Oxford Nanopore Technologies

MINION: PROTOCOL

Ligation sequencing DNA V14 (SQK-LSK114)

V GDE_9161_v114_revAC_24Sep2025

This protocol:

- Uses genomic DNA
- Has a library preparation time of ~65 minutes
- Can be used with a fragmentation step (optional)
- Requires no PCR
- Is compatible with R10.4.1 flow cells

For Research Use Only

FOR RESEARCH USE ONLY

Contents

1. Overview of the protocol

Introduction to the Ligation Sequencing Kit V14 (SQK-LSK114) protocol

This protocol describes how to carry out sequencing of a DNA sample using the Ligation Sequencing Kit V14 (SQK-LSK114). It is recommended that a Lambda control experiment is completed first to become familiar with the technology.

Steps in the sequencing workflow:

Prepare for your experiment

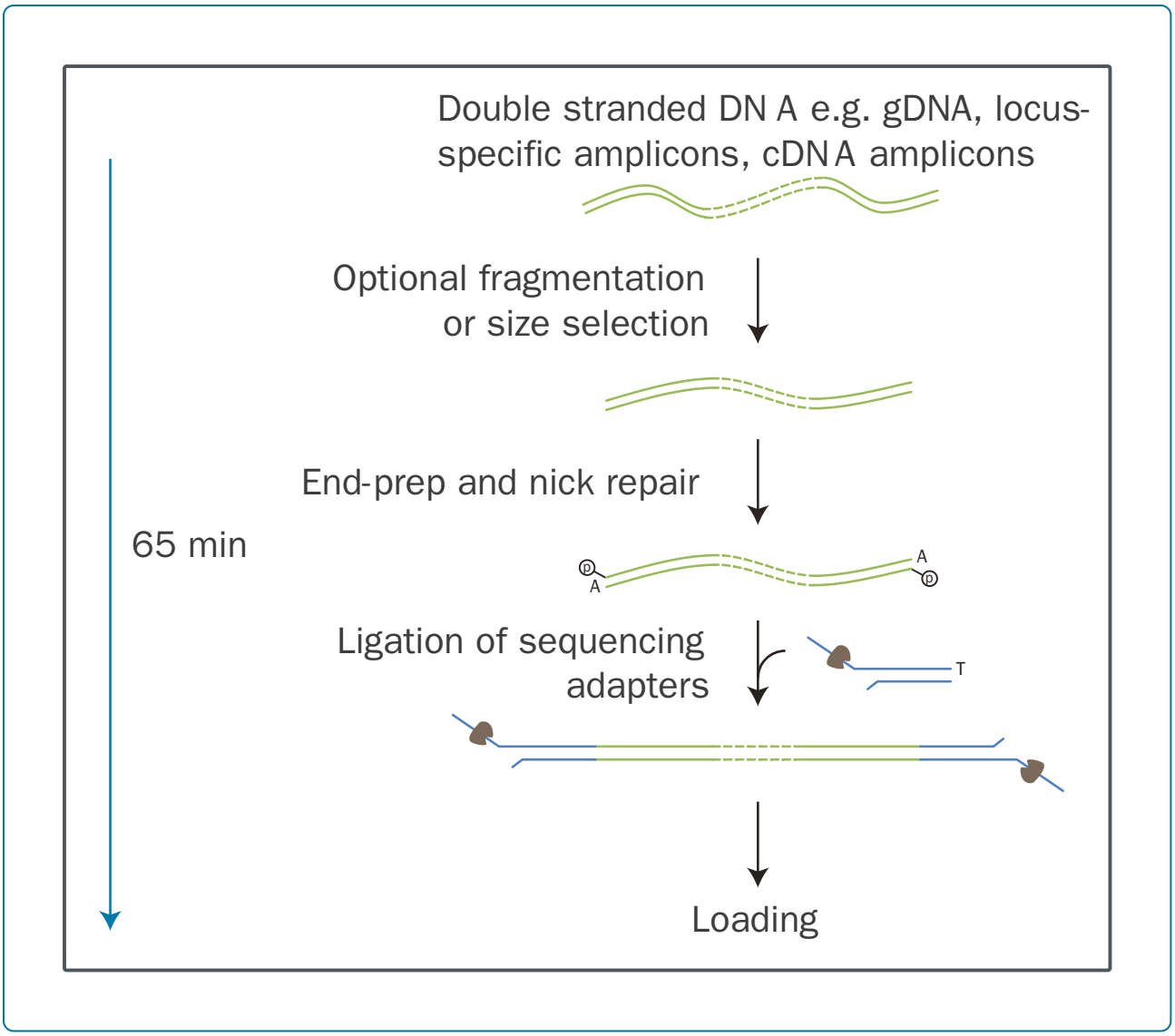
You will need to:

- 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

The Table below is an overview of the steps required in the library preparation, including timings and optional stopping points.

Library preparation	Process	Time	Stop option
DNA repair and end-prep	Repair the DNA and prepare the DNA ends for adapter attachment	35 minutes	4°C overnight
Adapter ligation and clean-up	Attach the sequencing adapters to the DNA ends	20 minutes	We recommend sequencing your library as soon as it is adapted. DNA library can be stored at 4°C for short-term storage or for repeated use (such as re-loading your flow cell) DNA library can be stored at -80°C for long-term storage.
Priming and loading the flow cell	Prime the flow cell and load the prepared library for sequencing	10 minutes	



Sequencing and analysis

You will need to:

- Start a sequencing run using the MinKNOW software which will collect raw data from the device and basecall reads.
- Start the EPI2ME software and select a bioinformatics workflow to analyse your data.

Compatibility of this protocol

This protocol should only be used in combination with:

- Ligation Sequencing Kit V14 (SQK-LSK114)
- Control Expansion (EXP-CTL001)
- R10.4.1 MinION Flow Cells (FLO-MIN114)
- Flow Cell Wash Kit (EXP-WSH004)
- MinION Mk1D - MinION Mk1D IT requirements document
- GridION - GridION IT requirements document

2. Equipment and consumables

Materials

1 µg (or 100-200 fmol) high molecular weight genomic DNA
OR >100 ng high molecular weight genomic DNA if performing DNA fragmentation
Ligation Sequencing Kit V14 (SQK-LSK114)

Consumables

MinION/GridION Flow Cell
Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
NEBNext® Companion Module v2 for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7672S or E7672L)
Freshly prepared 80% ethanol in nuclease-free water
Nuclease-free water (e.g. ThermoFisher, AM9937)
Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
1.5 ml Eppendorf DNA LoBind tubes
0.2 ml thin-walled PCR tubes
Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

MinION or GridION device
MinION/GridION Flow Cell Light Shield
Hula mixer (gentle rotator mixer)
Magnetic separation 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
Qubit™ fluorometer (or equivalent for QC check)

Adjust your sample input quantity depending on your initial DNA sample length:

Fragment library length	Starting input
Very short (<1 kb)	200 fmol
Short (1-10 kb)	100–200 fmol
Long (>10 kb)	1 µg

For more information on sample input and flow cell loading amounts for our ligation sequencing protocols please visit our know-how document.

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 v2 for Oxford Nanopore Technologies® Ligation Sequencing

We recommend buying the NEBNext® Companion Module v2 for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7672S or E7672L), which contains all the NEB reagents needed for use with the Ligation Sequencing Kit.

The previous version, NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L) is compatible, but the recommended v2 module offers more efficient dA-tailing and ligation, a result of the FFPEv2 DNA Repair Buffer and Salt-T4 DNA Ligase, respectively. A marked cost saving per sample preparation is also realised when using the v2 module.

Note: for our amplicon protocols, NEBNext FFPE DNA Repair Mix is not required and purchasing the required reagents separately is more cost effective.

Third-party reagents

We have validated and recommend the use of all the third-party reagents used in this protocol. Alternatives have not been tested by Oxford Nanopore Technologies.

For all third-party reagents, we recommend following the manufacturer's instructions to prepare the reagents for use.

Check your flow cell

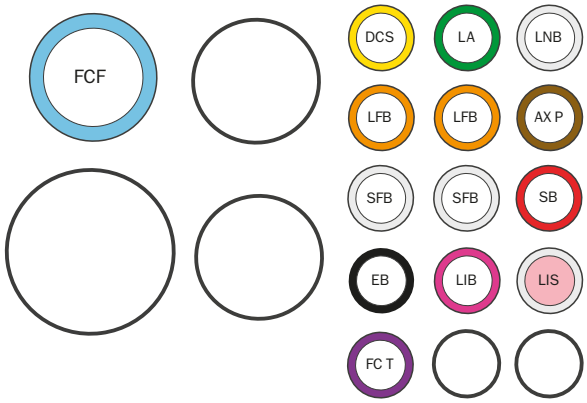
We highly recommend that you check the number of pores in your flow cell prior to starting a sequencing experiment. This should be done within 12 weeks of purchasing your MinION/GridION/PromethION Flow Cells or within four weeks of purchasing Flongle Flow Cells. Oxford Nanopore Technologies will replace any unused flow cell with fewer than the number of pores listed 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

We strongly recommend using the Ligation Buffer (LNB) supplied in the Ligation Sequencing Kit V14 rather than any third-party ligase buffers to ensure high ligation efficiency of the Ligation Adapter (LA).

Ligation Adapter (LA) included in this kit and protocol is not interchangeable with other sequencing adapters.

Ligation Sequencing Kit V14 (SQK-LSK114) contents



DCS : DNA Control Strand
LA : Ligation Adapter
LNB : Ligation Buffer
LFB : Long Fragment Buffer
SFB : Short Fragment Buffer
AXP : AMPure XP Beads
SB : Sequencing Buffer
EB : Elution Buffer
LIB : Library Beads
LIS : Library Solution
FCF : Flow Cell Flush
FCT : Flow Cell Tether

Note: This product contains AMPure XP reagent manufactured by Beckman Coulter, Inc. and can be stored at -20°C with the kit without detriment to reagent stability.

Note: The DNA Control Sample (DCS) is a 3.6 kb standard amplicon mapping the 3' end of the Lambda genome.

3. DNA repair and end-prep

Materials

1 µg (or 100-200 fmol) gDNA
DNA Control Sample (DCS)
AMPure XP Beads (AXP)

Consumables

NEBNext® FFPE DNA Repair Mix from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)
NEBNext® FFPE DNA Repair Buffer v2 from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)
NEBNext® Ultra II End Prep Enzyme Mix from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)
Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
Nuclease-free water (e.g. ThermoFisher, AM9937)
Freshly prepared 80% ethanol in nuclease-free water
Qubit™ Assay Tubes (Invitrogen, Q32856)
0.2 ml thin-walled PCR tubes
1.5 ml Eppendorf DNA LoBind tubes

Equipment

P1000 pipette and tips
P100 pipette and tips
P10 pipette and tips
Microfuge
Thermal cycler
Hula mixer (gentle rotator mixer)
Magnetic separation rack
Ice bucket with ice
Qubit™ fluorometer (or equivalent for QC check)

We recommend using the NEBNext® Companion Module v2 for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7672S or E7672L), which contains all the NEB reagents needed for use with the Ligation Sequencing Kit.

The previous version, NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L) is also compatible, but the recommended v2 module offers more efficient dA-tailing and ligation.

Check your flow cell

We recommend performing a flow cell check before starting your library prep to ensure you have a flow cell with sufficient pores for a good sequencing run.

See the flow cell check document for more information.

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

We recommend using the DNA Control Sample (DCS) in your library prep for troubleshooting purposes. However, you can omit this step and make up the extra 1 µl with your sample DNA.

2 Prepare the NEB 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. Vortex the FFPE DNA Repair Buffer v2 to ensure it is well mixed.
   Note: This buffer may contain a white precipitate. If this occurs, allow the mixture to come to room temperature and pipette the buffer several times to break up the precipitate, followed by a quick vortex to mix.
5. The FFPE DNA Repair Buffer v2 may have a yellow tinge and is fine to use if yellow.

3 Prepare the DNA in nuclease-free water:

1. Transfer 1 µg (or 100-200 fmol) input DNA into a 1.5 ml Eppendorf DNA LoBind tube.
2. Adjust the volume to 47 µl with nuclease-free water.
3. Mix thoroughly by pipetting up and down, or by flicking the tube.
4. 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 v2	7 µl
NEBNext FFPE DNA Repair Mix	2 µl
Ultra II End-prep Enzyme Mix	3 µl
Total	**60 µl**

5 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

6. **Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes. Then cool down to between 4°C and 20°C on the thermal cycler or place the samples on ice.**

7. **Resuspend the AMPure XP Beads (AXP) by vortexing.**

8. **Spin down and transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.**

9. **Add 60 µl of resuspended the AMPure XP Beads (AXP) 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. **Freshly prepare 500 µl of 80% 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 80% 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 by gently pipetting up and down or by flicking the tube. 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.**

Quantify 1 µl of eluted sample using a Qubit fluorometer.

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.

4. Adapter ligation and clean-up

Materials

Ligation Adapter (LA)
Ligation Buffer (LNB)
Long Fragment Buffer (LFB)
S Fragment Buffer (SFB)
AMPure XP Beads (AXP)
Elution Buffer (EB)

Consumables

Salt-T4® DNA Ligase (NEB, M0467)
1.5 ml Eppendorf DNA LoBind tubes
Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

Magnetic separation rack
Microfuge
Vortex mixer
P1000 pipette and tips
P100 pipette and tips
P20 pipette and tips
P10 pipette and tips
Qubit™ fluorometer (or equivalent for QC check)

We recommend using the Salt-T4® DNA Ligase (NEB, M0467).

Salt-T4® DNA Ligase (NEB, M0467) can be bought separately or is provided in the NEBNext® Companion Module v2 for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7672S or E7672L).

The Quick T4 DNA Ligase (NEB, E6057) available in the previous version NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L) is also compatible, but the new recommended reagent offers more efficient end ligation.

Although third-party ligase products may be supplied with their own buffer, the ligation efficiency of the Ligation Adapter (LA) is higher when using the Ligation Buffer (LNB) supplied in the Ligation Sequencing Kit.

1. **Spin down the Ligation Adapter (LA) and Salt-T4® DNA 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.**

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 **Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at room temperature and mix by vortexing. Then spin down and keep at room temperature.**

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

6 **Thoroughly mix the reaction by gently pipetting and briefly spinning down.**

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

8 **Resuspend the AMPure XP Beads (AXP) by vortexing.**

9 **Add 40 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by flicking the tube.**

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

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

12 **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.**

13 **Repeat the previous step.**

14 **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.**

15 **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.**

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

17 **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

Quantify 1 µl of eluted sample using a Qubit fluorometer.

18 **Depending on your DNA library fragment size, prepare your final library in 12 µl of Elution Buffer (EB).**

Fragment library length	Flow cell loading amount
Very short (<1 kb)	100 fmol
Short (1-10 kb)	35–50 fmol
Long (>10 kb)	300 ng

Note: If the library yields are below the input recommendations, load the entire library.

If required, we recommend using a mass to mol calculator such as the NEB calculator.

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

Library storage recommendations

We recommend storing libraries in Eppendorf DNA LoBind tubes at **4°C for short-term** storage or repeated use, for example, re-loading 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.

5. Priming and loading the MinION and GridION Flow Cell

Materials

Flow Cell Flush (FCF)
Flow Cell Tether (FCT)
Library Solution (LIS)
Library Beads (LIB)
Sequencing Buffer (SB)

Consumables

MinION/GridION Flow Cell
Nuclease-free water (e.g. ThermoFisher, AM9937)
Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
1.5 ml Eppendorf DNA LoBind tubes

Equipment

MinION or GridION device
MinION/GridION Flow Cell Light Shields

P1000 pipette and tips
P100 pipette and tips
P20 pipette and tips
P10 pipette and tips

Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).

Take the flow cell out of the fridge and leave it at room temperature for 20 minutes. This will improve visibility of the array during priming and sample loading.

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 (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.**

For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), add Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.

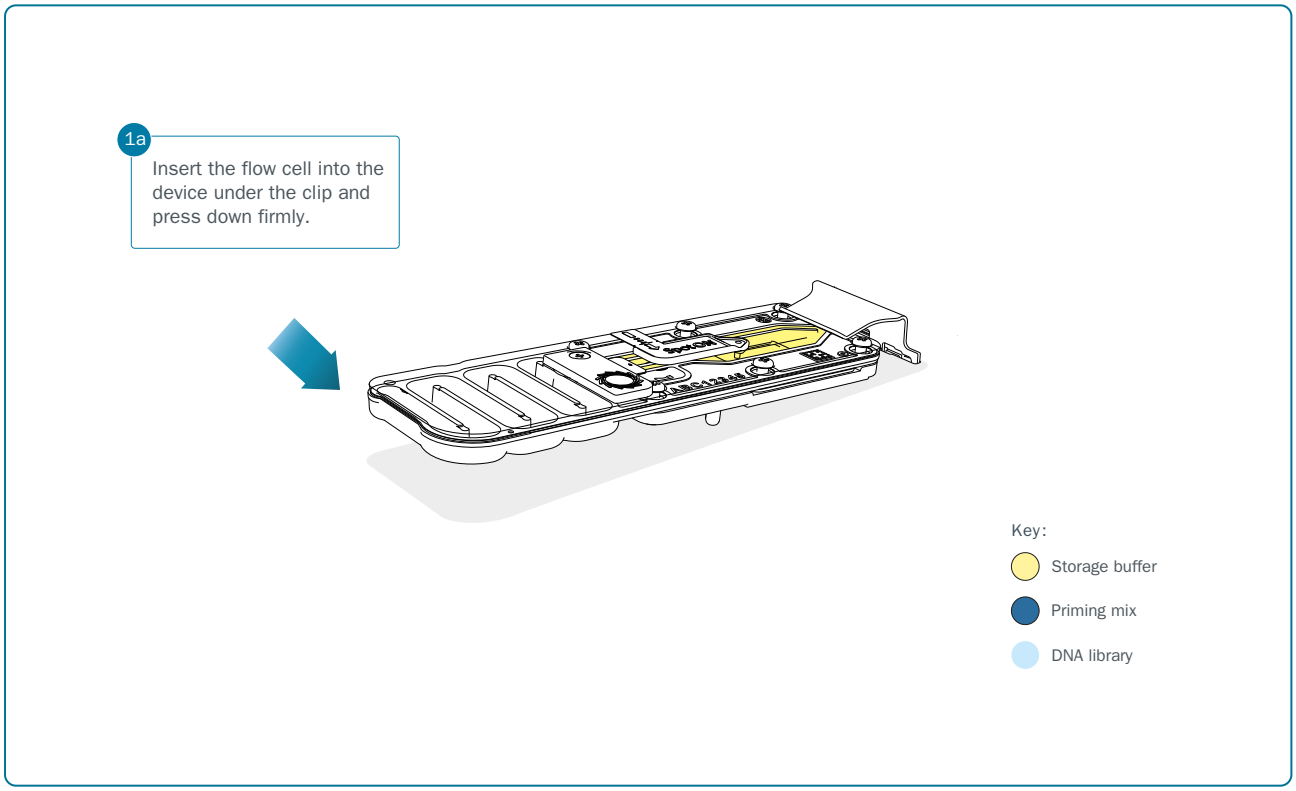
Note: We do not recommend using any other albumin type (e.g. recombinant human serum albumin).

2 **To prepare the flow cell priming mix with BSA, combine Flow Cell Flush (FCF) and Flow Cell Tether (FCT), as directed below. Mix by pipetting at room temperature.**

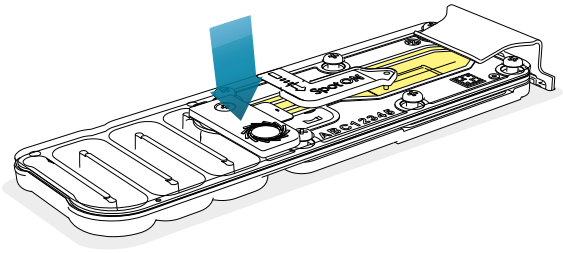
In a suitable tube for the number of flow cells, combine the following reagents:

Reagent	Volume per flow cell
Flow Cell Flush (FCF)	1,170 µl
Bovine Serum Albumin (BSA) at 50 mg/ml	5 µl
Flow Cell Tether (FCT)	30 µl
Total volume	**1,205 µl**

3 Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the priming port cover to ensure correct thermal and electrical contact.



1b Insert the flow cell into the device under the clip and press down firmly on the Priming port cover.



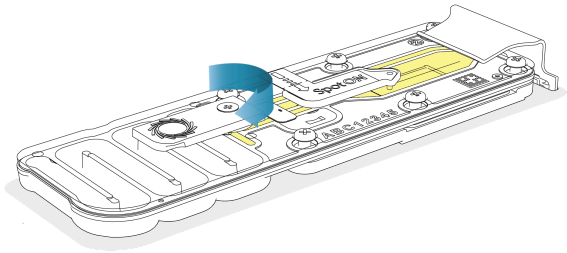
Complete a flow cell check to assess the number of pores available before loading the library.

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

See the flow cell check document for more information.

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

2 Slide open the **Priming port** cover.

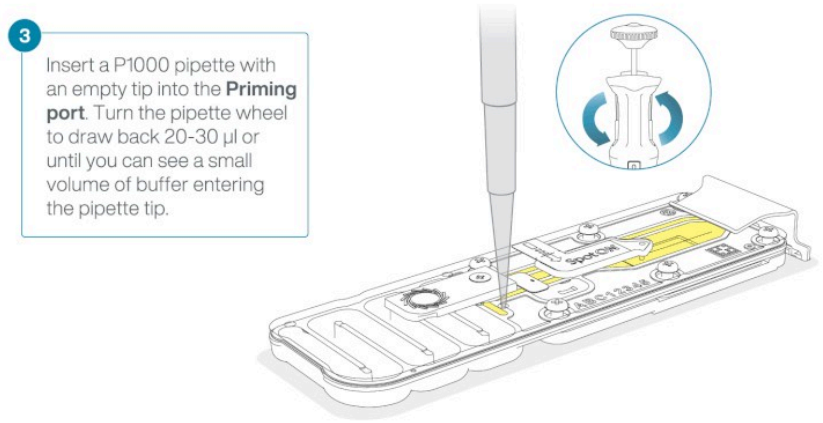


Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

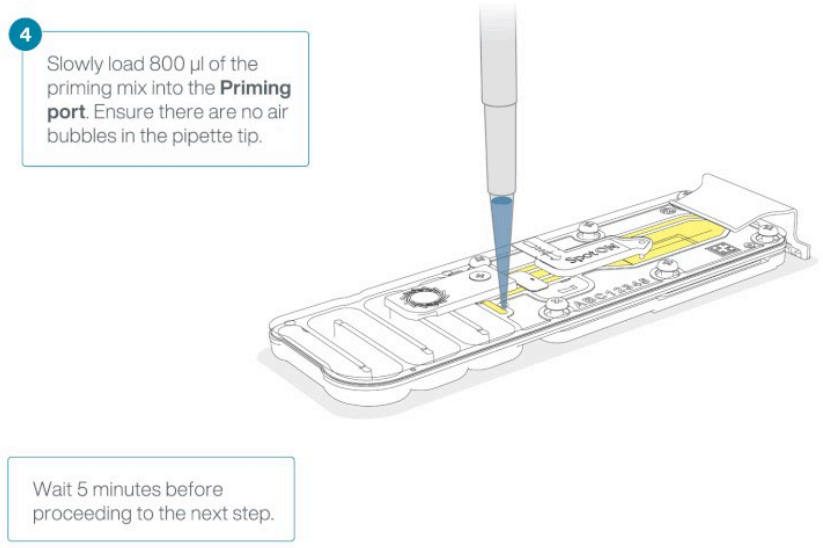
5 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:

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.**



7 **Thoroughly mix the contents of the Library Beads (LIB) by pipetting.**

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

We recommend using the Library Beads (LIB) for most sequencing experiments. However, the Library Solution (LIS) is available for more viscous libraries.

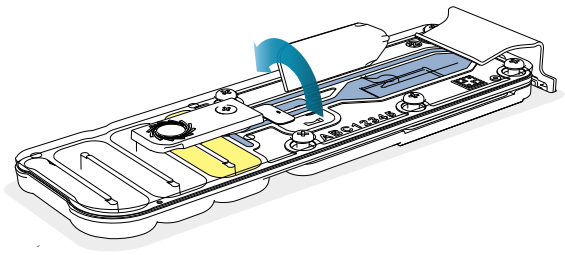
8 In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:

Reagent	Volume per flow cell
Sequencing Buffer (SB)	37.5 µl
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using	25.5 µl
DNA library	12 µl
Total	**75 µl**

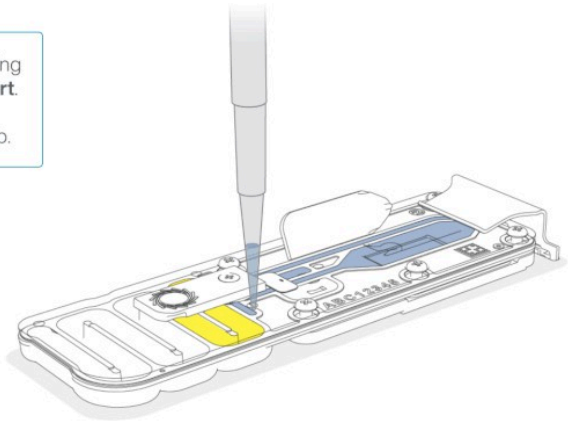
9 Complete the flow cell priming:

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.



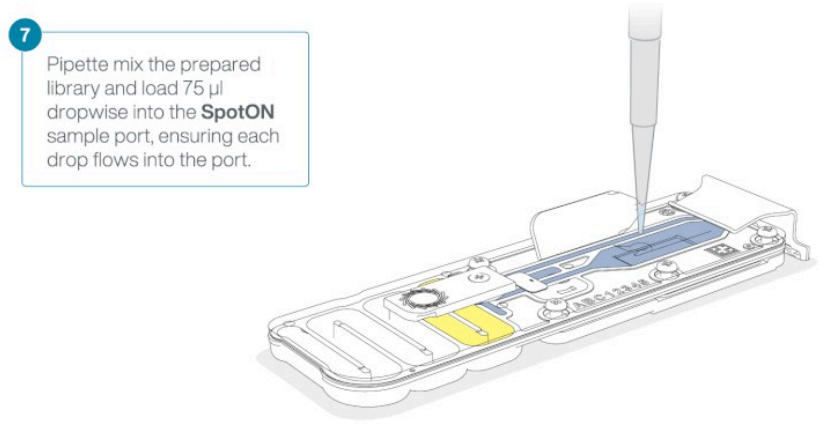
6 Load 200 µl of the priming mix into the **Priming Port**. Ensure there are no air bubbles in the pipette tip.



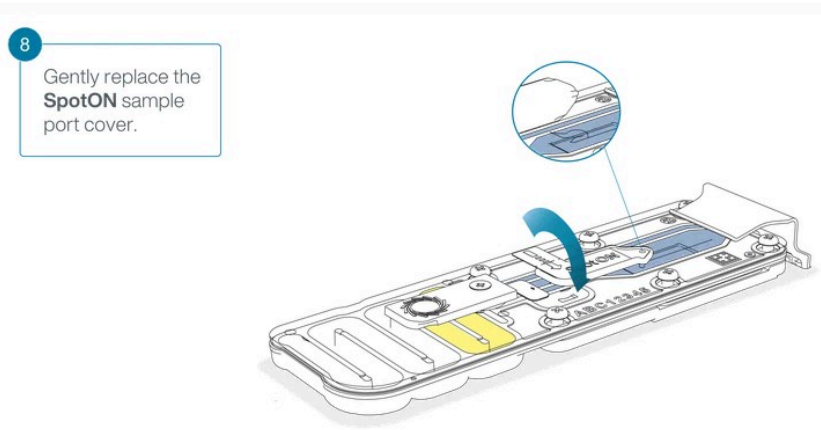
10 **Mix the prepared library gently by pipetting up and down just prior to loading.**

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**

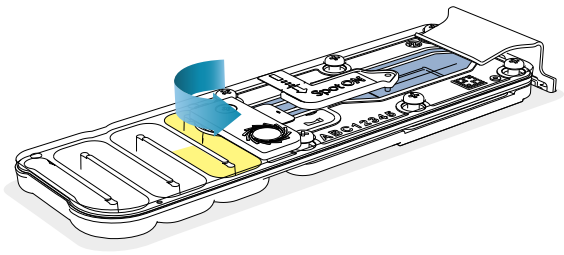
the next.



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



9 Gently close the **Priming port**.



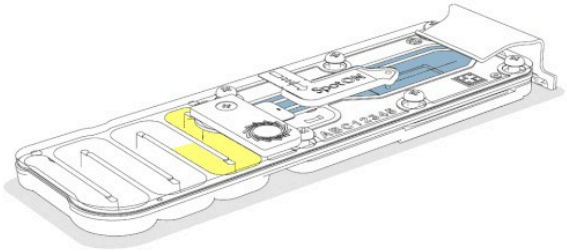
For optimal sequencing output, install the light shield on your flow cell as soon as the library has been loaded.

We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

13 Place the light shield onto the flow cell, as follows:

1. Carefully place the leading edge of the light shield against the clip. **Note:** Do not force the light shield underneath the clip.
2. Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.

10 Carefully align the **light shield** against the clip and lower onto the flow cell.



The MinION Flow Cell Light Shield is not secured to the flow cell. Therefore, careful handling is required after installation.

Close the device lid and set up a sequencing run on MinKNOW.

When a flow cell is inserted into the MinION Mk1D, the device lid will sit on top of the flow cell, leaving a small gap around the sides. This is normal and has no impact on the performance of the device.

Please refer to this FAQ regarding the device lid.

6. 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 or PromethION 24/48 sequencing device.

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

- MinION Mk1D 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 **Ligation Sequencing Kit V14 (SQK-LSK114)** 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** to initiate 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.

7. 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°C to +8°C.**

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

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 send the flow cell back to Oxford Nanopore.**

Instructions for returning flow cells can be found here.

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.

8. Downstream analysis

Post-basecalling analysis

There are several options for further analysing your basecalled data:

EPI2ME workflows

For in-depth data analysis, Oxford Nanopore Technologies offers a range of bioinformatics tutorials and workflows available in EPI2ME, which are available in the EPI2ME section of the Community. 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.

Research analysis tools

Oxford Nanopore Technologies' Research division has created a number of analysis tools, that 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.

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 resource centre and search for bioinformatics tools for your application. 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.

9. Issues during DNA/RNA extraction and library preparation

Below is a list of the most commonly encountered issues, with some suggested

causes and solutions.

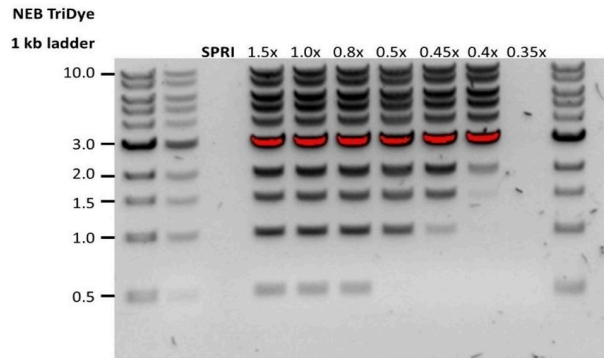
We also have an FAQ section available on the Nanopore Community Support section.

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.

10. 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 videos for how to load a MinION Flow Cell and how to load a PromethION Flow Cell.
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.

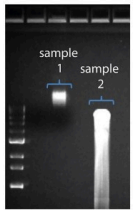
MinKNOW script failed

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.

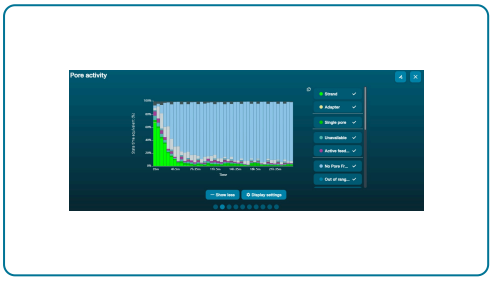
Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	Ensure the correct volume and concentration as stated on the appropriate protocol for your sequencing library is loaded onto the flow cell. Please quantify the library before loading and calculate fmols using tools like the Promega Biomath Calculator, choosing "dsDNA: µg to fmol"
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 tube for Kit 9, 10, 11, FCT for Kit 14, and FTU for ultra-long DNA kits). Make sure FLT/FCT/FTU was added to the buffer (FB for Kit 9, 10, 11, and FCF for Kit 14) 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 how to load a MinION Flow Cell or how to load a PromethION Flow Cell videos 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.

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 link for more information on MinION temperature control.