Overview of the protocol

Ligation Sequencing Kit features

This kit is recommended for users who:

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

> **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 remove short 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-LSK110). It is highly recommended that a Lambda control experiment is completed first to become familiar with the technology.

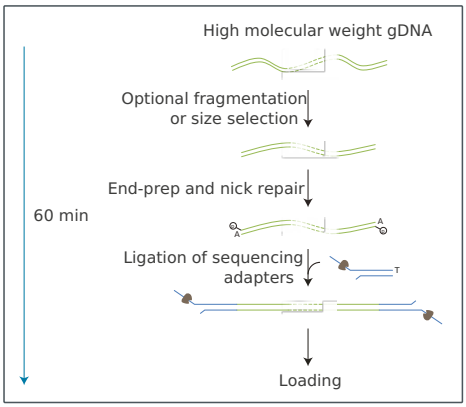
Steps in the sequencing workflow:

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-LSK110)
- Flongle Sequencing Expansion (EXP-FSE001)
- Control Expansion (EXP-CTL001)
- Flongle Flow Cell R9.4.1 (FLO-FLG001)

Equipment and consumables

Materials

- 500 ng high molecular weight genomic DNA
- OR 50+ ng high molecular weight genomic DNA if performing DNA fragmentation
- Ligation Sequencing Kit (SQK-LSK110)
- Flongle Flow Cell Priming Kit (EXP-FSE001)

Consumables

- Flongle device - flow cell and adapter
- Agencourt AMPure XP beads (Beckman Coulter™, A63881)
- NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (cat # E7180S or E7180L). Alternatively, you can use the three NEBNext® products below:
- NEBNext FFPE Repair Mix (M6630)
- NEBNext Ultra II End repair/dA-tailing Module (E7546)
- NEBNext Quick Ligation Module (E6056)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes

	• Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • Freshly prepared 70% ethanol in nuclease-free water
Equipment	• MinION • Hula mixer (gentle rotator mixer) • Magnetic separator, 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)

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 Flow Cell Priming Kit (EXP-FSE001) 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

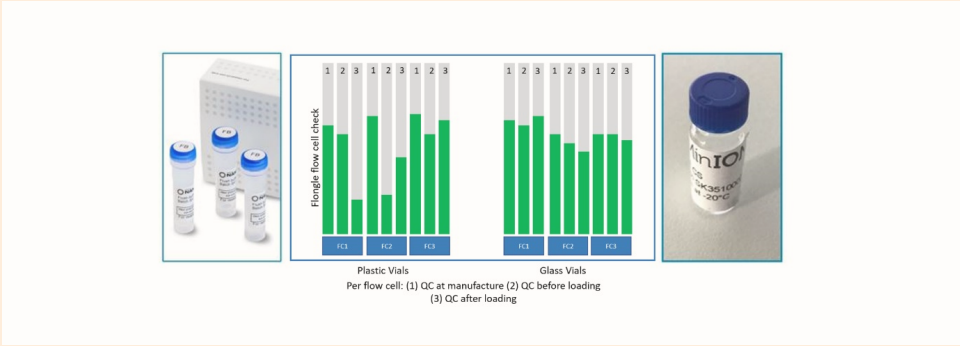
Following standard input recommendations, the protocol should produce enough final library (adapted DNA in EB) to load at least two Flongle flow cells. We recommend reserving enough library to load a second Flongle flow cell should you need to generate more data from a second flongle flow cell.

IMPORTANT

Flongle Flow Cell Priming Kit (EXP-FSE001)

There are three buffers that come into direct contact with a flow cell at point of loading (SBII: Sequencing Buffer II, FB: Flush Buffer and LB II: Loading Beads II or LS: Loading 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 Flow Cell Priming Kit (EXP-FSE001) 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 Flow Cell Priming Kit (EXP-FSE001) components
- Sequencing Buffer II (SBII)
- Flush Buffer (FB)
- Loading Beads II (LBII) or Loading Solution (LS)

Sequencing or Flow Cell Priming Kit components
- Flush Tether (FLT)

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 500 ng high molecular weight genomic DNA.

Although 500 ng (or 50-100 fmol) gDNA is recommended, users can start with lower input quantities (down to 50 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.

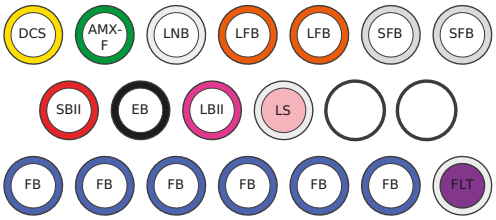
Chemical contaminants

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 theNEBNext® 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.

Ligation Sequencing Kit (SQK-LSK110) contents



DCS : DNA control strand
AMX-F : Adapter mix F
LNB : Ligation buffer
LFB : L fragment buffer
SFB : S fragment buffer
SBII : Sequencing buffer II
EB : Elution buffer
LBII : Loading beads II
LS : Loading solution
FB : Flush buffer
FLT : Flush tether

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
DNA CS	DCS	Yellow	1	35
Adapter Mix F	AMX-F	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 II	SBII	Red	1	500
Elution Buffer	EB	Black	1	200
Loading Beads II	LBII	Pink	1	360
Loading Solution	LS	White cap, pink sticker on label	1	360
Flush Buffer	FB	Blue	6	1,170
Flush Tether	FLT	Purple	1	200

Flongle Flow Cell Priming Kit contents (EXP-FSE001)

SBII : Sequencing Buffer II
LBII : Loading Beads II
LS : Loading Solution
FB : Flush Buffer

Name	Acronym	Cap colour	Number of vials	Fill volume per vial (µl)
Sequencing Buffer II	SBII	Blue	1	180
Loading Beads II	LBII	Blue	1	120
Loading Solution	LS	Blue	1	120
Flush Buffer	FB	Blue	2	702

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 Flow Cell Priming Kit (EXP-FSE001) to be 6 months from receipt by the customer.

Computer requirements and software

MinION Mk1B IT requirements

Unless you are using a MinIT device, 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 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 in real time and processes it into basecalls. You will be using MinKNOW for every sequencing experiment. MinKNOW can also demultiplex reads by barcode, and

basecall/demultiplex data after a sequencing run has completed.

MinKNOW use

For instructions on how to run the MinKNOW software, please refer to the relevant section in 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.

EPI2ME installation and use

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

Guppy (optional)

The Guppy command-line software can be used for basecalling and demultiplexing reads by barcode instead of MinKNOW. You can use it if you would like to re-analyse old data, or integrate basecalling into your analysis pipeline.

Guppy installation and use

If you would like to use the Guppy software, please refer to the Guppy 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 flow cells, or within four weeks of purchasing for 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

DNA repair and end-prep

~35 minutes

Materials	• gDNA in 23.5 µl nuclease-free water • DNA CS
Consumables	• 0.2 ml thin-walled PCR tubes • 1.5 ml Eppendorf DNA LoBind tubes • Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

- NEBNext FFPE DNA Repair Mix (M6630)
- NEBNext Ultra II End repair / dA-tailing Module (E7546)
- Agencourt AMPure XP beads
- Freshly prepared 70% ethanol in nuclease-free water

Equipment

- P1000 pipette and tips
- P100 pipette and tips
- P10 pipette and tips
- Thermal cycler at 20°C and 65°C
- Microfuge
- Hula mixer (gentle rotator mixer)
- Magnetic rack
- Ice bucket with ice

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 Flow Cell Priming Kit (EXP-FSE001) 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

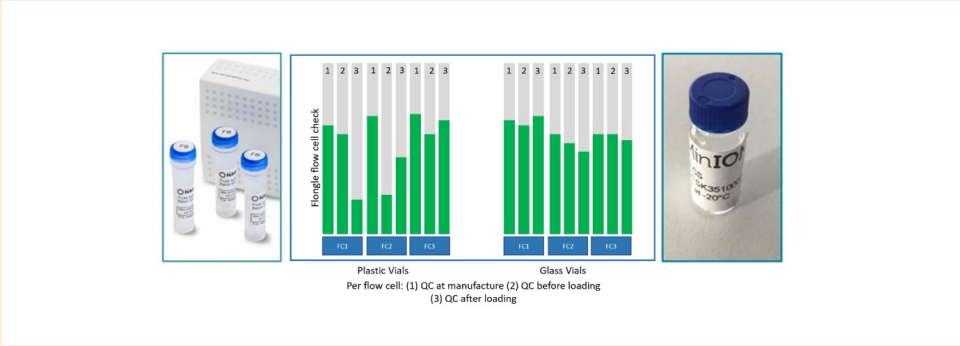
Following standard input recommendations, the protocol should produce enough final library (adapted DNA in EB) to load at least two Flongle flow cells. We recommend reserving enough library to load a second Flongle flow cell should you need to generate more data from a second flongle flow cell.

IMPORTANT

Flongle Flow Cell Priming Kit (EXP-FSE001)

There are three buffers that come into direct contact with a flow cell at point of loading (SBII: Sequencing Buffer II, FB: Flush Buffer and LB II: Loading Beads II or LS: Loading 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 Flow Cell Priming Kit (EXP-FSE001) 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 Flow Cell Priming Kit (EXP-FSE001) components
- Sequencing Buffer II (SBII)
- Flush Buffer (FB)
- Loading Beads II (LBII) or Loading Solution (LS)

Sequencing or Flow Cell Priming Kit components
- Flush Tether (FLT)

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 remove short fragments - instructions are available in the Size Selection section of Extraction methods.

1 Thaw DNA CS (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 perfomance, NEB recommend the following:

1. Thaw all reagents on ice.
2. Flick and/or invert reagent tube to ensure they are well mixed.
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 several seconds to ensure the reagent is thoroughly mixed.
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:

- Transfer 500 ng genomic DNA into a 1.5 ml Eppendorf DNA LoBind tube
- Adjust the volume to 23.5 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
DNA CS	0.5 µl
DNA	23.5 µl
NEBNext FFPE DNA Repair Buffer	1.75 µl
NEBNext FFPE DNA Repair Mix	1 µl
Ultra II End-prep reaction buffer	1.75 µl
Ultra II End-prep enzyme mix	1.5 µl
Total	**30 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 30 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 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

~30 minutes

Materials	• Adapter Mix F (AMX F) • Ligation Buffer (LNB) • Long Fragment Buffer (LFB) • Short Fragment Buffer (SFB) • Elution Buffer from the Oxford Nanopore kit (EB)
Consumables	• NEBNext Quick Ligation Module (E6056) • 1.5 ml Eppendorf DNA LoBind tubes • Agencourt AMPure XP beads
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 3rd party ligase is supplied with its own buffer, the ligation efficiency of Adapter Mix F (AMX-F) is higher when using Ligation Buffer supplied within the Ligation Sequencing Kit.

1 **Spin down the Adapter Mix F (AMX-F) 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, mix by vortexing, 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.

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:**

Reagent	Volume
DNA sample from the previous step	30 µl
Ligation Buffer (LNB)	12.5 µl
NEBNext Quick T4 DNA Ligase	5 µl
Adapter Mix F (AMX-F)	2.5 µl
Total	**50 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 20 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.**

13 **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.**

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 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.**

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

18 **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.

END OF STEP

The prepared library is used for loading into the flow cell. Store the library on ice until ready to load.

IMPORTANT

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. We recommend loading 3-20 fmol of this final prepared library onto the flow cell. Loading more than 50 fmol can have a detrimental effect on throughput. Dilute the library in EB or nuclease-free water up to a final volume of 5 µl.

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.
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-AUX002), available to purchase separately. This expansion also contains additional vials of Sequencing Buffer II (SBII) and Loading Beads II (LBII), required for loading the libraries onto flow cells.

Loading the Flongle flow cell

Materials

- Flongle Flow Cell Priming Kit (EXP-FSE001)

	• Flush Tether (FLT)
Consumables	• 1.5 ml Eppendorf DNA LoBind tubes • Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • Flongle flow cell
Equipment	• Flongle adapter • MinION or GridION • P200 pipette and tips • P10 pipette and tips

IMPORTANT

Flow cell deterioration/saturation

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IMPORTANT

Flongle Flow Cell Priming Kit (EXP-FSE001)

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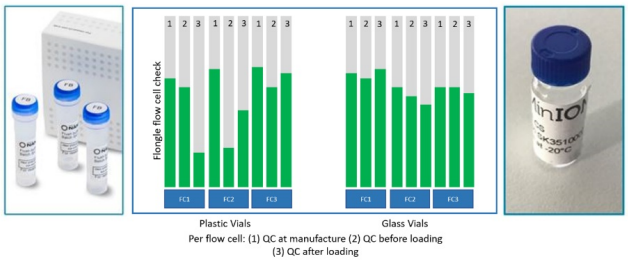

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- Sequencing Buffer II (SBII)
- Flush Buffer (FB)
- Loading Beads II (LBII) or Loading Solution (LS)

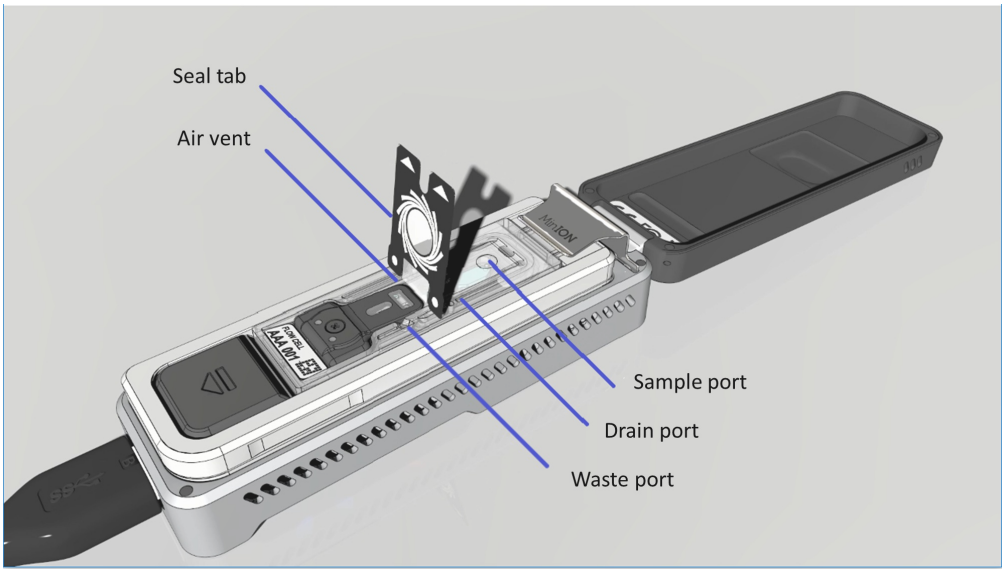
Sequencing or Flow Cell Priming Kit components
- Flush Tether (FLT)

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 II (SBII), Loading Beads II (LBII) and Flush Buffer (FB) from the Flongle Sequencing Expansion and Flush Tether (FLT) from the Ligation Sequencing Kit (SQK-LSK110) at room temperature.**

2 **Mix the Sequencing Buffer II (SBII), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing and spin down at room temperature.**

3 **In a fresh 1.5 ml Eppendorf DNA LoBind tube, mix 117 µl of Flush Buffer (FB) with 3 µl of Flush Tether (FLT) and mix by pipetting.**

4 **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.

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

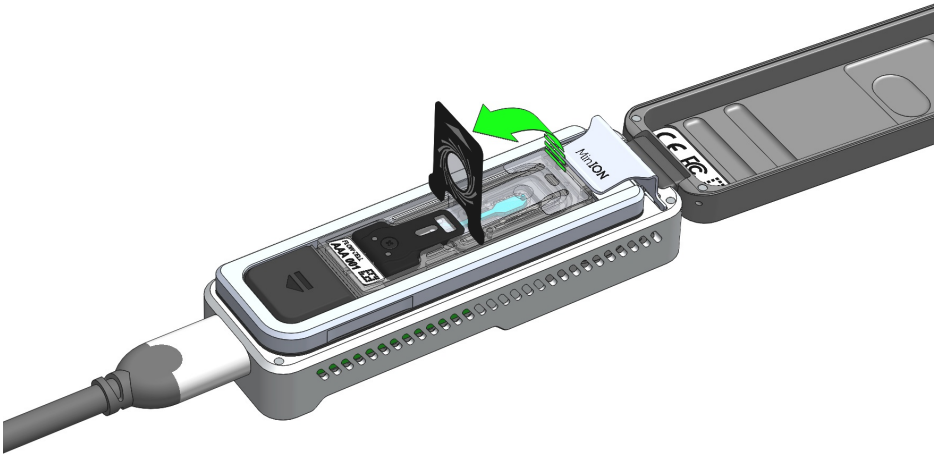
The flow cell should sit evenly and flat inside the adapter, to avoid any bubbles forming inside the fluidic compartments.

IMPORTANT

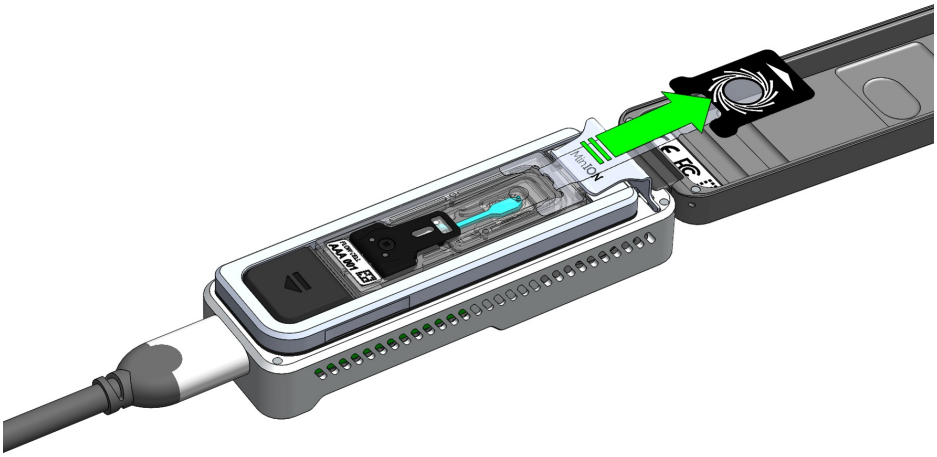
How to prime and load a Flongle flow cell

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

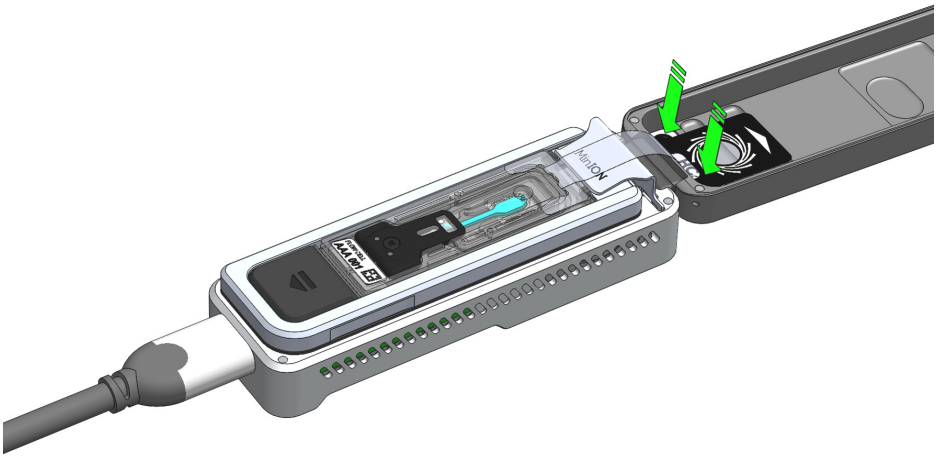
1. Lift up the seal tab:



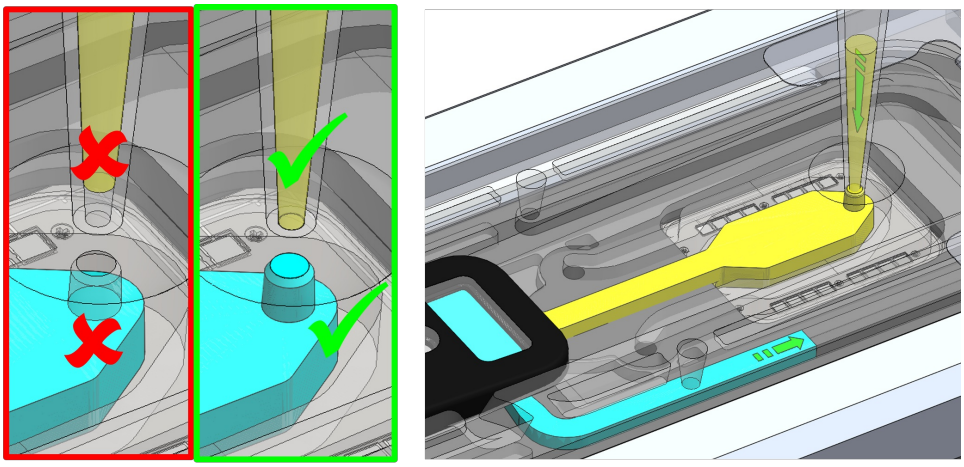
2. Pull the seal tab to open access to the sample port:



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



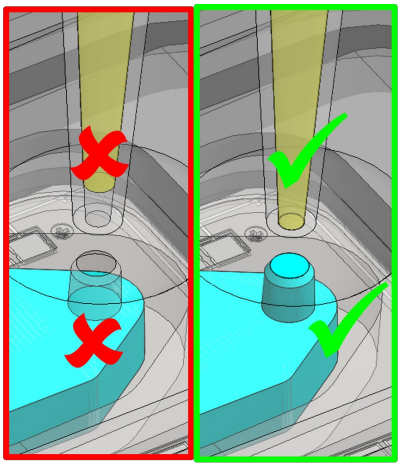
7 **To prime your flow cell with the mix of Flush Buffer (FB) and Flush Tether (FLT) that was prepared earlier, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 pipette tip inside the sample port and slowly dispense the priming fluid into the Flongle flow cell by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

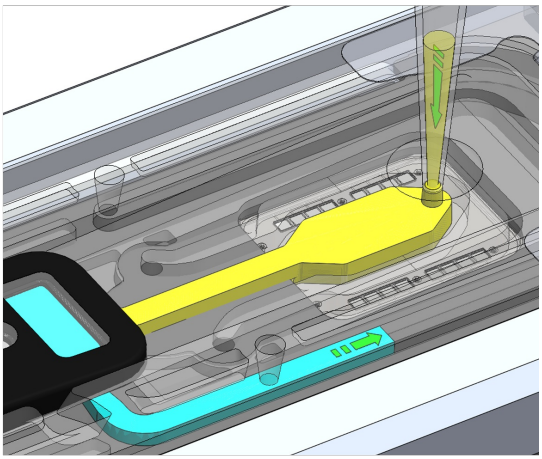


8 **Vortex the vial of Loading Beads II (LBII). Note that the beads settle quickly, so immediately prepare the Sequencing Mix in a fresh 1.5 ml Eppendorf DNA LoBind tube for loading the Flongle, as follows:**

Reagents	Volume
Sequencing Buffer II (SBII)	15 µl
Loading Beads II (LBII) mixed immediately before use, or Loading Solution (LS), if using. LS can be used instead of LBII when preparing libraries with the Ligation Sequencing Kit (SQK-LSK110)	10 µl
DNA library	5 µl
Total	**30 µl**

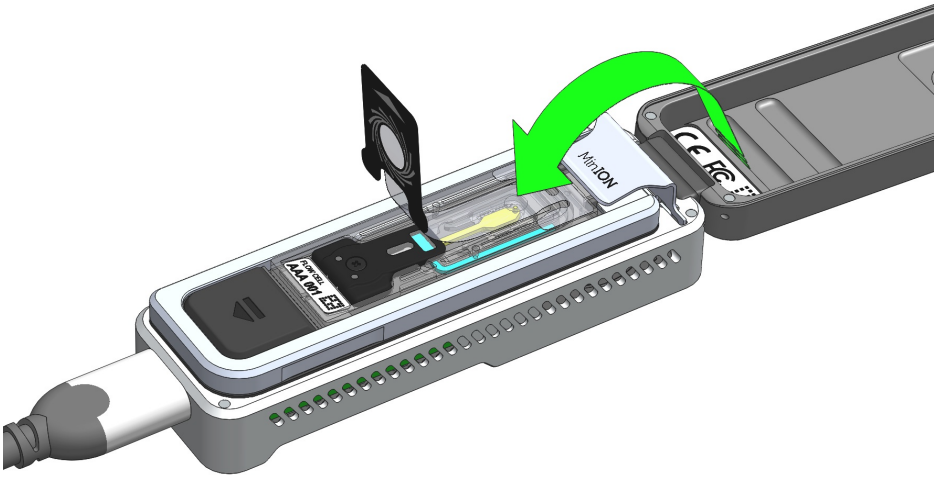
9 **To add the Sequencing Mix to the flow cell, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 tip inside the sample port and slowly dispense the Sequencing Mix into the flow cell by slowly pipetting down. We also recommend twisting the pipette plunger down to avoid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



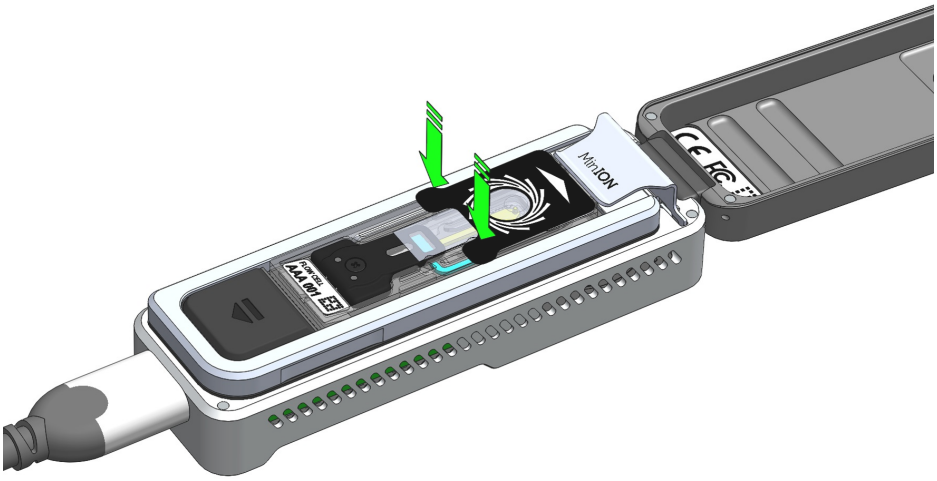


10 Seal the Flongle flow cell using the adhesive on the seal tab, as follows:

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.



11 Replace the sequencing platform lid.

Data acquisition and basecalling

Overview of nanopore data analysis

For a full overview of nanopore data analysis, which includes options for basecalling and post-basecalling analysis, please refer to the Data Analysis document.

How to start sequencing

The sequencing device control, data acquisition and real-time basecalling are carried out by the MinKNOW software. It is assumed you have already installed MinKNOW on your computer, or that you are using the MinIT device for data acquisition and basecalling. There are three options for how to carry out sequencing:

1. Data acquisition and basecalling in real-time using MinKNOW on a computer

Follow the instructions in the MinKNOW protocol beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section.

2. Data acquisition and basecalling in real-time using the GridION device

Follow the instructions in the GridION user manual.

3. Data acquisition and basecalling in real-time using the MinION Mk1C device

Follow the instructions in the MinION Mk1C user manual.

4. Data acquisition and basecalling in real-time using the MinIT device

Follow the instructions in the MinIT protocol.

5. Data acquisition using MinKNOW on a computer and basecalling at a later time using MinKNOW or Guppy

Follow the instructions in the MinKNOW protocol beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section. **When setting your experiment parameters, set the *Basecalling* tab to OFF.** After the sequencing experiment has completed, follow the instructions in the Post-run analysis section of the MinKNOW protocol or the Guppy protocol starting from the "Quick Start Guide for Guppy" section.

Downstream analysis

Post-basecalling analysis

There are several options for further analysing your basecalled data:

1. EPI2ME platform

The EPI2ME platform is a cloud-based data analysis service developed by Metrichor Ltd., a subsidiary of Oxford Nanopore Technologies. The EPI2ME platform offers a range of analysis workflows, e.g. for metagenomic identification, barcoding, alignment, and structural variant calling. The analysis requires no additional equipment or compute power, and provides an easy-to-interpret report with the results. For instructions on how to run an analysis workflow in EPI2ME, please follow the instructions in the EPI2ME protocol, beginning at the "Starting data analysis" step.

2. EPI2ME Labs tutorials and workflows

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

3. 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.

4. 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.

Issues during DNA/RNA extraction and library preparation

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

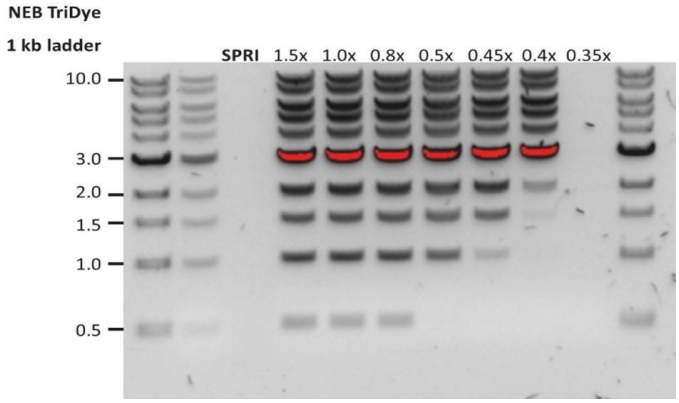
We also have an FAQ section available on the Nanopore Community Support section.

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 Know-how piece. 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 Know-how piece.
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 Know-how piece. 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.

The VolTRAX run terminated in the middle of the library prep

Observation	Possible cause	Comments and actions
The green light was switched off **or** **An adapter was used to connect the VolTRAX USB-C cable to the computer**	Insufficient power supply to the VolTRAX	The green LED signals that 3 A are being supplied to the device. This is the requirement for the full capabilities of the VolTRAX V2 device. Please use computers that meet the requirements listed on the VolTRAX V2 protocol.

The VolTRAX software shows an inaccurate amount of reagents loaded

Observation	Possible cause	Comments and actions
The VolTRAX software shows an inaccurate amount of reagents loaded	Pipette tips do not fit the VolTRAX cartridge ports	TRainin 20 µl or 30 µl and Gilson 10 µl, 20 µl or 30 µl pipette tips are compatible with loading reagents into the VolTRAX cartridge. Rainin 20 µl is the most suitable.
The VolTRAX software shows an inaccurate amount of reagents loaded	The angle at which reagents are pipetted into the cartridge is incorrect	The pipetting angle should be slightly greater than the cartridge inlet angle. Please watch the demo video included in the VolTRAX software before loading.

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.

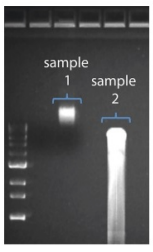
MinKNOW script failed

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.

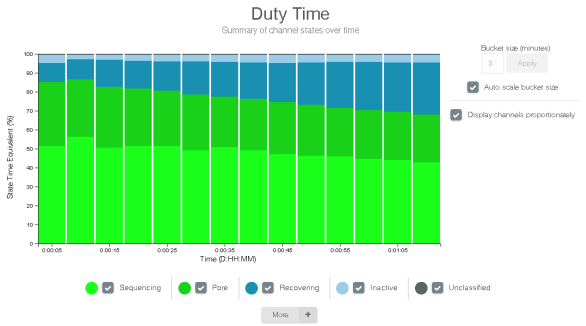
Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	5–50 fmol of good quality library can be loaded on to a MinION Mk1B/GridION 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 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.
Pore occupancy close to 0	No tether on the flow cell	Tethers are adding during flow cell priming (FLT tube). Make sure FLT was added to FB 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 recovering pores

Observation	Possible cause	Comments and actions
Large proportion of recovering pores (shown as dark blue in the channels panel and duty time plot)	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 "single pores". If the portion of recovering pores (unavailable pores in the extended view) stays large or increases: 1. A nuclease flush can be performed, or 2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.  The duty time plot above shows an increasing proportion of "recovering" pores over the course of a sequencing experiment

Large proportion of inactive pores

Observation	Possible cause	Comments and actions
Large proportion of inactive pores (shown as light blue in the channels panel and duty time 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 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 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	Fast fuel consumption is typically seen when the flow cell is overloaded with library (~5–50 fmol of library is recommended).	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" (37°C for Flow Cell Check, 34°C for sequencing on MinION Mk 1B/PromethION flow cells, and 35°C for sequencing on Flongle)	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

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.

MinIT – the MinKNOW interface is not shown in the web browser

Observation	Possible cause	Comments and actions
The MinKNOW interface is not shown in the web browser	Browser compatibility issue	Always use Google Chrome as the browser to view MinKNOW. Alternatively, instead of typing //mt-xxxxxx (x is a number) in the address bar, type in in the generic IP address, 10.42.0.1, which identifies the MinIT Wi-Fi router.
The MinKNOW interface is not shown in the web browser	The MinIT Wi-Fi was not used for connecting to the computer or mobile device	Make sure the computer or mobile device is using the MinIT Wi-Fi. It should be shown as MT-xxxxxx (x is a number) on the underside label on the MinIT:  Disable the Ethernet connection from the computer or mobile device as needed. If necessary, contact your IT department to determine if the MinIT Wi-Fi is blocked (MinIT generic IP: 10.42.0.1). Please white-list MinIT as needed.
The MinKNOW interface is not shown in the web browser	The MinIT was not on the same network that the computer was connected to.	Make sure that the wall sockets used by the Ethernet cables from the MinIT and computer belong to the same local network.

MinIT – the MinIT software cannot be updated

Observation	Possible cause	Comments and actions
The MinIT software cannot be updated	The firewall is blocking IPs for update	Please consult your IT department, as the MinIT software requires access to thefollowing AWS IP ranges. Access to the following IP addresses is also needed: 178.79.175.200 96.126.99.215

Observation	Possible cause	Comments and actions
The MinIT software cannot be updated	The device already has the latest version of the software	Occassionaly, the MinIT software admin page displays "updates available" even when the software is already up-to-date. Please compare the version listed on the admin page with the one on the Software Downloads page. Alternatively, SSH into the MinIT through a SSH Client (e.g. Bitvise or Putty, as described in the MinIT protocol) on a Windows computer or the terminal window on a Mac, run the command, *dpkg -l	grep minit*, to find out the version of the MinIT software and *sudo apt update* if an update is needed. If the issue still persists, please contact Technical Services with details of the error.