weight and has sheared

9. Assessing fragmentation

Post-fragmentation, the quality of the fragmented material may be assessed by different methods e.g. Agilent Bioanalzyer

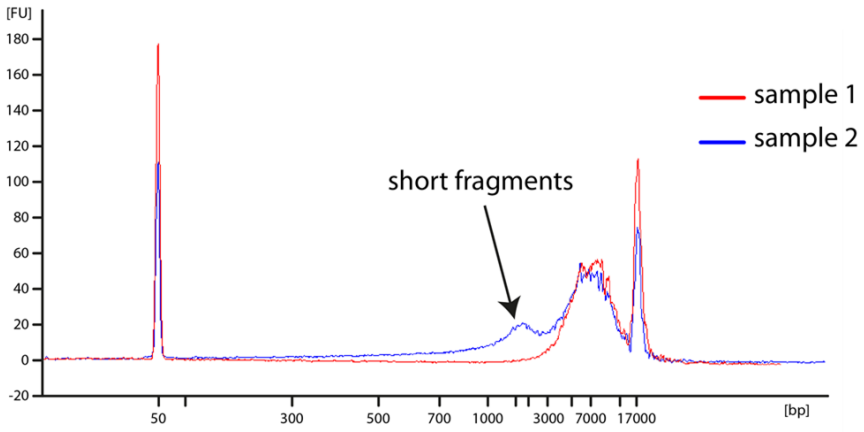


Figure DQFA: Agilent Bioanalyzer trace of two DNA samples after fragmentation

The figure above (figure DQFA) shows successful (sample 1) and unsuccessful (sample 2) fragmentation. Sample #2 contains a substantial proportion of low molecular weight fragments. This is possibly as a result of improper fragmentation, or these low MW weight fragments may have been present in the input sample.

1D Genomic DNA by ligation (SQK-LSK108)

Equipment and consumables

Print step

Materials

- Ligation Sequencing Kit 1D (SQK-LSK108)
- Library Loading Bead Kit (EXP-LLB001)

Consumables

- Agencourt AMPure XP beads
- NEBNext FFPE Repair Mix (M6630)
- NEBNext End repair / dA-tailing Module (E7546)
- NEB Blunt/TA Ligase Master Mix (M0367)
- Covaris g-TUBE
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 70% ethanol in nuclease-free water
- P1000 pipette tips
- P200 pipette tips
- P100 pipette tips
- P20 pipette tips
- P10 pipette tips
- P2 pipette tips

Equipment

- Hula mixer (gentle rotator mixer)
- Magnetic rack
- Microfuge
- Vortex mixer
- Heating block at 37° C capable of taking 1.5 ml tubes
- Thermal cycler at 20° C and 65° C
- P1000 pipette
- P200 pipette
- P100 pipette
- P20 pipette
- P10 pipette
- P2 pipette
- Ice bucket with ice
- Timer

1. Ligation Sequencing Kit 1D contents

Contents	Colour	No. of tubes
Adapter Mix (AMX1D)	green	3
Lambda DNA (LMD)	yellow	1
DNA CS (DCS)	yellow stripes	1
Elution Buffer (ELB)	black	1
Running Buffer FM (RBF)	red	6
Primers (PRM)	white	1
Adapter Bead Binding buffer (ABB)	orange	3
PCR Adapters (PCA)	blue	1

2. Library Loading Bead Kit contents

Contents	Colour	No. of tubes
Library Loading Beads (LLB)	pink	6

The kit contains Sephacryl beads. Sephacryl is a trademark of General Electric Company.

1D Genomic DNA by ligation (SQK-LSK108)

Computer requirements and software downloads

Print step

1. Software for nanopore sequencing

MinKNOW
The MinKNOW software controls the nanopore sequencing device, collects sequencing data in real-time and processes it into basecalls. You will be using MinKNOW for every sequencing experiment.

EPI2ME (optional)
The EPI2ME cloud-based platform performs further analysis of basecalled data, for example alignment to the Lambda genome, barcoding, or taxonomic classification. You will use the EPI2ME platform *only* if you would like further analysis of your data post-basecalling.

Albacore (optional)
The Albacore command-line software can be used for basecalling instead of MinKNOW. You can use it if you would like to re-basecall old data, or integrate basecalling into your analysis pipeline.

2. Important Software installation and updates

This section assumes that you have already installed the necessary software for your data analysis. If not, please proceed to the Downloads page in the Community and complete the installation.

Otherwise, please check that you are using the latest version of our software, as described below.

3. Check for software updates
    1. Open MinKNOW from the desktop icon, and follow the on-screen instructions to complete the automatic updates
    2. If using an EPI2ME workflow, open the EPI2ME Desktop Agent and follow the on-screen instructions to complete the automatic updates
4. Required disk space for data

A minimum of 1 TB storage space is recommended. To avoid the risk of losing experimental data due to running out of disk space, it is recommended that the SSD is always cleared of old read data before a run.

5. Important Disabling sleep modes

To ensure that the experiment runs to completion, all sleep modes (including screensavers and log-offs) should be disabled on the host computer.

1D Genomic DNA by ligation (SQK-LSK108)

Check your flow cell

Print step

Consumables

- MinION Flow Cell

Equipment

- MinION
- Host computer connected to the Internet with MinKNOW and the Desktop Agent installed

1. In this step, you will use the MinKNOW software to check the number of pores in your flow cell. This has to be done within 10 days of receiving your flow cell. Flow cells can then be stored until required. If there is a delay between receiving and using the flow cells, another check should be performed before library preparation begins.
2. How it works

Flow cells are shipped with a QC DNA molecule present in the buffer. This molecule produces a distinctive nanopore signal. The MinKNOW™ software uses this signal to validate the integrity of the nanopore array before use and provides the user with an estimate of the number of simultaneously available channels for the experiment. Active pores are reported in four groups, each of which may be used in turn when running long experiments e.g. a 48 hour sequencing run. Shorter experiments will use fewer than four groups.

3. Set up the MinION, Flow Cell and host computer

Figure SSFC: Assembled MinION and MinION SpotON Flow Cell connected to through the USB 3.0 port to the host computer

4. Once successfully plugged in, you will see a light and hear the fan.
5. Open the MinKNOW GUI from the desktop icon and establish a local or remote connection.
    - If running a MinION on the same host computer, plug the MinION into the computer. When the connection name appears under the **Local** tab, click **Connect**.
    - If running a MinION on a remote computer, first enter the name or IP address of the remote host under **Remote** and click **Connect**.
    - Plug a MinION and Flow Cell into the remote computer; the connection IDs will be displayed under **MinION Connection** and **Flowcell Connection**.
6. Enter the SampleID and FlowcellID being used, and click Submit.
    - Once a MinION and Flow Cell are connected, a **Label Experiment** dialogue box appears.
    - Click into the **Sample ID** box and name your sample using free text in alphanumeric format only, deleting any default **Sample_ID** that is present. **Warning: SampleID should not contain any personally identifiable information.**
    - Click into the FlowcellID box and enter the Flow Cell ID, which is the code found on a sticker on the top side of a Flow Cell.
7. Example Label Experiment dialogue box

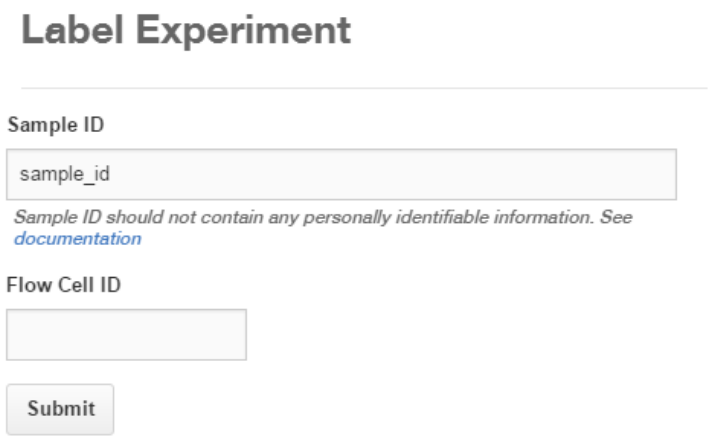


Figure PQNO: Label Experiment dialogue box.

The sample_id can be overwritten with a user-defined alphanumeric name. The Flowcell ID should be entered from the number found on a sticker on the top side of a Flow Cell.

8. Select the Platform QC script under Choose Operation, and start the script using the Execute button.

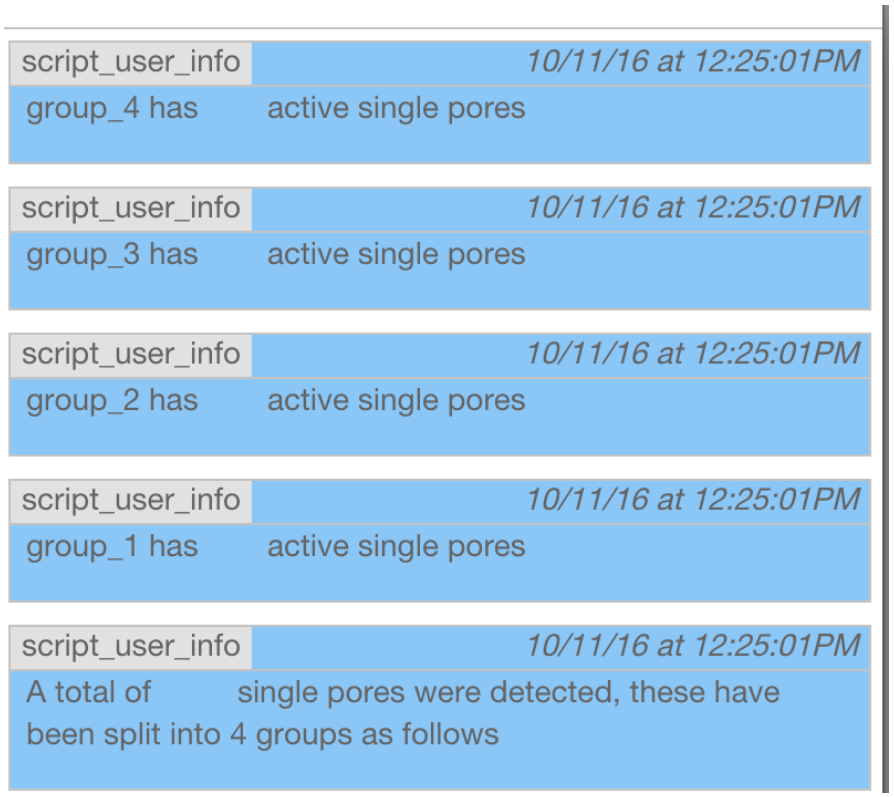
9. Check the number of active pores available for the experiment, reported in the message panel or in notifications when the check is complete.

   Once the check is complete, the software will return to the Connection page. To see the active pore report, click on **notifications**.

   If the total number of pores reported is lower than 800, please re-run the flow cell check using the instructions in this FAQ.

10. End of Step Flow cell check complete.

    The total number of pores available will be reported in the **notification panel.**



If the flow cell check is completed within 5 days of receipt, for the flow cell warranty will be activated.

Warranty for flow cells: 800 nanopores or above

1D Genomic DNA by ligation (SQK-LSK108)

DNA fragmentation (optional)

Print step

Optional for when experiment requires specific fragment sizes

~10 minutes

Materials

- 1-1.5 µg prepared DNA in 45 µl

Consumables

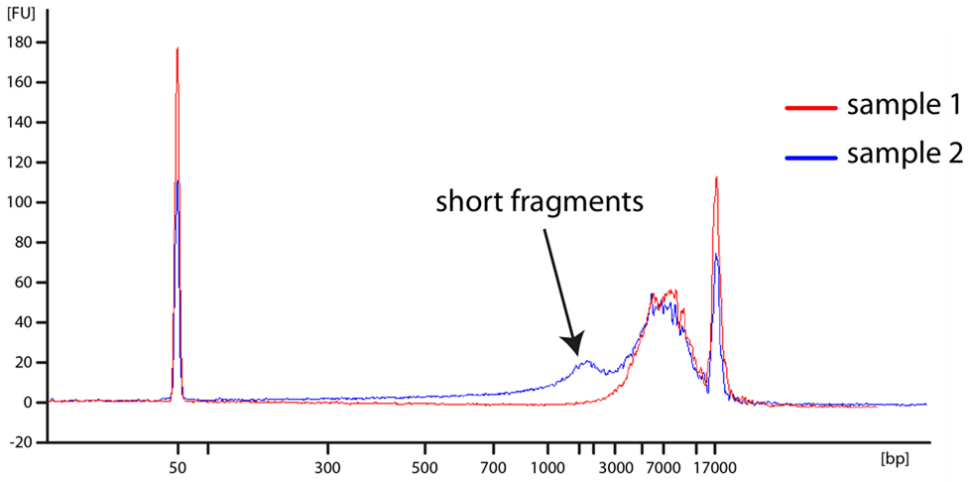
- Covaris g-TUBE

1. Covaris g-TUBE

   The standard Covaris g-TUBE protocol refers to using >4 µg genomic DNA. However, development work at Oxford Nanopore has shown that 1 µg genomic DNA in 46 µl fragments in the same way. Oxford Nanopore routinely uses an Eppendorf 5424 microfuge at 6000 rpm to generate average Lambda DNA fragments of 8 kb. When using different genomic DNA samples, wanting different fragment sizes and using different centrifuges, optimisation of spin speeds may be required. Please refer to the g-TUBE literature for more details.

2. Transfer 1-1.5 µg genomic DNA in 46 µl to the Covaris g-TUBE.
3. Spin the g-TUBE for 1 minute at room temperature at the speed for the fragment size required.
    - Spin the g-TUBE for 1 minute
    - Remove and check all the DNA has passed through the g-TUBE
    - If DNA remains in the upper chamber, spin again for 1 minute at the same speed
4. Invert the g-TUBE and spin again for 1 minute to collect the fragmented DNA.
    - Remove g-TUBE, invert the tube and replace into the centrifuge
    - Spin the g-TUBE for 1 minute
    - Remove and check the DNA has passed into the lower chamber
    - If DNA remains in the upper chamber, spin again for 1 minute
    - Remove g-TUBE
5. Transfer the 46 µl fragmented DNA to a clean 1.5 ml Eppendorf DNA LoBind tube.
6. Analyse 1 µl of the fragmented DNA for fragment size, quantity and quality.

    The success of the fragmentation process can be assessed by analysing 1 µl of sample using the Agilent Bioanalyzer. Below is an example of a successful fragmentation (sample 1) and an unsuccessful fragmentation (sample 2). The trace obtained for sample 2 shows a shoulder as a result of the presence of smaller fragments; this is indicative of substantial shearing/degradation of the input material and is likely to reduce the quality of the library preparation and the read length distribution.



7. End of Step 1-1.5 µg fragmented DNA in 45 µl is taken into the next step.

    If the experiment requires the removal of shorter fragments a 0.4 x Ampure bead clean up is recommended at this stage.

1D Genomic DNA by ligation (SQK-LSK108)

DNA repair (optional)

Print step

To repair nicks in the DNA to maximise read lengths

~30 minutes

Materials

- ~1.5 µg fragmented DNA in 45 µl

Consumables

- NEBNext FFPE Repair Mix (M6630)
- Agencourt AMPure XP beads
- 1.5 ml Eppendorf DNA LoBind tubes
- Freshly prepared 70% ethanol in nuclease-free water
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

Equipment

- Thermal cycler at 20 °C
- Ice bucket with ice
- Magnetic rack
- Hula mixer (gentle rotator mixer)
- Vortex mixer

1. Important Use of magnetic beads

   After washing the beads with the DNA bound it is important that they are allowed to dry to ensure that all the ethanol has been removed. However, over drying will result in some of the DNA not eluting efficiently leading to reduced recovery.

2. DNA repair

   The FFPE DNA repair step is recommended when there is a chance that the genomic DNA contains nicks or other damage which may compromise the success of the end-prep step. FFPE DNA repair mix has been shown to improve read lengths compared to other DNA repair methods.

3. Perform FFPE DNA repair treatment using NEB M6630.

Reagent	Volume
1-1.5 µg fragmented** DNA	45µl
Nuclease-free water	8.5 µl
FFPE Repair Buffer	6.5 µl
FFPE Repair Mix	2 µl
Total	62 µl

4. **Fragmentation is not necessary. When attempting to obtain long reads g-TUBE fragmentation can be omitted and FFPE repair performed directly on the high molecular weight input material.
5. Mix gently by flicking the tube, and spin down.
6. Incubate the reaction for 15 minutes at 20 °C.
7. Prepare the AMPure XP beads for use; resuspend by vortexing.

8. Add 62 µl of the resuspended beads to the FFPE-repair reaction and mix gently by flicking the tube.
9. Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
10. Rotator mixer

    The agitation is gentle and the contents of the tube may not appear to move at all. This allows the DNA to bind to the beads.

11. Prepare 500 µl of fresh 70% ethanol in nuclease-free water.
12. Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
13. Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard. Repeat.
14. Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Briefly allow to dry.
15. Remove the tube from the magnetic rack and resuspend pellet in 46 µl nuclease-free water. Incubate for 2 minutes at room temperature.
16. Pellet beads on magnet until the eluate is clear and colourless.
17. Remove and retain 46 µl of eluate in a clean 1.5 ml Eppendorf DNA LoBind tube.
18. Quantify 1 µl of fragmented and repaired DNA using a Qubit fluorometer - recovery aim > 1 µg.
19. End of Step Take 1 µg of FFPE repaired DNA in 45 µl into End-prep.

1D Genomic DNA by ligation (SQK-LSK108)

End-prep

Print step

End-repair and dA-tail of double-stranded DNA fragments

~50 minutes

Materials

- ~1 µg fragmented and repaired DNA in 45 µl

Consumables

- NEBNext End repair / dA-tailing Module (E7546)
- Freshly prepared 70% ethanol in nuclease-free water
- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Agencourt AMPure XP beads

Equipment

- Thermal cycler at 20° C and 65° C
- Magnetic rack
- Hula mixer (gentle rotator mixer)
- Vortex mixer

- Ice bucket with ice

1. Perform end repair and dA-tailing of fragmented DNA as follows:

   End-prep demo

   Mix the following reagents in a 1.5 ml Eppendorf DNA LoBind tube:

Reagent	Volume
~1 µg DNA (fragmented genomic DNA, amplicon or cDNA)	45 µl
Ultra II End-prep reaction buffer	7 µl
Ultra II End-prep enzyme mix	3 µl
Nuclease-free water	5 µl
Total	60 µl

   If using the barcoding approach the pooled input DNA should be ~1 µg in 45 µl, whether genomic, amplicon or cDNA.

2. Mix gently by flicking the tube, and spin down.
3. Transfer the sample to a 0.2 ml PCR tube, and incubate for 5 minutes at 20 °C and 5 minutes at 65 °C using the thermal cycler.

   If condensation is observed in the tube after the thermocycling, briefly spin down the tube contents in a microfuge.

4. Prepare the AMPure XP beads for use; resuspend by vortexing.
5. Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube.
6. Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting.

   End-prep cleanup demo

7. Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
8. Prepare 500 µl of fresh 70% ethanol in nuclease-free water.
9. Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
10. Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard. Repeat.
11. Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Briefly allow to dry.
12. Remove the tube from the magnetic rack and resuspend pellet in 31 µl nuclease-free water. Incubate for 2 minutes at room temperature.
13. Pellet beads on magnet until the eluate is clear and colourless.

14. Remove and retain 31 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.
15. Quantify 1 µl of end-prepped DNA using a Qubit fluorometer - recovery aim > 700 ng.

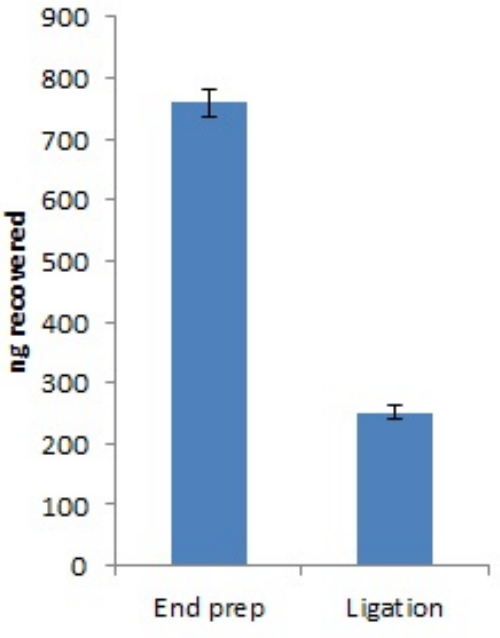


Figure NSRL: Typical DNA recovery levels at the end of two steps during the library preparation.

16. End of Step Take forward approximately 700 ng of end-prepped DNA in 30 µl into adapter ligation.

1D Genomic DNA by ligation (SQK-LSK108)

Adapter ligation

Print step

Addition of Adapters

Materials

- Adapter Mix (AMX1D)

Consumables

- NEB Blunt/TA Ligase Master Mix (M0367)
- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

Equipment

- Microfuge
- Ice bucket with ice

1. Fragment size and adapter ligation

   The amount of adapter has been optimised for fragment sizes greater or equal to 8 kb. If the fragments are generally smaller than 3 kb, adjustments should be made to use 0.2 pmoles of DNA in the adapter ligation step.

2. Thaw and prepare the kit reagents as follows:

Contents	On ice	At room temperature
Adapter Bead Binding Buffer (ABB)		✓
Elution Buffer (ELB)		✓
Adapter Mix 1D (AMX1D)	✓	
Running Buffer with Fuel Mix (RBF)	✓	
Blunt/TA Ligation Master Mix	✓	

3. Check the contents of each tube are clear of any precipitate and are thoroughly mixed before setting up the reaction.
   - Mix the contents of each tube by flicking
   - Check that there is no precipitate present (DTT in the Blunt/TA Master Mix can sometimes form a precipitate)
   - Spin down briefly before accurately pipetting the contents in the reaction
4. Taking the end-prepped DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition.

   Adapter ligation and tethering demo

Reagent	Volume
End-prepped DNA	30 µl
Adapter Mix	20 µl
Blunt/TA Ligation Master Mix	50 µl
Total	100 µl

5. Mix gently by flicking the tube, and spin down.
6. Incubate the reaction for 10 minutes at room temperature.
7. End of Step Adapted DNA library.

   The adapted DNA library is now ready to be purified prior to loading into the flow cell. The purification removes any excess proteins, nucleotides and salts etc. from the DNA library.

[1D Genomic DNA by ligation (SQK-LSK108)]

AMPure XP bead binding

Print step

Materials

- Adapter Bead Binding (ABB)
- Elution Buffer (ELB)

Consumables

- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Agencourt AMPure XP beads

Equipment

- Magnetic rack
- Vortex mixer
- Microfuge
- Ice bucket with ice

1. Prepare the AMPure XP beads for use; resuspend by vortexing.
2. Add 40 µl of resuspended AMPure XP beads to the adapter ligation reaction from the previous step and mix by pipetting.
3. Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
4. Place on magnetic rack, allow beads to pellet and pipette off supernatant.
5. Add 140 µl of the Adapter Bead Binding buffer to the beads. Close the tube lid, and resuspend the beads by flicking the tube. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant. Repeat.
6. Important Agitating the beads by flicking results in a more efficient removal of free adapter, compared to adding the wash buffer and immediately aspirating.
7. Remove the tube from the magnetic rack and resuspend pellet in 15 µl Elution Buffer. Incubate for 10 minutes at room temperature.
8. Pellet beads on magnet until the eluate is clear and colourless.
9. Remove and retain 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.
    - Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube
    - Dispose of the pelleted beads
10. End of Step The prepared library is used for loading into the flow cell. Store the library on ice until ready to load.
11. Quantify 1 µl of fragmented and repaired DNA using a Qubit fluorometer - recovery aim ~430 ng.

1D Genomic DNA by ligation (SQK-LSK108)

Priming and loading the SpotON flow cell

Print step

Priming and loading of the sensory array in the flow cell

~10 minutes

Materials

- Running Buffer with Fuel Mix (RBF)
- Library Loading Bead Kit (EXP-LLB001)

Consumables

- SpotON Flow Cell

Equipment

- MinION

1. Important Thoroughly mix the contents of the RBF tube by vortexing or pipetting, and spin down briefly.
2. Flip back the MinION lid and slide the priming port cover clockwise so that the priming port is visible.

Priming and loading the SpotON Flow Cell

Priming and loading: The steps for priming and loading the SpotON Flow Cell. Written instructions are given below. The library is loaded dropwise without putting the pipette tip firmly into the port.

Take care to avoid introducing any air during pipetting.

3. Important Care must be taken when drawing back buffer from the flow cell. The array of pores must be covered by buffer at all times. Removing more than 20-30 µl risks damaging the pores in the array.
4. After opening the priming port, check for small bubble under the cover. Draw back a small volume to remove any bubble (a few µls):
    1. Set a P1000 pipette to 200 µl
    2. Insert the tip into the priming port
    3. Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip

Visually check that there is continuous buffer from the priming port across the sensor array.

5. Prepare the flow cell priming mix in a clean 1.5 ml Eppendorf DNA LoBind tube.

Reagent	Volume
RBF	576 µl
Nuclease-free water	624 µl
Total	**1200 µl**

6. Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes.
7. Thoroughly mix the contents of the RBF and LLB tubes by pipetting.
8. Tip Using the Library Loading Beads

Demo of how to use the Library Loading Beads.

9. Prepare the library for loading as follows:

Reagent	Volume
RBF	35.0 µl
LLB	25.5 µl
Nuclease-free water	2.5 µl
DNA library	12 µl
Total	**75.0 µl**

10. Complete the flow cell priming:
    1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
    2. Load **200 µl** of the priming mix into the flow cell via the priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.
11. Mix the prepared library gently by pipetting up and down just prior to loading.
12. Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.
13. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.

1D Genomic DNA by ligation (SQK-LSK108)

Starting a sequencing run

Print step
~5 minutes

1. Double–click the MinKNOW icon located on the desktop to open the MinKNOW GUI.
2. Wait for the MinKNOW GUI to open

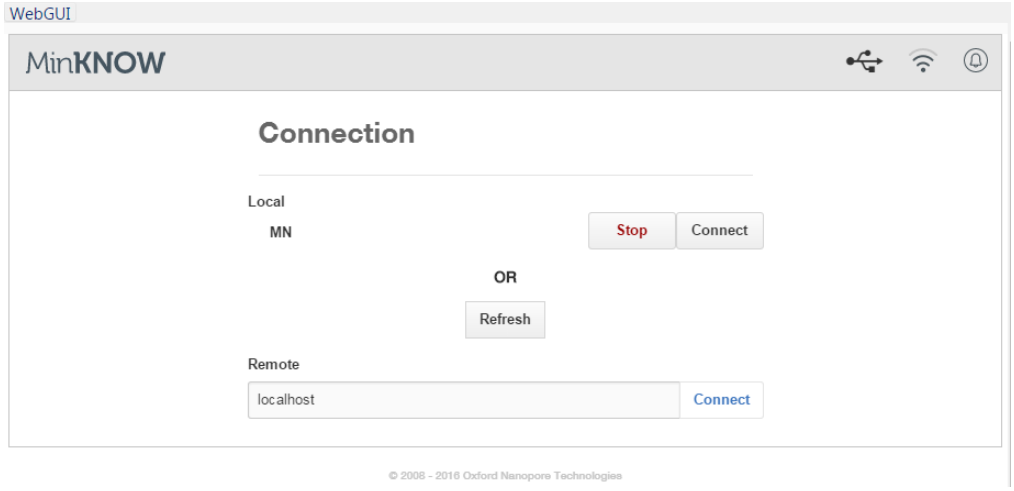


Figure MGCP: **MinKNOW GUI.** The Connection page allows the user to set up the connection with the MinION attached to the host computer, or set up connections to MinIONs which are available on the network to allow these devices to be controlled remotely when starting or stopping the experiment.

Full explanation of the Connection page is provided here.

3. Select the local MinION, and click Connect.

   Alternatively, connect to a remote host by completing the Remote box and clicking Connect.

4. Enter the SampleID and FlowcellID being used, and click Submit.
   - Once a MinION and Flow Cell are connected, a **Label Experiment** dialogue box appears.
   - Click into the **Sample ID** box and name your sample using free text in alphanumeric format only, deleting any default **Sample_ID** that is present. **Warning: SampleID should not contain any personally identifiable information.**
   - Click into the FlowcellID box and enter the Flow Cell ID, which is the code found on a sticker on the top side of a Flow Cell.
5. Select the appropriate protocol script

   To select the correct script there are five steps:

   - Experiment type: Choose **Sequencing Run** under "Choose Operation"
   - Flow Cell product code: Choose the Flow Cell type under "Flow cell product code"
   - Sequencing kit: Choose **SQK-LSK108** under Sequencing Kit
   - Choose whether or not live basecalling is enabled
   - The most appropriate script will appear in the drop-down menu.

6. Start the script using the Execute button at the bottom of the Connections page.
7. Allow the script to run to completion.
    - The MinKNOW Experiment page will indicate the progression of the script
    - Monitor messages in the Message panel in the MinKNOW GUI
8. The basecalled read files are stored in :\data\reads

[1D Genomic DNA by ligation (SQK-LSK108)]

Progression of MinKNOW protocol script

Print step

1. Tip As the MinKNOW script progresses, you can check the following:
    - Number of active pores
    - Heatsink temperature
    - Development of the read histogram
    - Pore occupancy
    - Local basecalling report
2. Check the number of active pores reported in the MUX scan are similar (within 10-15%) to those reported at the end of the Platform QC
    - If there is a significant reduction in the numbers, restart MinKNOW.
    - If the numbers are still significantly different, close down the host computer and reboot.
    - When the numbers are similar to those reported at the end of the Platform QC, restart the experiment on the Connection page. There is no need to load any additional library after restart.
3. Check the heatsink temperature is approximately 34 °C.
4. MinION temperature control

    The MinION is able to maintain a heatsink temperature of 34 °C on a typical lab bench when the local ambient conditions are between 19.5 °C and 24.5 °C. However, there are a number of external factors which can disrupt the local conditions and which need to be taken into account, for example warm air expelled from laptops, or cool air from a fan or air conditioning system increasing airflow around the MinION.

    The MinION takes approximately 10 minutes to get to temperature.

5. Monitor the development of the read length histogram.
6. Example Read length histogram

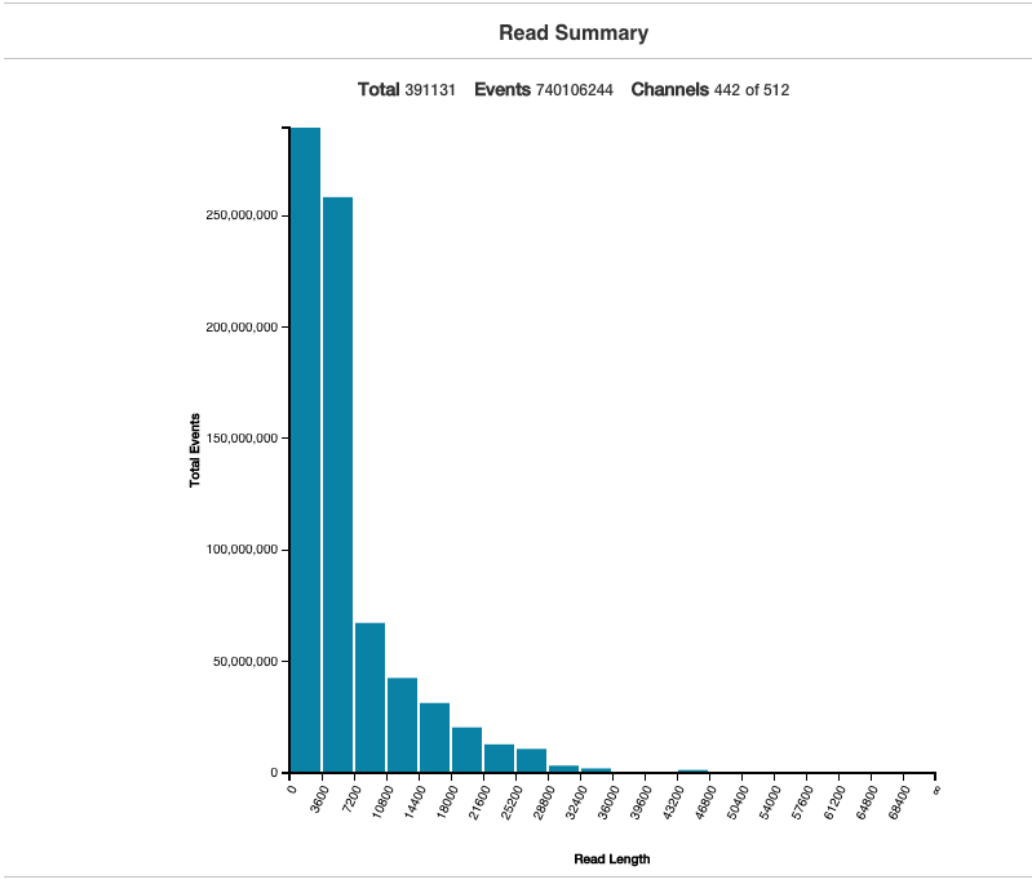


Figure LDRH: Read length histogram. The histogram will reflect expected read lengths for the experimental design being used.

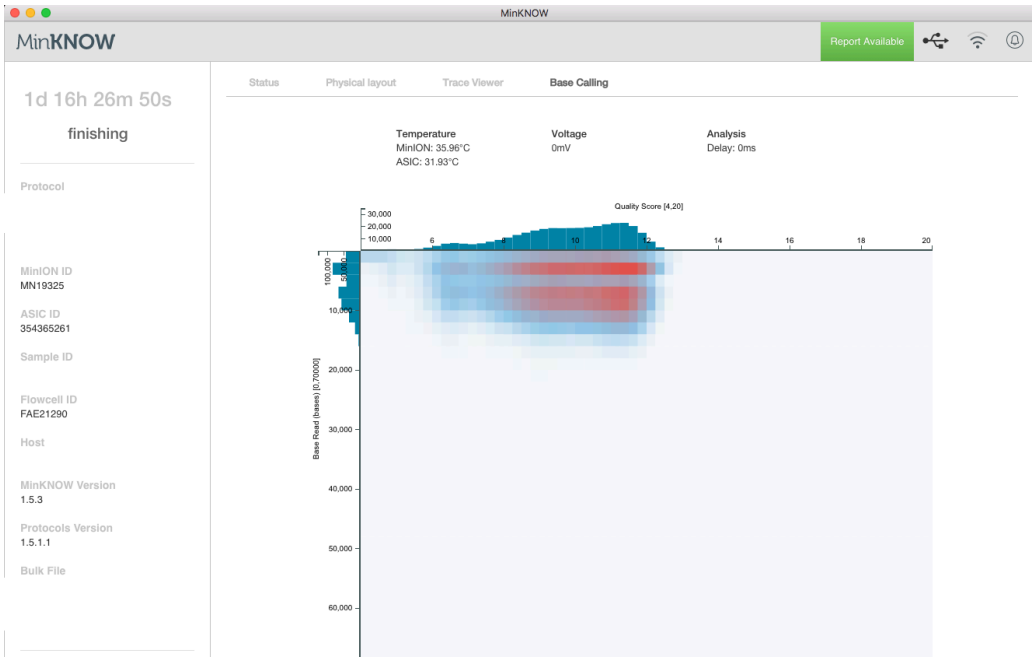
The Read Summary includes the total number of **Events**. An event is describes a series of raw data points where the contents of the pore are constant, a full description is provided here. MinKNOW groups events into reads by detecting the abrupt signal change when DNA enters and leaves the nanopore.

7. Check pore occupancy by looking at the panel at the top of the Status or Physical Layout views.
8. Example Physical Layout screen

Figure LDCP: Physical Layout screen. A good library will be indicated by a high proportion of light and dark green channels. The combination of light and dark green indicate the number of active pores at any point in time and the dark green indicate the proportion of pores in strand (or sequencing) at a particular time point. A low proportion of dark green channels will reduce the throughput of the sequencing.

9. Example Local basecalling report in the MinKNOW GUI

The progress of the local basecalling can be monitored via the **Base Calling** panel on the experiment page of the MinKNOW GUI. The histogram shows the number of reads against q-score, with red boxes representing a higher number of reads.



10. End of Step End of sequencing protocol script.

    The length of the sequencing scripts are indicated during selection, e.g. 48 Hr. However, if live basecalling is selected, a minimum-spec laptop may not keep up with the speed of data acquisition, and not all reads will be basecalled by the end of the experiment. The reads that had not finished being basecalled during the experiment will continue to be basecalled in Catch-Up mode.

    The reads from the experiment will be found in the location set during installation of the software. The read file structure is described here.

    If the Desktop Agent is running simultaneously to MinKNOW, the full report will also be available. If it is being run at a different times, the reads will be present in the data/reads folder ready to be processed.

1D Genomic DNA by ligation (SQK-LSK108)

Further analysis with EPI2ME (optional)

Print step

Setting up and starting the upload and download of reads

~5 minutes

Consumables

- EPI2ME account

Equipment

- Host computer connected to the Internet with MinKNOW and the Desktop Agent installed

1. Open the Desktop Agent using the desktop shortcut.
2. Click on the New Workflow tab in the Desktop Agent and select the workflow to be used in the analysis.

3. Check the correct settings are selected in the Desktop Agent.

   Check Input Directory, Uploaded Directory, Download Directory, Split downloads into folders, and Download Mode. You can also configure the q-score cut off (this defaults to 6 unless changed).

4. Click "Start Run" to start data analysis.
5. Follow the progression of upload and download of read files in the Desktop Agent.
6. Click on VIEW REPORT.
   - Click on VIEW REPORT to navigate to the EPI2ME website, this can be done at any point during data exchange
   - Return to the Desktop Agent to see progression of the exchange
7. When the upload and download numbers are the same, the data exchange is complete. The processed reads will be in downloads folder in the selected location on the host computer.

   Figure MADF: Folder structure on the host computer. As the reads are uploaded to the cloud, they are transferred into the uploaded folder. Once they have been processed by the workflow, the reads are returned to the downloads folder.

1D Genomic DNA by ligation (SQK-LSK108)

Close down MinKNOW and the Desktop Agent

Print step
~2 minutes

1. Quit Desktop Agent using the close x.
2. Quit MinKNOW by closing down the web GUI.
3. Disconnect the MinION.

1D Genomic DNA by ligation (SQK-LSK108)

Prepare the flow cell for re-use or return to Oxford Nanopore.

Print step

Materials

- Wash Kit (EXP-WSH002)

1. If you would like to reuse the flow cell, follow the Wash Kit instructions and store the washed flow cell at 2-8 °C, OR

   The Wash Kit protocol is available here.

2. Follow the returns procedure by washing out the MinION Flow Cell ready to send back to Oxford Nanopore.

   Instructions for returning flow cells can be found here.