PROTOCOL

Ligation sequencing gDNA (SQK-LSK109) VGDE_9063_v109_revAP_25May2022

- This protocol uses genomic DNA
- High yield
- Library preparation time ~60 minutes
- Fragmentation optional
- No PCR

For Research Use Only

This is a Legacy product This kit is soon to be discontinued and we recommend all customers to upgrade to the latest chemistry for their relevant kit which is available on the Store. If customers require further support for any ongoing critical experiments using a Legacy product, please contact Customer Support via email: support@nanoporetech.com.

FOR RESEARCH USE ONLY

Contents

1. Overview of the protocol

IMPORTANT

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Ligation Sequencing Kit features

This kit is recommended for users who:

- want to optimise their sequencing experiment for throughput
- would like to utilise upstream processes such as size selection, whole genome amplification, or enrichment for long reads

IMPORTANT

Optional fragmentation and size selection

By default, the protocol contains no DNA fragmentation step, however in some cases it may be advantageous to fragment your sample. For example, when working with lower amounts of input gDNA (100 ng – 500 ng), fragmentation will increase the number of DNA molecules and therefore increase throughput. Instructions are available in the DNA Fragmentation section of Extraction methods.

Additionally, we offer several options for size-selecting your DNA sample to enrich for long fragments - instructions are available in the Size Selection section of Extraction methods.

Introduction to the Ligation Sequencing protocol for gDNA

This protocol describes how to carry out sequencing of a DNA sample using the Ligation Sequencing Kit (SQK-LSK109). 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

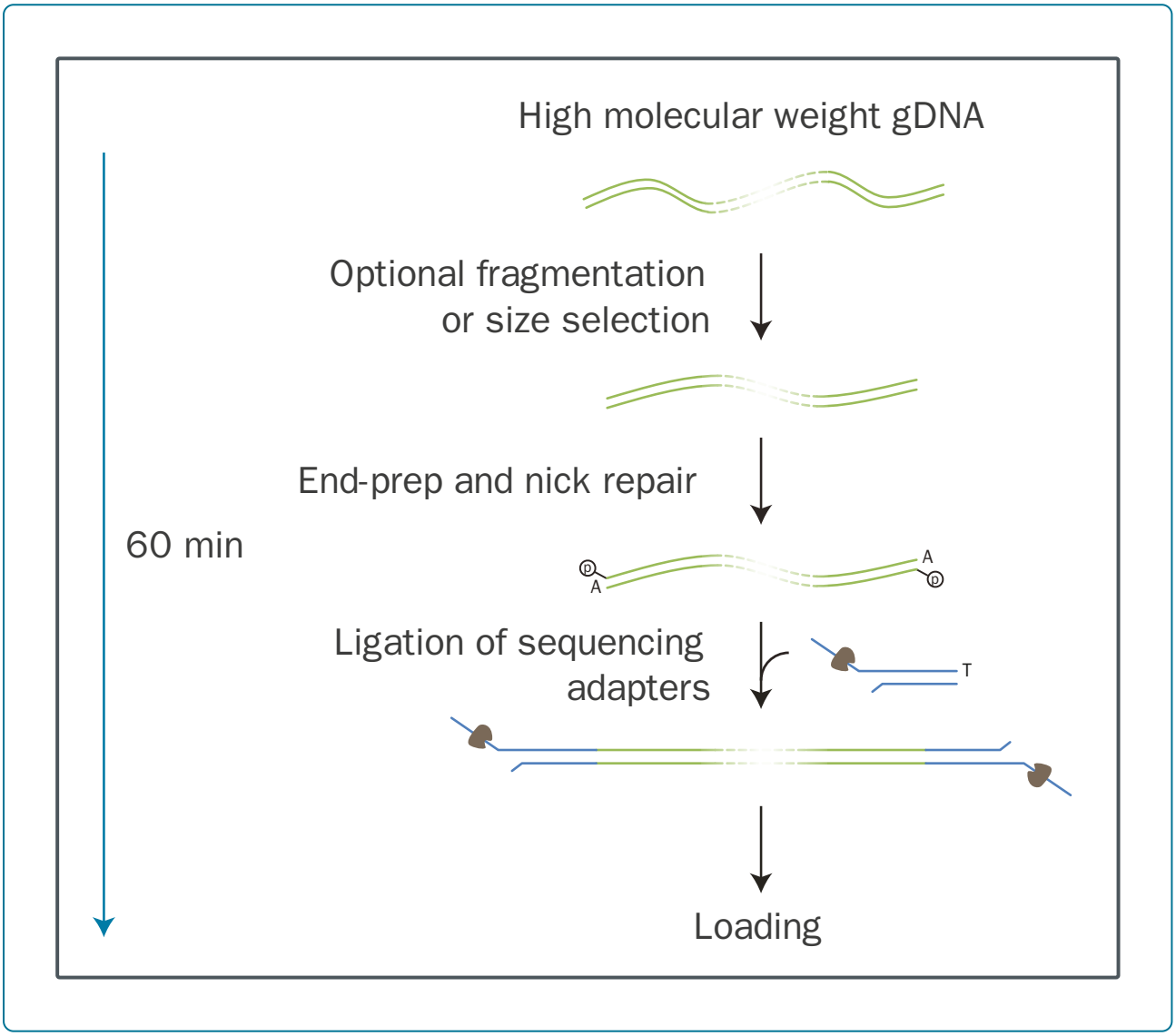
You will need to:

- Extract your DNA, and check its length, quantity and purity. **The quality checks performed during the protocol are essential in ensuring experimental success.**
- 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:

- Repair the DNA, and 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)

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Ligation Sequencing Kit (SQK-LSK109)
- Control Expansion (EXP-CTL001)
- FLO-MIN106 (R9.4.1) flow cells
- Flow Cell Wash Kit (EXP-WSH004)
- PCR Barcoding Kit (EXP-PBC001 and EXP-PBC096)
- Native Barcoding Kits (EXP-NBD104 and EXP-NBD114)

2. Equipment and consumables

Materials

1 µg (or 100-200 fmol) gDNA
OR 100+ ng high molecular weight genomic DNA if performing DNA fragmentation
Ligation Sequencing Kit (SQK-LSK109)
Flow Cell Priming Kit (EXP-FLP002)

Consumables

Agencourt AMPure XP beads (Beckman Coulter, A63881)
NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L). Alternatively, you can use the NEBNext® products below:
NEBNext FFPE Repair Mix (NEB, M6630)
NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
NEBNext Quick Ligation Module (NEB, E6056)
1.5 ml Eppendorf DNA LoBind tubes
0.2 ml thin-walled PCR tubes
Nuclease-free water (e.g. ThermoFisher, AM9937)
Freshly prepared 70% ethanol in nuclease-free water

Equipment

Hula mixer (gentle rotator mixer)
Magnetic rack, suitable for 1.5 ml Eppendorf tubes
Microfuge
Vortex mixer
Thermal cycler
P1000 pipette and tips
P200 pipette and tips
P100 pipette and tips
P20 pipette and tips
P10 pipette and tips
P2 pipette and tips
Ice bucket with ice
Timer

Optional equipment	Agilent Bioanalyzer (or equivalent) Qubit fluorometer (or equivalent for QC check) Eppendorf 5424 centrifuge (or equivalent)

For this protocol, you will need 1 µg (or 100-200 fmol) gDNA.

Although 1 µg (or 100-200 fmol) gDNA is recommended, users can start with lower input quantities (down to 100 ng) if performing DNA fragmentation to increase the number of DNA molecules in the sample, or if amplifying the sample by PCR.

Input DNA

How to QC your input DNA

It is important that the input DNA meets the quantity and quality requirements. Using too little or too much DNA, or DNA of poor quality (e.g. highly fragmented or containing RNA or chemical contaminants) can affect your library preparation.

For instructions on how to perform quality control of your DNA sample, please read the Input DNA/RNA QC protocol.

Chemical contaminants

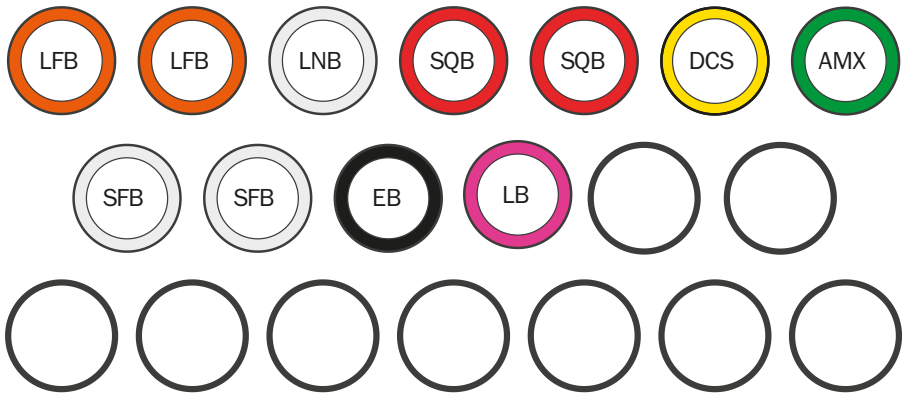
Depending on how the DNA is extracted from the raw sample, certain chemical contaminants may remain in the purified DNA, which can affect library preparation efficiency and sequencing quality. Read more about contaminants on the Contaminants page of the Community.

NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing

For customers new to nanopore sequencing, we recommend buying the NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (catalogue number E7180S or E7180L), which contains all the NEB reagents needed for use with the Ligation Sequencing Kit.

Please note, for our amplicon protocols, NEBNext FFPE DNA Repair Mix and NEBNext FFPE DNA Repair Buffer are not required.

Ligation Sequencing Kit (SQK-LSK109) contents

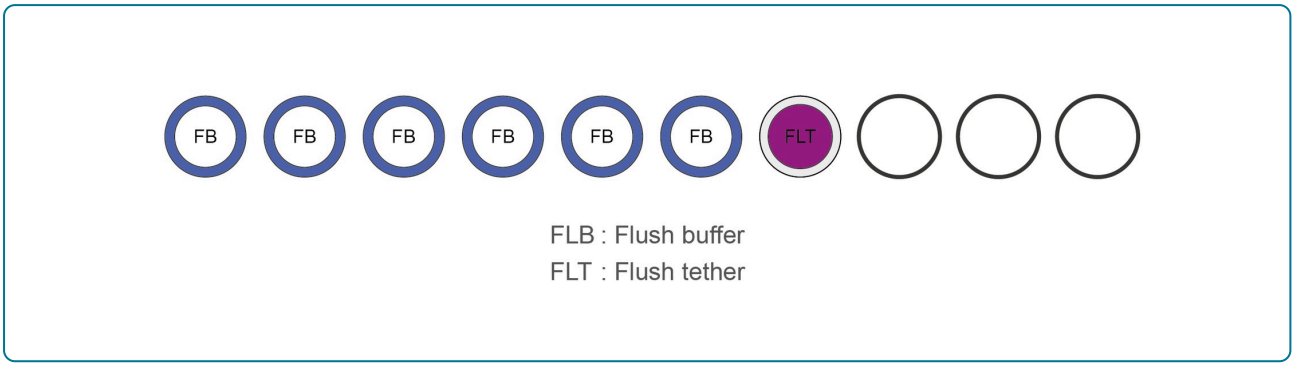


LFB : L fragment buffer
LNB : Ligation buffer
SQB : Sequencing buffer
DCS : DNA control strand
AMX : Adapter mix

SFB : S fragment buffer
EB : Elution buffer
LB : Loading beads

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
DNA CS	DCS	Yellow	1	50
Adapter Mix	AMX	Green	1	40
Ligation Buffer	LNB	Clear	1	200
L Fragment Buffer	LFB	White cap, orange stripe on label	2	1,800
S Fragment Buffer	SFB	Grey	2	1,800
Sequencing Buffer	SQB	Red	2	300
Elution Buffer	EB	Black	1	200
Loading Beads	LB	Pink	1	360

Flow Cell Priming Kit (EXP-FLP002) contents



Name	Acronym	Cap colour	No. of vial	Fill volume per vial (µl)
Flush Buffer	FB	Blue	6	1,170
Flush Tether	FLT	Purple	1	200

3. Computer requirements and software

MinION Mk1B IT requirements

Sequencing on a MinION Mk1B requires a high-spec computer or laptop to keep up with the rate of data acquisition. Read more in the MinION Mk1B IT Requirements document.

MinION Mk1C IT requirements

The MinION Mk1C contains fully-integrated compute and screen, removing the need for any accessories to generate and analyse nanopore data. Read more in the MinION Mk1C IT requirements document.

Software for nanopore sequencing

MinKNOW

The MinKNOW software controls the nanopore sequencing device, collects sequencing data and basecalls in real time. You will be using MinKNOW for every sequencing experiment to sequence, basecall and demultiplex if your samples were barcoded.

For instructions on how to run the MinKNOW software, please refer to the MinKNOW protocol.

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.

For instructions on how to create an EPI2ME account and install the EPI2ME Desktop Agent, please refer to the EPI2ME Platform protocol.

Check your flow cell

We highly recommend that you check the number of pores in your flow cell prior to starting a sequencing experiment. This should be done within three months of purchasing for MinION/GridION/PromethION or within four weeks of purchasing Flongle Flow Cells. Oxford Nanopore Technologies will replace any flow cell with fewer than the number of pores in the table below, when the result is reported within two days of performing the flow cell check, and when the storage recommendations have been followed. To do the flow cell check, please follow the instructions in the Flow Cell Check document.

Flow cell	Minimum number of active pores covered by warranty
Flongle Flow Cell	50
MinION/GridION Flow Cell	800
PromethION Flow Cell	5000

4. DNA repair and end-prep

Materials

gDNA in 47 µl nuclease-free water
DNA Control Sample (DCS)

Consumables

0.2 ml thin-walled PCR tubes
1.5 ml Eppendorf DNA LoBind tubes
Nuclease-free water (e.g. ThermoFisher, AM9937)
NEBNext FFPE DNA Repair Mix (NEB, M6630)
NEBNext® Ultra II End Repair / dA-tailing Module (NEB, E7546)
Agencourt AMPure XP beads (Beckman Coulter™, A63881)
Freshly prepared 70% ethanol in nuclease-free water

Equipment

P1000 pipette and tips
P100 pipette and tips
P10 pipette and tips
Thermal cycler
Microfuge
Hula mixer (gentle rotator mixer)
Magnetic rack
Ice bucket with ice

IMPORTANT

Optional fragmentation and size selection

By default, the protocol contains no DNA fragmentation step, however in some cases it may be advantageous to fragment your sample. For example, when working with lower amounts of input gDNA (100 ng – 500 ng), fragmentation will increase the number of DNA molecules and therefore increase throughput. Instructions are available in the DNA Fragmentation section of Extraction methods.

Additionally, we offer several options for size-selecting your DNA sample to enrich for long fragments - instructions are available in the Size Selection section of Extraction methods.

1 Thaw DNA Control Sample (DCS) at room temperature, spin down, mix by pipetting, and place on ice.

2 Prepare the NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice.

For optimal performance, NEB recommend the following:

1. Thaw all reagents on ice.
2. Flick and/or invert the reagent tubes to ensure they are well mixed.
   Note: Do not vortex the FFPE DNA Repair Mix or Ultra II End Prep Enzyme Mix.
3. Always spin down tubes before opening for the first time each day.
4. The Ultra II End Prep Buffer and FFPE DNA Repair Buffer may have a little precipitate. Allow the mixture to come to room temperature and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for 30 seconds to solubilise any precipitate.
   Note: It is important the buffers are mixed well by vortexing.
5. The FFPE DNA Repair Buffer may have a yellow tinge and is fine to use if yellow.

3 Prepare the DNA in nuclease-free water:

- For R9.4.1 flow cells, transfer 1 µg (or 100-200 fmol) genomic DNA into a 1.5 ml Eppendorf DNA LoBind tube.
- Adjust the volume to 47 µl with nuclease-free water
- Mix thoroughly by flicking the tube
- Spin down briefly in a microfuge

4 In a 0.2 ml thin-walled PCR tube, mix the following:

Between each addition, pipette mix 10-20 times.

Reagent	Volume
DNA from the previous step	47 µl
DNA CS (optional)	1 µl
NEBNext FFPE DNA Repair Buffer	3.5 µl
NEBNext FFPE DNA Repair Mix	2 µl
Ultra II End-prep Reaction Buffer	3.5 µl
Ultra II End-prep Enzyme Mix	3 µl
Total	**60 µl**

5 **Ensure the components are thoroughly mixed by pipetting, and spin down.**

6 **Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.**

IMPORTANT

AMPure XP bead clean-up

It is recommended that the repaired/end-prepped DNA sample is subjected to the following clean-up with AMPure XP beads. This clean-up can be omitted for simplicity and to reduce library preparation time. However, it has been observed that omission of this clean-up can: reduce subsequent adapter ligation efficiency, increase the prevalence of chimeric reads, and lead to an increase in pores being unavailable for sequencing. If omitting the clean-up step, proceed to the next section.

7 **Resuspend the AMPure XP beads by vortexing.**

8 **Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.**

9 **Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by flicking the tube.**

10. Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.

11. Prepare 500 µl of fresh 70% ethanol in nuclease-free water.

12. Spin down the sample and pellet on a magnet until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant.

13. Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

14. Repeat the previous step.

15. Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

16. Remove the tube from the magnetic rack and resuspend the pellet in 61 µl nuclease-free water. Incubate for 2 minutes at room temperature.

17. Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

18. Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

CHECKPOINT

Quantify 1 µl of eluted sample using a Qubit fluorometer.

END OF STEP

Take forward the repaired and end-prepped DNA into the adapter ligation step. However, at this point it is also possible to store the sample at 4°C overnight.

5. Adapter ligation and clean-up

Materials	Adapter Mix (AMX) Ligation Buffer (LNB) Long Fragment Buffer (LFB) Short Fragment Buffer (SFB) Elution Buffer (EB)
Consumables	NEBNext Quick Ligation Module (NEB, E6056) 1.5 ml Eppendorf DNA LoBind tubes Agencourt AMPure XP beads (Beckman Coulter™, A63881)
Equipment	Magnetic rack Microfuge Vortex mixer P1000 pipette and tips P100 pipette and tips P20 pipette and tips P10 pipette and tips

IMPORTANT

Although the recommended third-party ligase is supplied with its own buffer, the ligation efficiency of Adapter Mix (AMX) is higher when using Ligation Buffer supplied within the Ligation Sequencing Kit.

1. **Spin down the Adapter Mix (AMX) and Quick T4 Ligase, and place on ice.**

2. **Thaw Ligation Buffer (LNB) at room temperature, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.**

3. **Thaw the Elution Buffer (EB) at room temperature and mix by vortexing. Then spin down and place on ice.**

IMPORTANT

Depending on the wash buffer (LFB or SFB) used, the clean-up step after adapter ligation is designed to either enrich for DNA fragments of >3 kb, or purify all fragments equally.

- To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB)
- To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB)

4 **To enrich for DNA fragments of 3 kb or longer, thaw one tube of Long Fragment Buffer (LFB) at room temperature, mix by vortexing, spin down and place on ice.**

5 **To retain DNA fragments of all sizes, thaw one tube of Short Fragment Buffer (SFB) at room temperature, mix by vortexing, spin down and place on ice.**

6 **In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order:**

Between each addition, pipette mix 10 - 20 times.

Reagent	Volume
DNA sample from the previous step	60 µl
Ligation Buffer (LNB)	25 µl
NEBNext Quick T4 DNA Ligase	10 µl
Adapter Mix (AMX)	5 µl
Total	**100 µl**

7 **Ensure the components are thoroughly mixed by pipetting, and spin down.**

8 **Incubate the reaction for 10 minutes at room temperature.**

IMPORTANT

If you have omitted the AMPure purification step after DNA repair and end-prep, do not incubate the reaction for longer than 10 minutes.

9. Resuspend the AMPure XP beads by vortexing.

10. Add 40 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube.

11. Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.

12. Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless.

13. Wash the beads by adding either 250 µl Long Fragment Buffer (LFB) or 250 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.

14. Repeat the previous step.

15. Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

16. Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB). Spin down and incubate for 10 minutes at room temperature. For high molecular weight DNA, incubating at 37°C can improve the recovery of long fragments.

17. Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

18 Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

Dispose of the pelleted beads

CHECKPOINT

Quantify 1 µl of eluted sample using a Qubit fluorometer.

IMPORTANT

We recommend loading 5-50 fmol of the final prepared library onto a flow cell.

Loading more than the maximal recommended amount of DNA can have a detrimental effect on output as higher quantities of DNA results in a larger number of ligated DNA ends with loaded motor protein. This depletes fuel in the Sequencing Buffer, regardless of whether or not the DNA fragments are being sequenced. This leads to fuel depletion and speed drop-off early in the sequencing run. Dilute the libraries in Elution Buffer if required.

If you are using the Flongle for sample prep development, we recommend loading 3-20 fmol instead.

END OF STEP

The prepared library is used for loading into the flow cell. Store the library on ice or at 4°C until ready to load.

TIP

Library storage recommendations

We recommend storing libraries in Eppendorf DNA LoBind tubes at **4°C for short term** storage or repeated use, for example, reloading flow cells between washes. For single use and **long-term storage** of more than 3 months, we recommend storing libraries at **-80°C** in Eppendorf DNA LoBind tubes. For further information, please refer to the DNA library stability Know-How document.

OPTIONAL ACTION

If quantities allow, the library may be diluted in Elution Buffer (EB) for splitting across multiple flow cells.

Additional buffer for doing this can be found in the Sequencing Auxiliary Vials expansion (EXP-AUX001), available to purchase separately. This expansion also contains additional vials of Sequencing Buffer (SQB) and Loading Beads (LB), required for loading the libraries onto flow cells.

6. Priming and loading the SpotON flow cell

Materials	Flow Cell Priming Kit (EXP-FLP002) Loading Beads (LB) Sequencing Buffer (SQB)
Consumables	1.5 ml Eppendorf DNA LoBind tubes Nuclease-free water (e.g. ThermoFisher, AM9937)
Equipment	MinION Mk1B or Mk1C SpotON Flow Cell P1000 pipette and tips P100 pipette and tips P20 pipette and tips P10 pipette and tips

TIP

Priming and loading a flow cell

We recommend all new users watch the 'Priming and loading your flow cell' video before your first run.

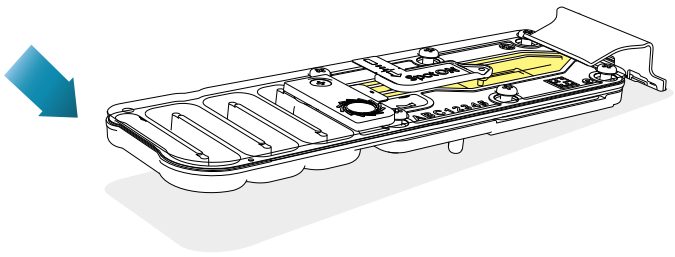
1. **Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature before mixing the reagents by vortexing, and spin down at room temperature.**

2. **To prepare the flow cell priming mix, add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at room temperature.**

3 Open the MinION device lid and slide the flow cell under the clip.

Press down firmly on the flow cell to ensure correct thermal and electrical contact.

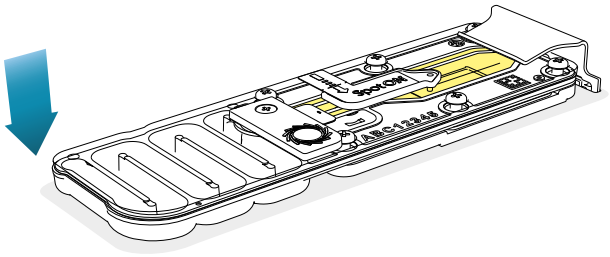
1a Insert the flow cell into the device under the clip and press down firmly.



Key:

- Storage buffer
- Priming mix
- DNA library

1b Insert the flow cell into the device under the clip and press down firmly.



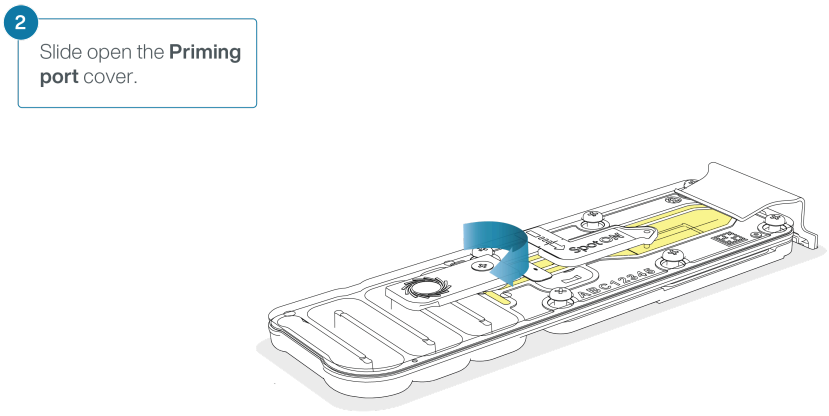
OPTIONAL ACTION

Complete a flow cell check to assess the number of pores available before loading the library.

This step can be omitted if the flow cell has been checked previously.

See the flow cell check instructions in the MinKNOW protocol for more information.

4 Slide the flow cell priming port cover clockwise to open the priming port.



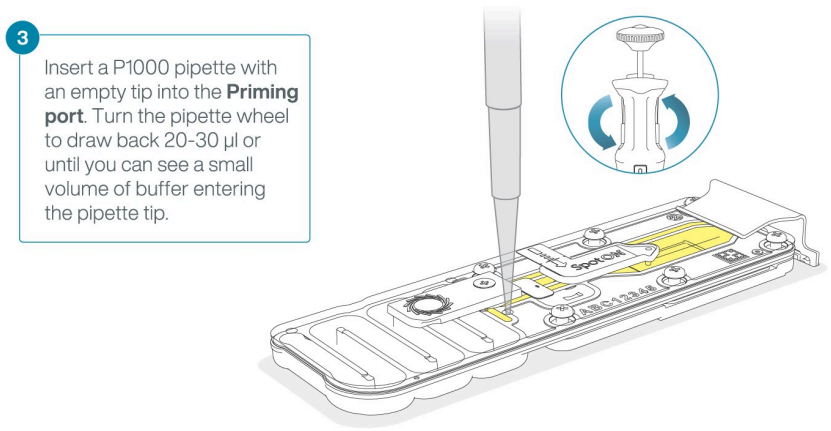
IMPORTANT

Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

5 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:

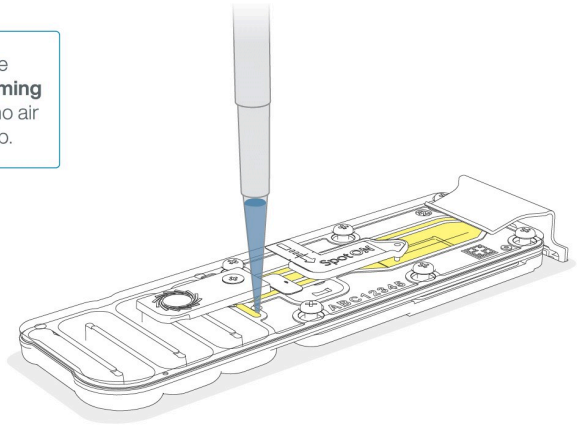
1. Set a P1000 pipette to 200 µl

2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 µl, to draw back 20-30 µl, or until you can see a small volume of buffer entering the pipette tip
   __Note:__ Visually check that there is continuous buffer from the priming port across the sensor array.



6 Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes. During this time, prepare the library for loading by following the steps below.

4 Slowly load 800 µl of the priming mix into the **Priming port**. Ensure there are no air bubbles in the pipette tip.



Wait 5 minutes before proceeding to the next step.

7 Thoroughly mix the contents of the Loading Beads (LB) by pipetting.

IMPORTANT

The Loading Beads (LB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

8 In a new tube, prepare the library for loading as follows:

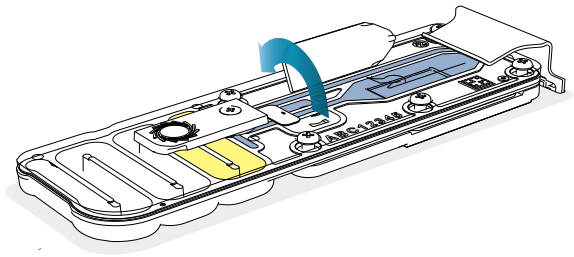
Reagent	Volume per flow cell
Sequencing Buffer (SQB)	37.5 µl
Loading Beads (LB), mixed immediately before use	25.5 µl
DNA library	12 µl
Total	**75 µl**

Note: Load the library onto the flow cell immediately after adding the Sequencing Buffer (SQB) and Loading Beads (LB) because the fuel in the buffer will start to be consumed by the adapter.

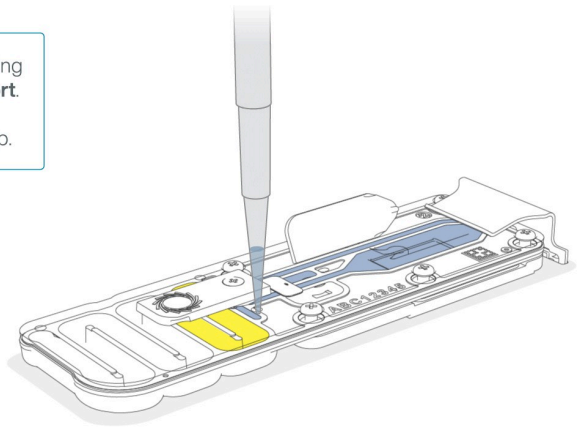
9 Complete the flow cell priming:

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 priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.

5 Gently flip open the SpotON sample port cover.



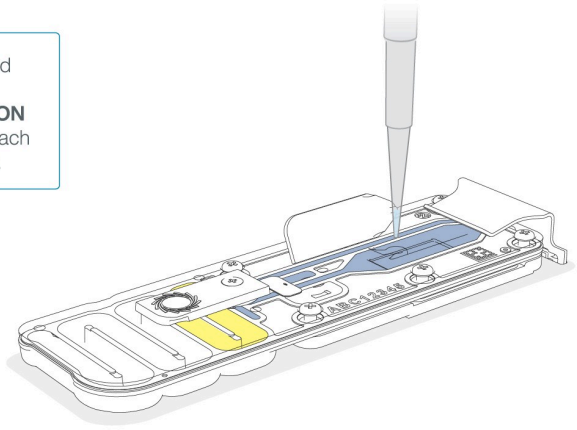
6 Load 200 µl of the priming mix into the **Priming Port**. Ensure there are no air bubbles in the pipette tip.



10 Mix the prepared library gently by pipetting up and down just prior to loading.

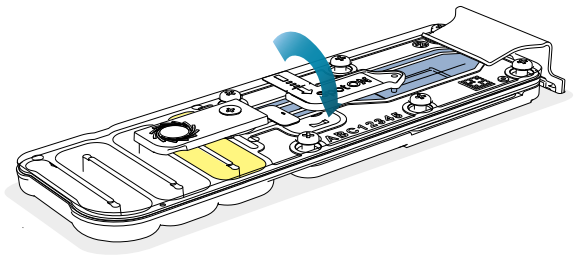
11 Add 75 µl of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

7 Pipette mix the prepared library and load 75 µl dropwise into the **SpotON** sample port, ensuring each drop flows into the port.

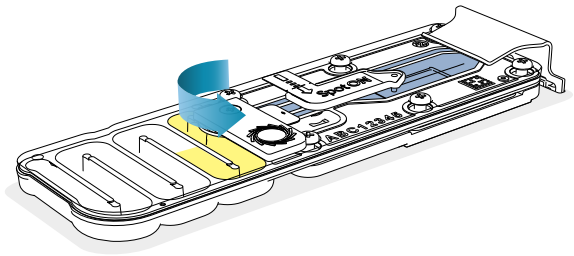


12 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION device lid.

8 Gently replace the **SpotON** sample port cover.



9 Gently close the **Priming port**.



7. Data acquisition and basecalling

How to start sequencing

Once you have loaded your flow cell, the sequencing run can be started on MinKNOW, our sequencing software that controls the device, data acquisition and real-time basecalling. For more detailed information on setting up and using MinKNOW, please see the MinKNOW protocol.

MinKNOW can be used and set up to sequence in multiple ways:

- On a computer either directly or remotely connected to a sequencing device.
- Directly on a GridION, MinION Mk1C or PromethION 24/48 sequencing device.

For more information on using MinKNOW on a sequencing device, please see the device user manuals:

- MinION Mk1B user manual
- MinION Mk1C user manual
- GridION user manual

To start a sequencing run on MinKNOW:

1. Navigate to the start page and click **Start sequencing.**

2. Fill in your experiment details, such as name and flow cell position and sample ID.

3. Select the **sequencing kit used in the library preparation** on the Kit page.

4. Configure the sequencing and output parameters for your sequencing run or keep to the default settings on the Run configuration tab.

Note: If basecalling was turned off when a sequencing run was set up, basecalling can be performed post-run on MinKNOW. For more information, please see the MinKNOW protocol.

5. Click **Start** on the Review page to start the sequencing run.

Data analysis after sequencing

After sequencing has completed on MinKNOW, the flow cell can be reused or returned, as outlined in the Flow cell reuse and returns section.

After sequencing and basecalling, the data can be analysed. For further information about options for basecalling and post-basecalling analysis, please refer to the Data Analysis document.

In the Downstream analysis section, we outline further options for analysing your data.

8. Flow cell reuse and returns

Materials Flow Cell Wash Kit (EXP-WSH004)

1 **After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Flow Cell Wash Kit protocol and store the washed flow cell at 2-8°C.**

The Flow Cell Wash Kit protocol is available on the Nanopore Community.

TIP

We recommend you to wash the flow cell as soon as possible after you stop the run. However, if this is not possible, leave the flow cell on the device and wash it the next day.

2 **Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore.**

Instructions for returning flow cells can be found here.

Note: All flow cells must be flushed with deionised water before returning the product.

IMPORTANT

If you encounter issues or have questions about your sequencing experiment, please refer to the Troubleshooting Guide that can be found in the online version of this protocol.

9. Downstream analysis

Post-basecalling analysis

There are several options for further analysing your basecalled data:

1. EPI2ME workflows

For in-depth data analysis, Oxford Nanopore Technologies offers a range of bioinformatics tutorials and workflows available in EPI2ME. The platform provides a vehicle where workflows deposited in GitHub by our Research and Applications teams can be showcased with descriptive texts, functional bioinformatics code and example data.

2. Research analysis tools

Oxford Nanopore Technologies' Research division has created a number of analysis tools, which are available in the Oxford Nanopore GitHub repository. The tools are aimed at advanced users, and contain instructions for how to install and run the software. They are provided as-is, with minimal support.

3. Community-developed analysis tools

If a data analysis method for your research question is not provided in any of the resources above, please refer to the Bioinformatics section of the Resource centre. Numerous members of the Nanopore Community have developed their own tools and pipelines for analysing nanopore sequencing data, most of which are available on GitHub. Please be aware that these tools are not supported by Oxford Nanopore Technologies, and are not guaranteed to be compatible with the latest chemistry/software configuration.

10. Issues during DNA/RNA extraction and library preparation

Below is a list of the most commonly encountered issues, with some suggested causes and solutions.

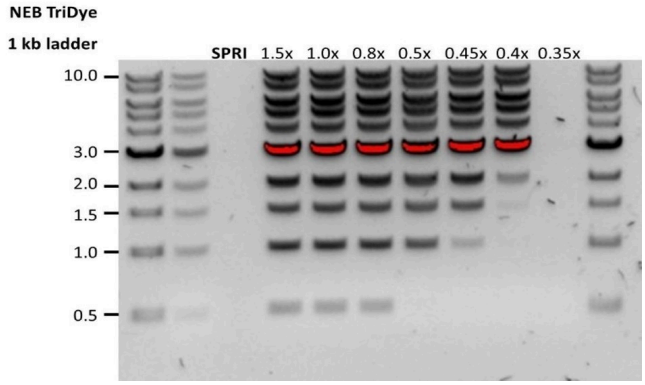
We also have an FAQ section available on the Nanopore Community Support section.

If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via LiveChat in the Nanopore Community.

Low sample quality

Observation	Possible cause	Comments and actions
Low DNA purity (Nanodrop reading for DNA OD 260/280 is <1.8 and OD 260/230 is <2.0–2.2)	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the Contaminants document. Please try an alternative extraction method that does not result in contaminant carryover. Consider performing an additional SPRI clean-up step.
Low RNA integrity (RNA integrity number <9.5 RIN, or the rRNA band is shown as a smear on the gel)	The RNA degraded during extraction	Try a different RNA extraction method. For more info on RIN, please see the RNA Integrity Number document. Further information can be found in the DNA/RNA Handling page.
RNA has a shorter than expected fragment length	The RNA degraded during extraction	Try a different RNA extraction method. For more info on RIN, please see the RNA Integrity Number document. Further information can be found in the DNA/RNA Handling page. We recommend working in an RNase-free environment, and to keep your lab equipment RNase-free when working with RNA.

Low DNA recovery after AMPure bead clean-up

Observation	Possible cause	Comments and actions
Low recovery	DNA loss due to a lower than intended AMPure beads-to-sample ratio	1. AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample. 2. When the AMPure beads-to-sample ratio is lower than 0.4:1, DNA fragments of any size will be lost during the clean-up.
Low recovery	DNA fragments are shorter than expected	The lower the AMPure beads-to-sample ratio, the more stringent the selection against short fragments. Please always determine the input DNA length on an agarose gel (or other gel electrophoresis methods) and then calculate the appropriate amount of AMPure beads to use. 
Low recovery after end-prep	The wash step used ethanol <70%	DNA will be eluted from the beads when using ethanol <70%. Make sure to use the correct percentage.

11. Issues during the sequencing run

Below is a list of the most commonly encountered issues, with some suggested causes and solutions.

We also have an FAQ section available on the Nanopore Community Support section.

If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via LiveChat in the Nanopore Community.

Fewer pores at the start of sequencing than after Flow Cell Check

Observation	Possible cause	Comments and actions
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	An air bubble was introduced into the nanopore array	After the Flow Cell Check it is essential to remove any air bubbles near the priming port before priming the flow cell. If not removed, the air bubble can travel to the nanopore array and irreversibly damage the nanopores that have been exposed to air. The best practice to prevent this from happening is demonstrated in this video.
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	The flow cell is not correctly inserted into the device	Stop the sequencing run, remove the flow cell from the sequencing device and insert it again, checking that the flow cell is firmly seated in the device and that it has reached the target temperature. If applicable, try a different position on the device (GridION/PromethION).
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	Contaminations in the library damaged or blocked the pores	The pore count during the Flow Cell Check is performed using the QC DNA molecules present in the flow cell storage buffer. At the start of sequencing, the library itself is used to estimate the number of active pores. Because of this, variability of about 10% in the number of pores is expected. A significantly lower pore count reported at the start of sequencing can be due to contaminants in the library that have damaged the membranes or blocked the pores. Alternative DNA/RNA extraction or purification methods may be needed to improve the purity of the input material. The effects of contaminants are shown in the Contaminants Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover.

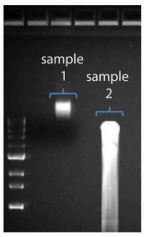
MinKNOW script failed

Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support. If you do not have another sequencing device available, we recommend storing the flow cell and the loaded library at 4°C and contact Technical Support for further storage guidance.

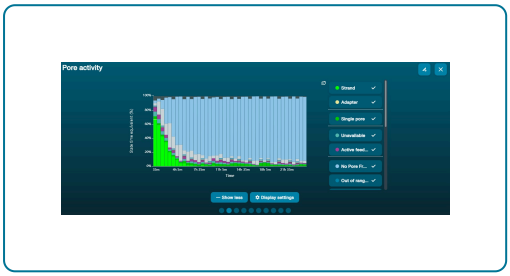
Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	Ensure you load the recommended amount of good quality library in the relevant library prep protocol onto your flow cell. Please quantify the library before loading and calculate mols using tools like the Promega Biomath Calculator, choosing "dsDNA: µg to pmol"
Pore occupancy close to 0	The Ligation Sequencing Kit was used, and sequencing adapters did not ligate to the DNA	Make sure to use the NEBNext Quick Ligation Module (E6056) and Oxford Nanopore Technologies Ligation Buffer (LNB, provided in the sequencing kit) at the sequencing adapter ligation step, and use the correct amount of each reagent. A Lambda control library can be prepared to test the integrity of the third-party reagents.
Pore occupancy close to 0	The Ligation Sequencing Kit was used, and ethanol was used instead of LFB or SFB at the wash step after sequencing adapter ligation	Ethanol can denature the motor protein on the sequencing adapters. Make sure the LFB or SFB buffer was used after ligation of sequencing adapters.
Pore occupancy close to 0	No tether on the flow cell	Tethers are adding during flow cell priming (FLT/FCT tube). Make sure FLT/FCT was added to FB/FCF before priming.

Shorter than expected read length

Observation	Possible cause	Comments and actions
Shorter than expected read length	Unwanted fragmentation of DNA sample	Read length reflects input DNA fragment length. Input DNA can be fragmented during extraction and library prep. 1. Please review the Extraction Methods in the Nanopore Community for best practice for extraction. 2. Visualise the input DNA fragment length distribution on an agarose gel before proceeding to the library prep.  In the image above, Sample 1 is of high molecular weight, whereas Sample 2 has been fragmented. 3. During library prep, avoid pipetting and vortexing when mixing reagents. Flicking or inverting the tube is sufficient.

Large proportion of unavailable pores

Observation	Possible cause	Comments and actions
Large proportion of unavailable pores (shown as blue in the channels panel and pore activity plot)  The pore activity plot above shows an increasing proportion of "unavailable" pores over time.	Contaminants are present in the sample	Some contaminants can be cleared from the pores by the unblocking function built into MinKNOW. If this is successful, the pore status will change to "sequencing pore". If the portion of unavailable pores stays large or increases: 1. A nuclease flush using the Flow Cell Wash Kit (EXP-WSH004) can be performed, or 2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.

Large proportion of inactive pores

Observation	Possible cause	Comments and actions
Large proportion of inactive/unavailable pores (shown as light blue in the channels panel and pore activity plot. Pores or membranes are irreversibly damaged)	Air bubbles have been introduced into the flow cell	Air bubbles introduced through flow cell priming and library loading can irreversibly damage the pores. Watch the Priming and loading your flow cell video for best practice
Large proportion of inactive/unavailable pores	Certain compounds co-purified with DNA	Known compounds, include polysaccharides, typically associate with plant genomic DNA. 1. Please refer to the Plant leaf DNA extraction method. 2. Clean-up using the QIAGEN PowerClean Pro kit. 3. Perform a whole genome amplification with the original gDNA sample using the QIAGEN REPLI-g kit.
Large proportion of inactive/unavailable pores	Contaminants are present in the sample	The effects of contaminants are shown in the Contaminants Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover.

Reduction in sequencing speed and q-score later into the run

Observation	Possible cause	Comments and actions
Reduction in sequencing speed and q-score later into the run	For Kit 9 chemistry (e.g. SQK-LSK109), fast fuel consumption is typically seen when the flow cell is overloaded with library (please see the appropriate protocol for your DNA library to see the recommendation).	Add more fuel to the flow cell by following the instructions in the MinKNOW protocol. In future experiments, load lower amounts of library to the flow cell.

Temperature fluctuation

Observation	Possible cause	Comments and actions
Temperature fluctuation	The flow cell has lost contact with the device	Check that there is a heat pad covering the metal plate on the back of the flow cell. Re-insert the flow cell and press it down to make sure the connector pins are firmly in contact with the device. If the problem persists, please contact Technical Services.

Failed to reach target temperature

Observation	Possible cause	Comments and actions
MinKNOW shows "Failed to reach target temperature"	The instrument was placed in a location that is colder than normal room temperature, or a location with poor ventilation (which leads to the flow cells overheating)	MinKNOW has a default timeframe for the flow cell to reach the target temperature. Once the timeframe is exceeded, an error message will appear and the sequencing experiment will continue. However, sequencing at an incorrect temperature may lead to a decrease in throughput and lower q-scores. Please adjust the location of the sequencing device to ensure that it is placed at room temperature with good ventilation, then re-start the process in MinKNOW. Please refer to this FAQ for more information on MinION Mk 1B temperature control.

Guppy – no input .fast5 was found or basecalled

Observation	Possible cause	Comments and actions
No input .fast5 was found or basecalled	*input_path* did not point to the .fast5 file location	The *--input_path* has to be followed by the full file path to the .fast5 files to be basecalled, and the location has to be accessible either locally or remotely through SSH.
No input .fast5 was found or basecalled	The .fast5 files were in a subfolder at the *input_path* location	To allow Guppy to look into subfolders, add the *--recursive* flag to the command

Guppy – no Pass or Fail folders were generated after basecalling

Observation	Possible cause	Comments and actions
No Pass or Fail folders were generated after basecalling	The *--qscore_filtering* flag was not included in the command	The *--qscore_filtering* flag enables filtering of reads into Pass and Fail folders inside the output folder, based on their strand q-score. When performing live basecalling in MinKNOW, a q-score of 7 (corresponding to a basecall accuracy of ~80%) is used to separate reads into Pass and Fail folders.

Guppy – unusually slow processing on a GPU computer

Observation	Possible cause	Comments and actions
Unusually slow processing on a GPU computer	The *--device* flag wasn't included in the command	The *--device* flag specifies a GPU device to use for accelerate basecalling. If not included in the command, GPU will not be used. GPUs are counted from zero. An example is *--device cuda:0 cuda:1*, when 2 GPUs are specified to use by the Guppy command.