

Overview of the protocol

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

This protocol describes how to carry out sequencing of amplicon 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.

For information about Kit 14 chemistry, please see the [Kit 14 sequencing and duplex basecalling info sheet](#).

Steps in the sequencing workflow:

Prepare for your experiment

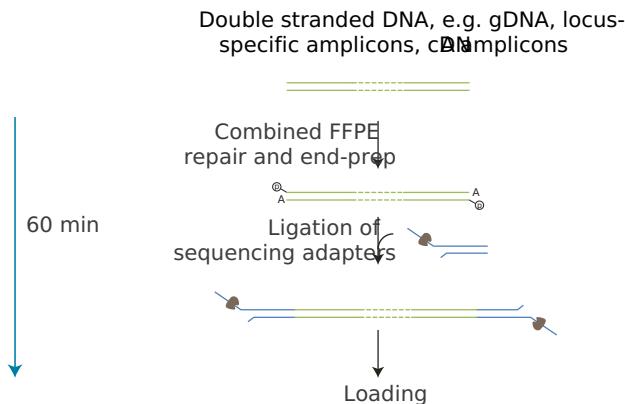
You will need to:

- 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 stopping points.

Library preparation step	Process	Time	Stop option
End-prep	Prepare the DNA ends for adapter attachment.	35 minutes	4°C overnight
Adapter ligation and clean-up	Attach sequencing adapters to the DNA ends and perform a bead clean-up.	30 minutes	4°C short-term storage or for repeated use, such as re-loading your flow cell. -80°C for single-use long-term storage. We strongly recommend sequencing your library as soon as it is adapted.
Priming and loading the flow cell	Prime the flow cell and load the prepared library for sequencing.	5 minutes	



Sequencing and analysis

You will need to:

- Start a sequencing run using the MinKNOW software which will collect raw data and convert it into basecalled reads

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Ligation Sequencing Kit V14 (SQK-LSK114)
- Control Expansion (EXP-CTL001)
- Flongle Sequencing Expansion (EXP-FSE002)
- R10.4.1 Flongle Flow Cells (FLO-FLG114)
- MinION Mk1B - [MinION Mk1B IT requirements document](#)
- MinION Mk1C - [MinION Mk1C IT requirements document](#)
- GridION - [GridION IT requirements document](#)

Equipment and consumables

Materials

- 50-100 fmol of amplicon DNA
- Ligation Sequencing Kit V14 (SQK-LSK114)
- Flongle Sequencing Expansion (EXP-FSE002)

Consumables

- Flongle Flow Cell
 - NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L).
- Alternatively, you can use the NEBNext® products below:
- 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 80% ethanol in nuclease-free water
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)

Equipment

- Flongle adapter
- MinION or GridION device
- 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
- Qubit fluorometer (or equivalent for QC check)

IMPORTANT

Flow cell deterioration/saturation

At Oxford Nanopore we look to continuously improve our production processes to deliver a more robust product. In the case of Flongle, we are seeing the stability of the flow cells we ship improve. However for a small number of flow cells, upon loading, the flow cell rapidly deteriorates. This can be seen as saturation in the MinKNOW GUI. We are working hard to resolve this, however in the meantime we suggest the following loading recommendations and to use the buffers from the Flongle Sequencing Expansion (EXP-FSE002) shipped with your Flongle flow cells. If you do see rapid deteriorate/saturation on your flow cell, please contact support@nanoporetech.com for assistance.

Loading recommendations

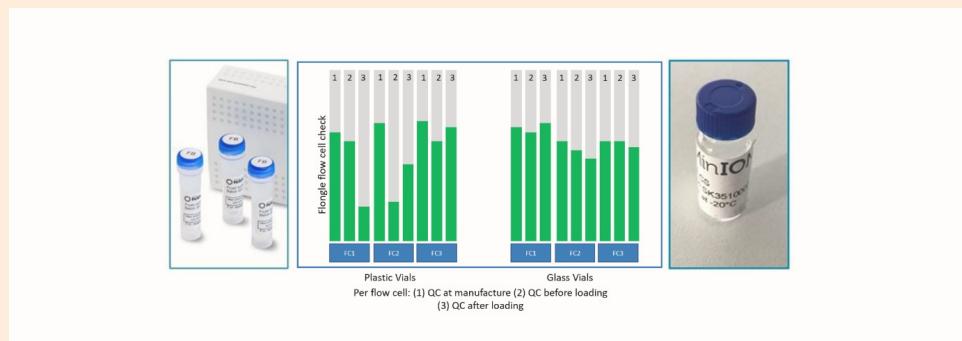
Following standard input recommendations, the protocol should produce enough final library (adapted DNA in EB) to load at least two Flongle flow cells. We recommend reserving enough library to load a second Flongle flow cell should you need to generate more data from a second flongle flow cell.

IMPORTANT

Flongle Sequencing Expansion (EXP-FSE002)

There are three buffers that come into direct contact with a flow cell at point of loading (SB: Sequencing Buffer, FCF: Flow Cell Flush and LIB: Library Beads or LIS: Library Solution). When looking at these buffers, we found that there are a very low level of contaminants seeping out of the plastic vials that impacts the robustness of the Flongle flow cell system (MinION and PromethION are not impacted by this).

We have found that when storing these buffers in glass vials instead of plastic, incidence of deterioration is reduced.



To rapidly deploy this to Flongle users, we have produced a Flongle Sequencing Expansion (EXP-FSE002) with these three components in glass vials, which can perform 12 Flongle flow cell loads in total.

To load a library onto your Flongle flow cell, you will need to use the following components:

Flongle Sequencing Expansion (EXP-FSE002) components

- Sequencing Buffer (SB)
- Flow Cell Flush (FCF)
- Library Beads (LIB) or Library Solution (LIS)

Sequencing Kit components

- Flow Cell Tether (FCT)

Oxford Nanopore Technologies deem the useful life of the Flow Cell Expansion to be 6 months from receipt by the customer.

For this protocol, you will need 50-100 fmol amplicon DNA.

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 [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.

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.

Flow cell check

We strongly recommend performing a flow cell check before loading a DNA or RNA library to assess the number of pores available.

Oxford Nanopore Technologies will replace any flow cell that falls below the warranty number of active pores within three months of purchase, provided that you report the results within two days of performing the flow cell check and you have followed the storage recommendations.

Flow cell	Minimum number of active pores covered by warranty
Flongle Flow Cell (FLO-FLG001)	50
MinION/GridION Flow Cell	800
PromethION Flow Cell	5000

IMPORTANT

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

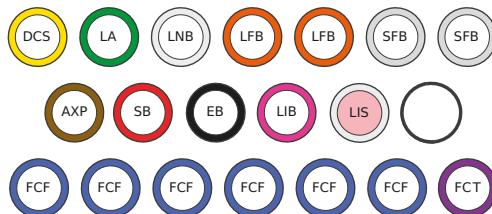
IMPORTANT

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

Ligation Sequencing Kit V14 (SQK-LSK114) contents

Note: We are in the process of reformatting our kits with single-use tubes into a bottle format.

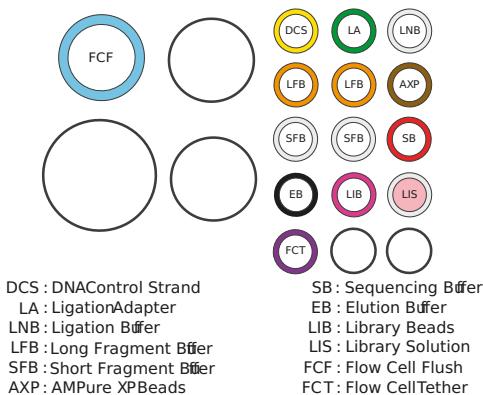
Single-use tubes format:



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

Bottle format:



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.

Flongle Sequencing Expansion (EXP-FSE002) contents



SB : Sequencing Buffer
 LIB : Library Beads
 LIS : Library Solution
 FCF : Flow Cell Flush

Name	Acronym	Cap colour	Number of vials	Fill volume per vial (µl)
Sequencing Buffer	SB	Blue	1	250
Library Beads	LIB	Blue	1	200
Library Solution	LIS	Blue	1	200

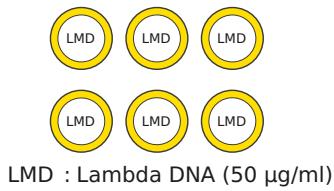
Name	Acronym	Cap colour	Number of vials	Fill volume per vial (µl)
Flow Cell Flush	FCF	Blue	1	1,600

Oxford Nanopore Technologies deem the useful life of the Flow Cell Expansion to be 6 months from receipt by the customer.

IMPORTANT

Please note that Oxford Nanopore Technologies deem the useful life of the Flongle Sequencing Expansion (EXP-FSE002) to be 6 months from receipt by the customer.

Control Expansion (EXP-CTL001) contents



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

End-prep

~35 minutes

Materials

- 50-100 fmol of amplicon DNA
- DNA Control Sample (DCS)
- AMPure XP Beads (AXP)

Consumables

- NEBNext® Ultra II End Repair / dA-tailing Module (NEB, E7546)
- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes
- Freshly prepared 80% ethanol in nuclease-free water
- Qubit™ Assay Tubes (Invitrogen, Q32856)

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
- Qubit fluorometer (or equivalent for QC check)

Check your flow cell.

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

See the [flow cell check instructions](#) in the MinKNOW protocol for more information.

IMPORTANT

Flow cell deterioration/saturation

At Oxford Nanopore we look to continuously improve our production processes to deliver a more robust product. In the case of Flongle, we are seeing the stability of the flow cells we ship improve. However for a small number of flow cells, upon loading, the flow cell rapidly deteriorates. This can be seen as saturation in the MinKNOW GUI. We are working hard to resolve this, however in the meantime we suggest the following loading recommendations and to use the buffers from the Flongle Sequencing Expansion (EXP-FSE002) shipped with your Flongle flow cells. If you do see rapid deteriorate/saturation on your flow cell, please contact support@nanoporetech.com for assistance.

Loading recommendations

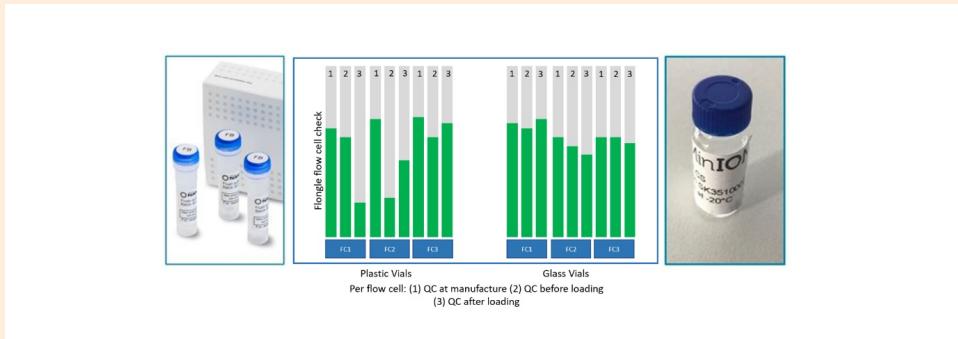
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IMPORTANT

Flongle Sequencing Expansion (EXP-FSE002)

There are three buffers that come into direct contact with a flow cell at point of loading (SB: Sequencing Buffer, FCF: Flow Cell Flush and LIB: Library Beads or LIS: Library Solution). When looking at these buffers, we found that there are a very low level of contaminants seeping out of the plastic vials that impacts the robustness of the Flongle flow cell system (MinION and PromethION are not impacted by this).

We have found that when storing these buffers in glass vials instead of plastic, incidence of deterioration is reduced.



To rapidly deploy this to Flongle users, we have produced a Flongle Sequencing Expansion (EXP-FSE002) with these three components in glass vials, which can perform 12 Flongle flow cell loads in total.

To load a library onto your Flongle flow cell, you will need to use the following components:

Flongle Sequencing Expansion (EXP-FSE002) components

- Sequencing Buffer (SB)
- Flow Cell Flush (FCF)
- Library Beads (LIB) or Library Solution (LIS)

Sequencing Kit components

- Flow Cell Tether (FCT)

Oxford Nanopore Technologies deem the useful life of the Flow Cell Expansion to be 6 months from receipt by the customer.

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 (25 ng-125 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 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 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 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.

IMPORTANT

Do not vortex the NEBNext Ultra II End Prep Enzyme Mix.

IMPORTANT

It is important that the NEBNext Ultra II End Prep Reaction Buffer is mixed well by vortexing.

Check for any visible precipitate; vortexing for at least 30 seconds may be required to solubilise all precipitate.

3 Prepare the amplicon DNA in nuclease-free water:

- Transfer 50-100 fmol of amplicon DNA into a 1.5 ml Eppendorf DNA LoBind tube
- Adjust the volume to 24.5 µl with nuclease-free water
- Mix thoroughly by flicking the tube to avoid unwanted shearing
- Spin down briefly in a microfuge

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

Reagent	Volume
DCS	0.5 µl
DNA	24.5 µl
Ultra II End-prep Reaction Buffer	3.5 µl
Ultra II End-prep Enzyme Mix	1.5 µl
Total	30 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.**
- 7 Resuspend the AMPure XP Beads (AXP) by vortexing.**
- 8 Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.**
- 9 Add 30 µl of resuspended 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 Prepare 500 µl of fresh 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 31 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 31 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

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.

Adapter ligation and clean-up

~20 minutes

Materials

- Ligation Adapter (LA)
- Ligation Buffer (LNB) from the Ligation Sequencing Kit
- Long Fragment Buffer (LFB)
- Short Fragment Buffer (SFB)
- AMPure XP Beads (AXP)
- Elution Buffer (EB) from the Oxford Nanopore sequencing kit

Consumables

- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- NEBNext Quick Ligation Module (NEB, E6056)
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- Magnetic 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)

IMPORTANT

Although the recommended third-party ligase is supplied with its 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 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 Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at room temperature and mix by vortexing. Then spin down and place on ice.

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

Reagent	Volume
DNA sample from the previous step	30 µl
Ligation Buffer (LNB)	12.5 µl
NEBNext Quick T4 DNA Ligase	5 µl
Ligation Adapter (LA)	2.5 µl
Total	50 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 20 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 125 µl Long Fragment Buffer (LFB) or 125 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, 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 pellet in 7 µl Elution Buffer (EB). 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 7 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 Prepare your final library to 5-10 fmol in 5 µl of Elution Buffer (EB).

IMPORTANT

We recommend loading 5-10 fmol of this final prepared library onto the R10.4.1 flow cell.

Following standard input recommendations, the protocol should produce enough final library (adapter DNA in EB) to load at least two Flongle flow cells. We recommend reserving enough library to load onto a second flow cell. Loading more than 10 fmol can have a detrimental effect on output and reduce the rate of duplex read capture. Dilute the library in EB or nuclease-free water to a final volume of 5 µl.

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, 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.

Loading the Flongle Flow Cell

~5 minutes

Materials

- Flongle Sequencing Expansion (EXP-FSE002)
- Flow Cell Tether (FCT)

Consumables

- Flongle Flow Cell
- 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- Flongle adapter
- MinION or GridION device
- P200 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

IMPORTANT

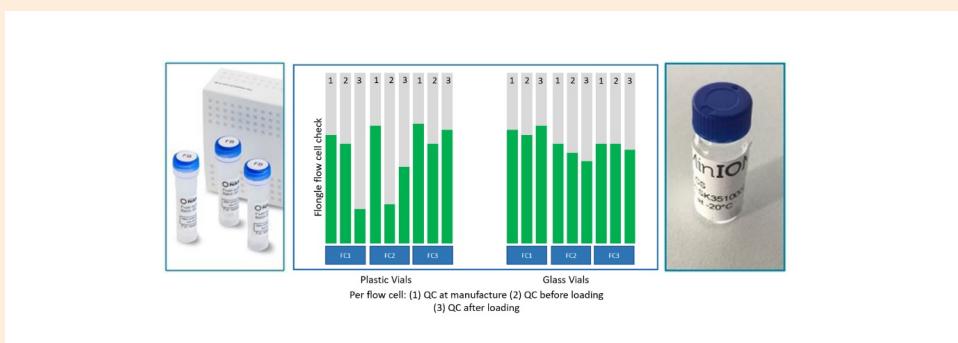
Please note, this kit is only compatible with R10.4.1 flow cells (FLO-FLG114).

IMPORTANT

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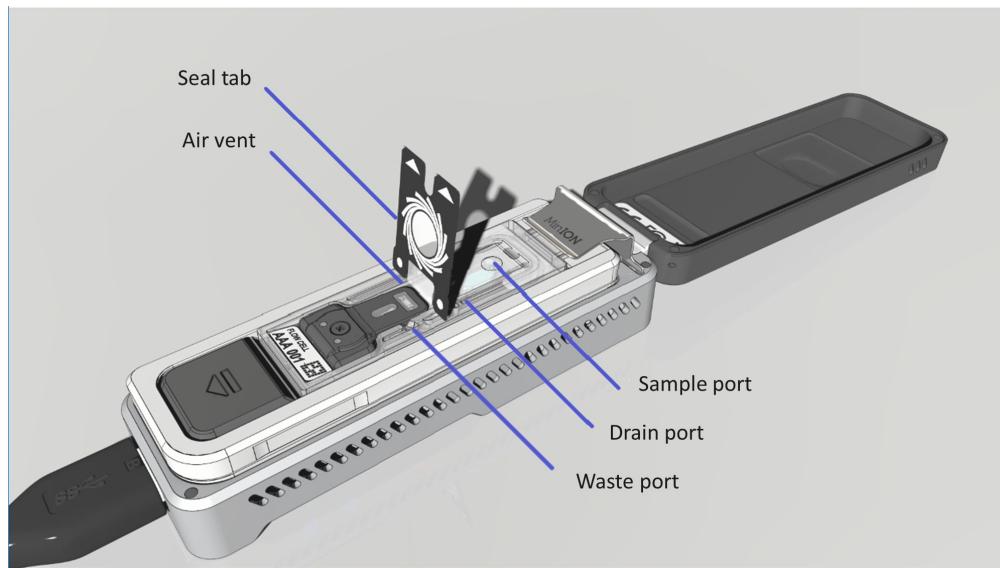
Oxford Nanopore Technologies deem the useful life of the Flow Cell Expansion to be 6 months from receipt by the customer.

IMPORTANT

Do NOT touch the reverse side of the Flongle flow cell array or the contact pads on the Flongle adapter. ALWAYS wear gloves when handling Flongle flow cells and adapters to avoid damage to the flow cell or adapter.



The diagram below shows the components of the Flongle flow cell:



The seal tab, air vent, waste channel, drain port and sample port are visible here. The sample port, drain port and air vent only become accessible once the seal tab is peeled back.

- 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.**

2 In a fresh 1.5 ml Eppendorf DNA LoBind tube, mix 117 µl of Flow Cell Flush (FCF) with 3 µl of Flow Cell Tether (FCT) and mix by pipetting.

3 Place the Flongle adapter into the MinION or one of the five GridION positions.

The adapter should sit evenly and flat on the MinION Mk1B or GridION platform. This ensures the flow cell assembly is flat during the next stage.

IMPORTANT

The adapter needs to be plugged into your device, and the device should be plugged in and powered on before inserting the Flongle flow cell.



4 Place the flow cell into the Flongle adapter, and press the flow cell down until you hear a click.

The flow cell should sit evenly and flat inside the adapter, to avoid any bubbles forming inside the fluidic compartments.



IMPORTANT

How to prime and load a Flongle flow cell

5 Peel back the seal tab from the Flongle flow cell, up to a point where the sample port is exposed, as follows:

1. Lift up the seal tab:



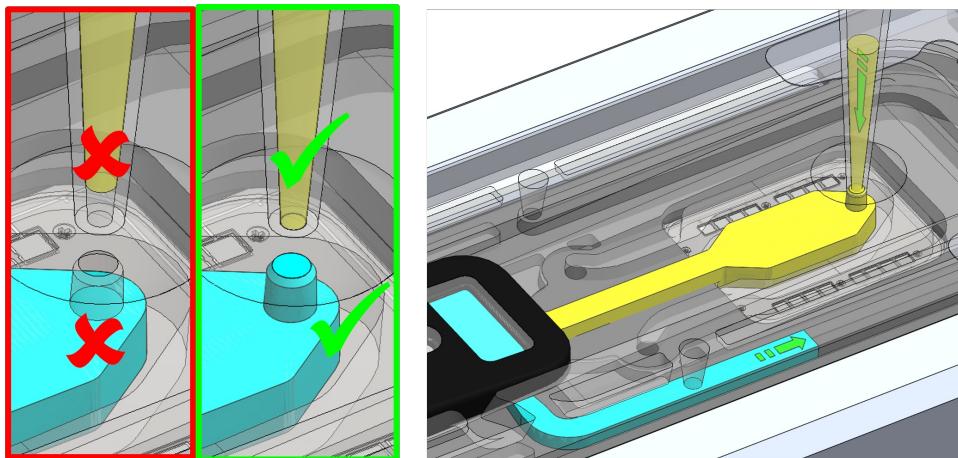
2. Pull the seal tab to open access to the sample port:



3. Hold the seal tab open by using adhesive on the tab to stick to the MinION Mk 1B lid:



- 6 To prime your flow cell with the mix of Flow Cell Flush (FCF) and Flow Cell Tether (FCT) that was prepared earlier, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 pipette tip inside the sample port and slowly dispense the 120 µl of priming fluid into the Flongle flow cell by slowly pipetting down. We also recommend twisting the pipette plunger down to avoid flushing the flow cell too vigorously.**



IMPORTANT

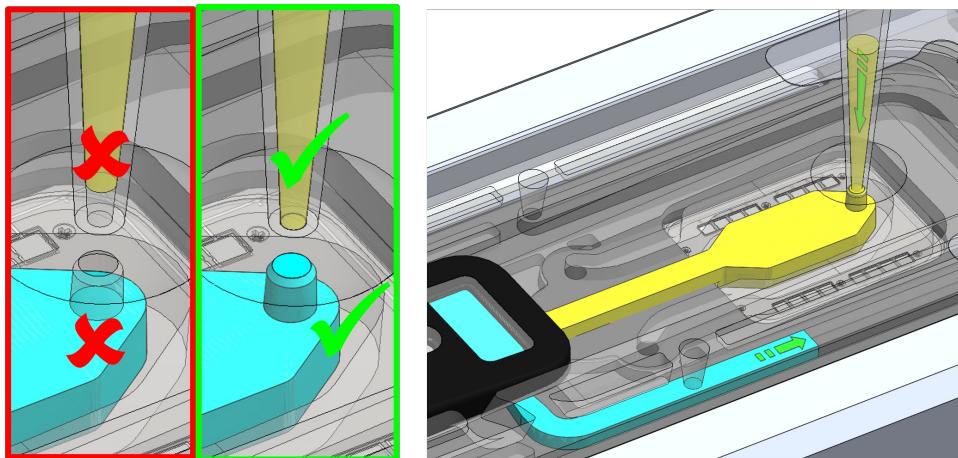
The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

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

- 7 Vortex the vial of Library Beads (LIB). Note that the beads settle quickly, so immediately prepare the Sequencing Mix in a fresh 1.5 ml Eppendorf DNA LoBind tube for loading the Flongle, as follows:**

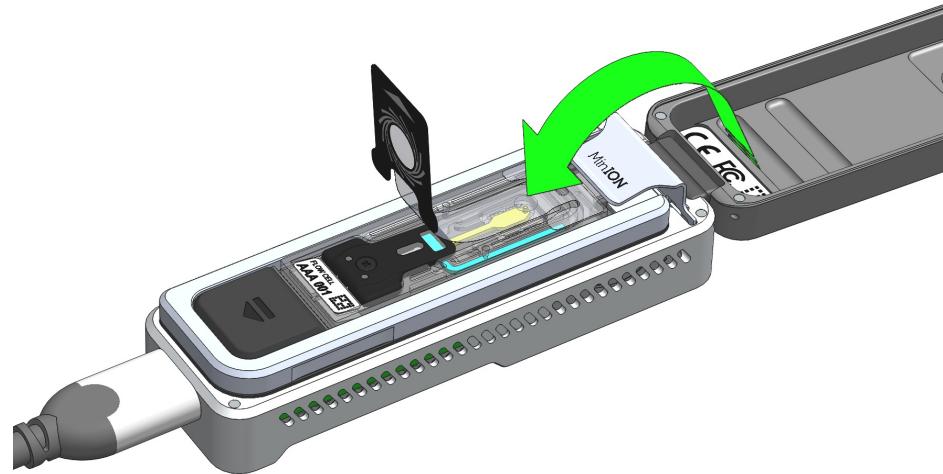
Reagents	Volume
Sequencing Buffer (SB)	15 µl
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using.	10 µl
DNA library	5 µl
Total	30 µl

8 To add the Sequencing Mix to the flow cell, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 tip inside the sample port and slowly dispense the Sequencing Mix into the flow cell by slowly pipetting down. We also recommend twisting the pipette plunger down to avoid flushing the flow cell too vigorously.

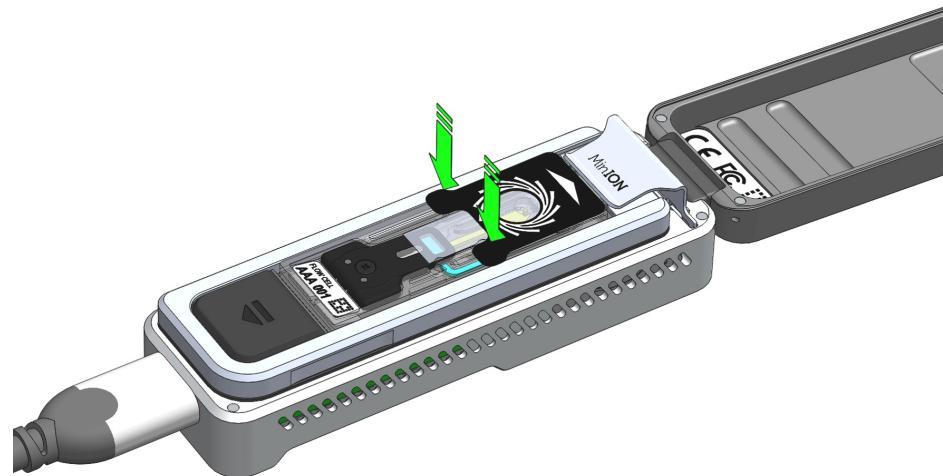


9 Seal the Flongle flow cell using the adhesive on the seal tab, as follows:

1. Stick the transparent adhesive tape to the sample port.



2. Replace the top (Wheel icon section) of the seal tab to its original position.



END OF STEP

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

Data acquisition and basecalling

How to start sequencing

Once you have loaded your flow cell, the sequencing run can be started on MinNOW, 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:

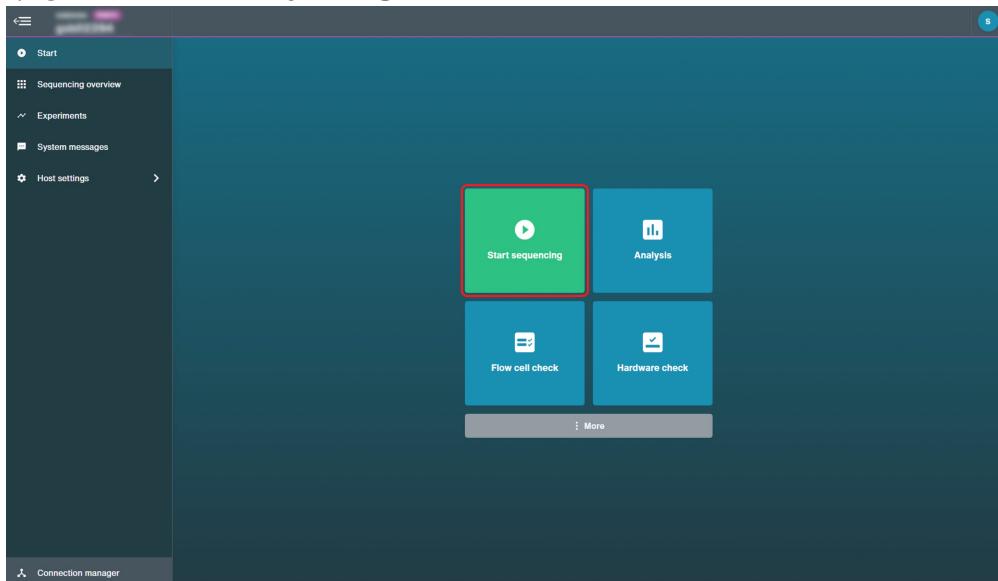
- On a computer either directly or remotely connected to a sequencing device.
- Directly on a GridION, MinION Mk1C or PromethION 24/48 sequencing device.

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

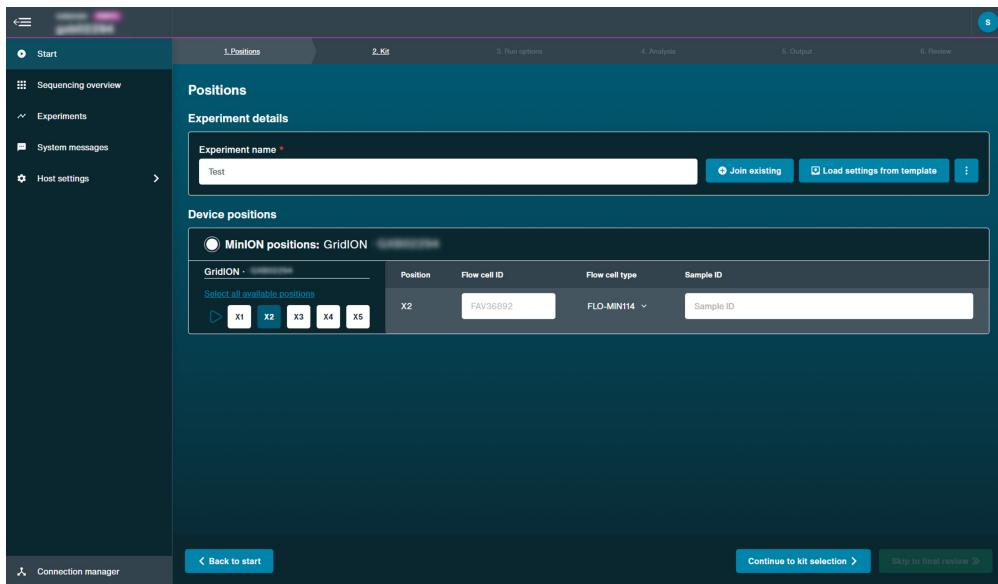
- [MinION Mk1C user manual](#)
- [MinION Mk1B user manual](#)
- [GridION user manual](#)

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.

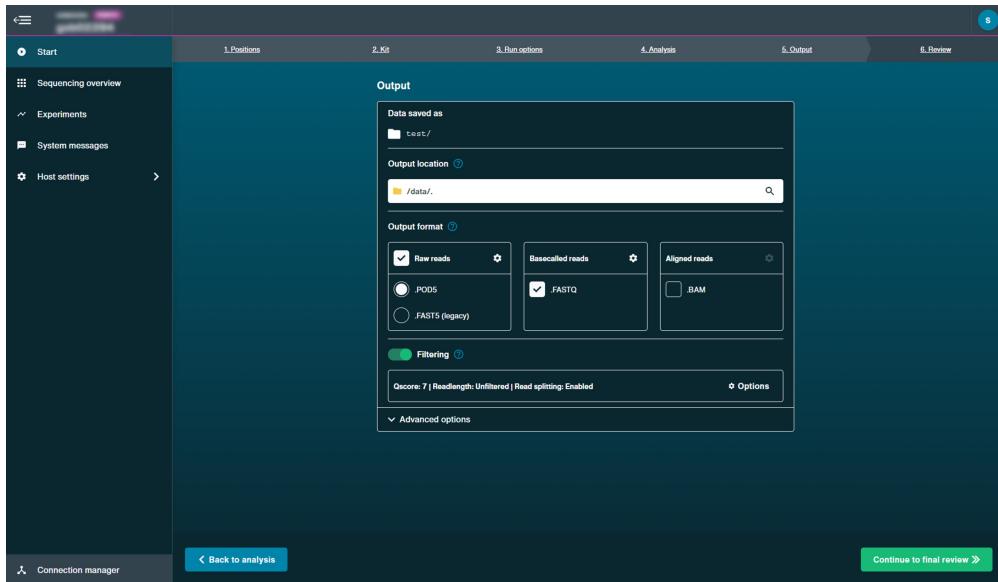


3. Select the sequencing kit used in the library preparation on the Kit page.

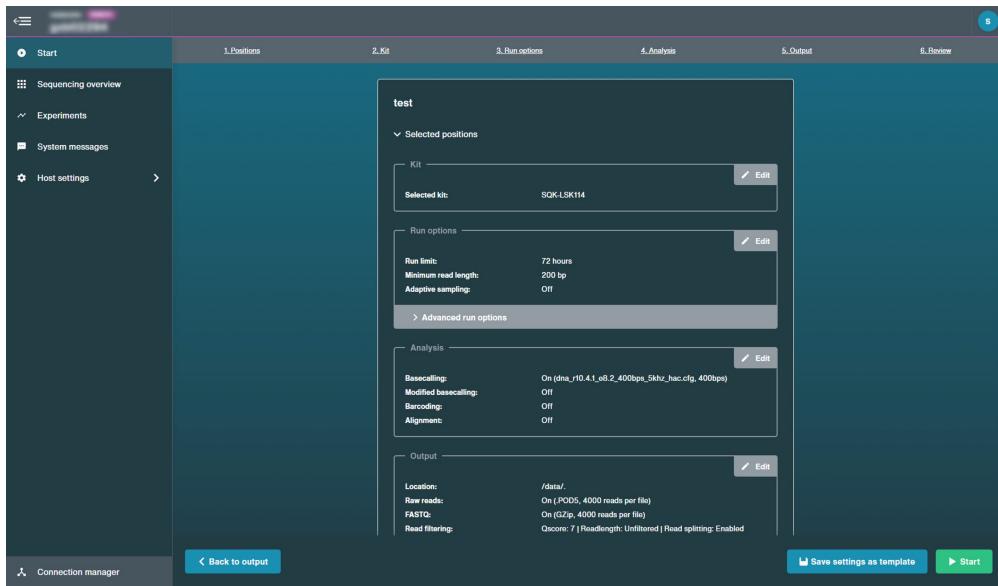
4. Configure the sequencing parameters for your sequencing run or keep to the default settings on the Run options and Analysis tabs.

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. On the Output page, set up the output parameters or keep to the default settings.



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



Duplex basecalling

Kit 14 chemistry has improved duplex basecalling which requires rebasecalling on [Dorado](#) after simplex basecalling has been performed on MinKNOW.

For detailed information on setting up your sequencing run, for both simplex and duplex basecalling, please see the [Kit 14 sequencing and duplex basecalling info sheet](#).

Note: When running Dorado, we recommend stopping other basecalling for the best performance by maximising memory available to Dorado. This can be stopped and restarted when Dorado has finished via the GUI on MinKNOW.

Data analysis after sequencing

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.

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.

Observation	Possible cause	Comments and actions
Low recovery	DNA fragments are shorter than expected	<p>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.</p>
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.

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 .

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	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

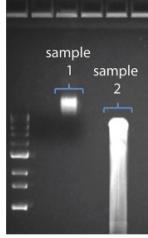
Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support. If you do not have another sequencing device available, we recommend storing the flow 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 SQK-LSK110 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.

Observation	Possible cause	Comments and actions
Pore occupancy close to 0	No tether on the flow cell	Tethers are added during flow cell priming (FLT tube for Kit 9, 10, 11, and FCT for Kit 14). Make sure FLT/FCT 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	<p>Read length reflects input DNA fragment length. Input DNA can be fragmented during extraction and library prep.</p> <ol style="list-style-type: none"> 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.  <p>In the image above, Sample 1 is of high molecular weight, whereas Sample 2 has been fragmented.</p> <ol style="list-style-type: none"> 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)	Contaminants are present in the sample	<p>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:</p> <ol style="list-style-type: none"> 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.

The pore activity plot above shows an increasing proportion of "unavailable" pores over time.



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	<p>Known compounds, include polysaccharides, typically associate with plant genomic DNA.</p> <ol style="list-style-type: none"> 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.