

# Overview of the protocol

## IMPORTANT

### This is an Early Access product

For more information about our Early Access programmes, please see [this article on product release phases](#)

Please ensure you always use the most recent version of the protocol.

## Ligation Sequencing Kit V14 features

This kit is recommended for users who:

- Want to achieve median raw read accuracy of Q20+ (99%) and above.
- Want to optimise their sequencing experiment for output.
- Require control over read length.
- Would like to utilise upstream processes such as size selection, whole genome amplification, or enrichment for long reads.
- Want to achieve duplex basecalling. For more information, please see the [Kit 14 sequencing and duplex basecalling](#) info sheet.

## IMPORTANT

### Kit 14 sequencing and duplex basecalling info sheet

The Kit 14 chemistry is a new development from Oxford Nanopore Technologies with improved duplex basecalling, which requires a different set of tools. For more information, please see the [Kit 14 sequencing and duplex basecalling](#) info sheet. We strongly recommend that you read it before proceeding with Kit 14 chemistry sequencing experiments and basecalling duplex data.

## 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.

### Steps in the sequencing workflow:

#### Prepare for your experiment

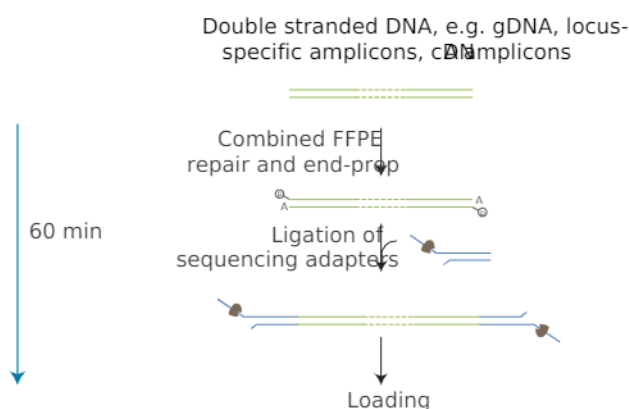
You will need to:

- 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 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)

# Equipment and consumables

## Materials

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

## Consumables

- Flongle device - flow cell and adapter
- NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (cat # E7180S or E7180L). Alternatively, you can use the NEBNext® products below:

- 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 80% ethanol in nuclease-free water
- Qubit™ Assay Tubes (ThermoFisher, Q32856)
- Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)

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**Equipment**

- 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
- Qubit fluorometer (or equivalent for QC check)

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**Optional Equipment**

- Agilent Bioanalyzer (or equivalent)
- 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 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](mailto: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 [input DNA/RNA QC protocol](#).

### Chemical contaminants

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

Community.

### NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing

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

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

### 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.

#### 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) used in this kit and protocol is not interchangeable with other sequencing adapters.

### Ligation Sequencing Kit V14 (SQK-LSK114) contents



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

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
DNA Control Strand	DCS	Yellow	1	35
Ligation Adapter	LA	Green	2	40
AMPure XP Beads	AXP	Amber	1	1,200
Ligation Buffer	LNB	White	1	200

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Long Fragment Buffer	LFB	Orange	2	1,800
Short Fragment Buffer	SFB	Clear	2	1,800
Sequencing Buffer	SB	Red	1	700
Elution Buffer	EB	Black	1	1,200
Library Beads	LIB	Pink	1	600
Library Solution	LIS	White cap, pink sticker on label	1	600
Flow Cell Flush	FCF	Blue	6	1,170
Flow Cell Tether	FCT	Purple	1	200

**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.

#### 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
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.

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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



LMD : Lambda DNA (50 µg/ml)

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Lambda DNA	LMD	Yellow	6	90

## Computer requirements and software

### 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](#)

### MinION Mk1B IT requirements

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

### 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

## End-prep

~35 minutes

## Materials

- 500 ng (or 50-100 fmol) amplicon DNA
- DNA Control Sample (DCS)
- AMPure XP Beads (AXP)

## Consumables

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

## 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
- Qubit fluorometer (or equivalent for QC check)

### IMPORTANT

#### Flow cell deterioration/saturation

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#### Loading recommendations

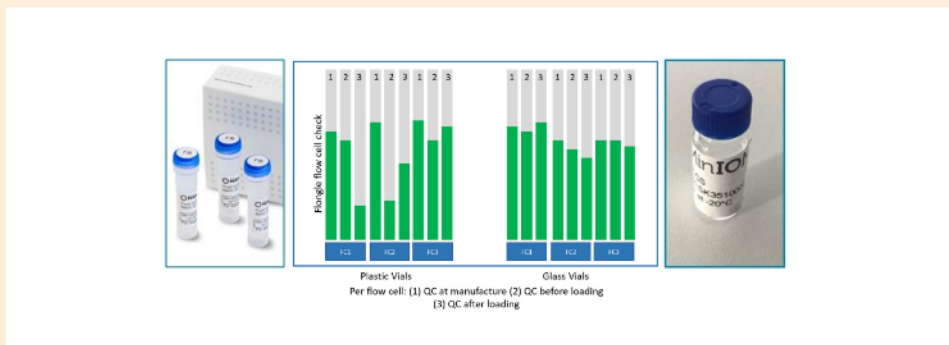
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To rapidly deploy this to Flongle users, we have produced a Flongle Sequencing Expansion (EXP-FSE002) with these three components in glass vials, which can perform 12 Flongle flow cell loads in total.

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 500 ng (or 50-100 fmol) 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
<b>Total</b>	<b>30 µl</b>

## 5 Ensure the reaction is thoroughly mixed by gently pipetting and spin down briefly.

## 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

Quantify 1  $\mu$ l 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

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

#### 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, 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.

- 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, mix by vortexing, 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 $\mu$ l
Ligation Buffer (LNB)	12.5 $\mu$ l
NEBNext Quick T4 DNA Ligase	5 $\mu$ l
Ligation Adapter (LA)	2.5 $\mu$ l
<b>Total</b>	<b>50 <math>\mu</math>l</b>

**6 Ensure the reaction is thoroughly mixed by gently pipetting and spin down briefly.**

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

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

**9 Add 20  $\mu$ l 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.**

**12 Wash the beads by adding either 125  $\mu$ l Long Fragment Buffer (LFB) or 125  $\mu$ l 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  $\mu$ l 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  $\mu$ l of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Dispose of the pelleted beads

Quantify 1  $\mu$ l of eluted sample using a Qubit fluorometer.

### 18 Make up your library to 5 $\mu$ l at 5-10 fmol.

#### 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  $\mu$ l.

#### END OF STEP

**The prepared library is used for loading into the flow cell. Store the library on ice 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.

#### Optional Action

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

Depending on how many flow cells the library will be split across, more Elution Buffer (EB) than what is supplied in the kit will be required.

## Loading the Flongle flow cell

### Materials

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

### 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**

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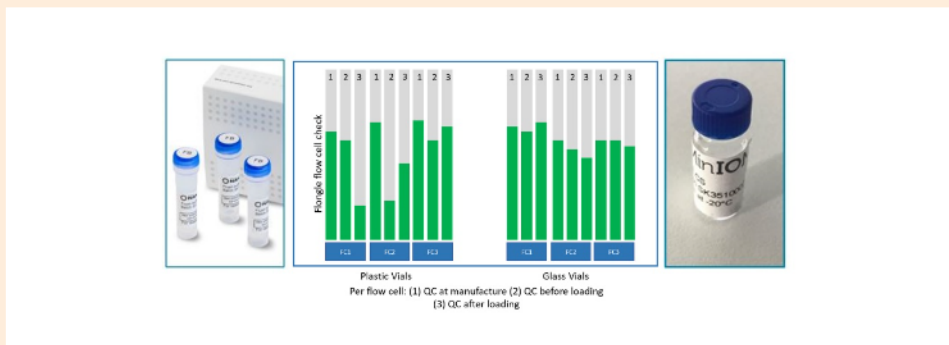
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- Sequencing Buffer (SB)
- Flow Cell Flush (FCF)
- Library Beads (LIB) or Library Solution (LIS)

#### Sequencing Kit components

- Flow Cell Tether (FCT)

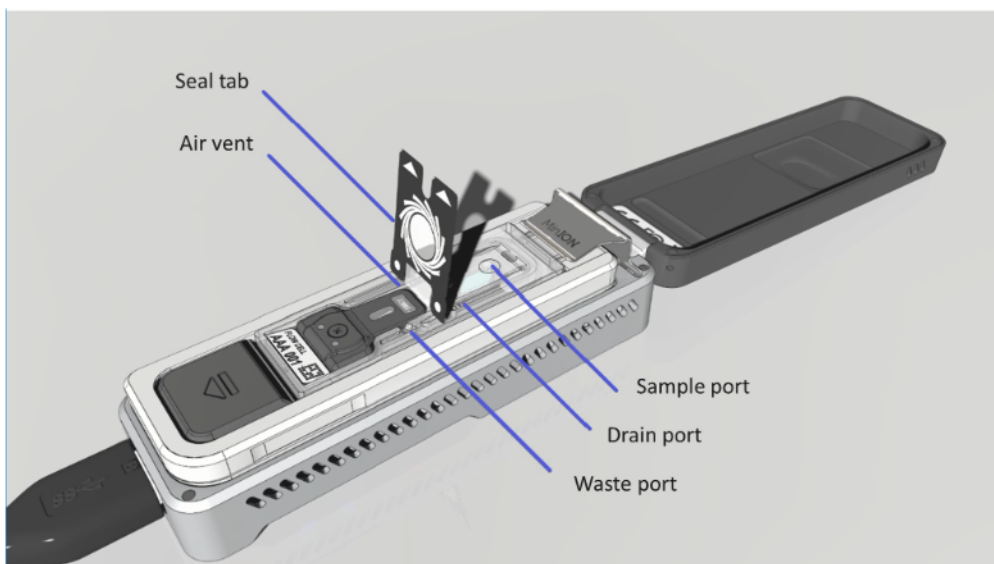
Oxford Nanopore Technologies deem the useful life of the Flow Cell Expansion to be 6 months from receipt by the customer.

### IMPORTANT

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.

### Using the Library Solution

We recommend using the Library Beads (LIB) for loading your library onto the flow cell for most sequencing experiments. However, if

you have previously used water to load your library, you must use Library Solution (LIS) instead of water.

**Note:** Some customers have noticed that viscous libraries can be loaded more easily when not using Library Beads.

- 1 Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and one tube of Flow Cell Flush (FCF) at room temperature. Mix by vortexing and spin down.**
- 2 In a fresh 1.5 ml Eppendorf DNA LoBind tube, mix 117  $\mu$ l of Flow Cell Flush (FCF) with 3  $\mu$ l 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.

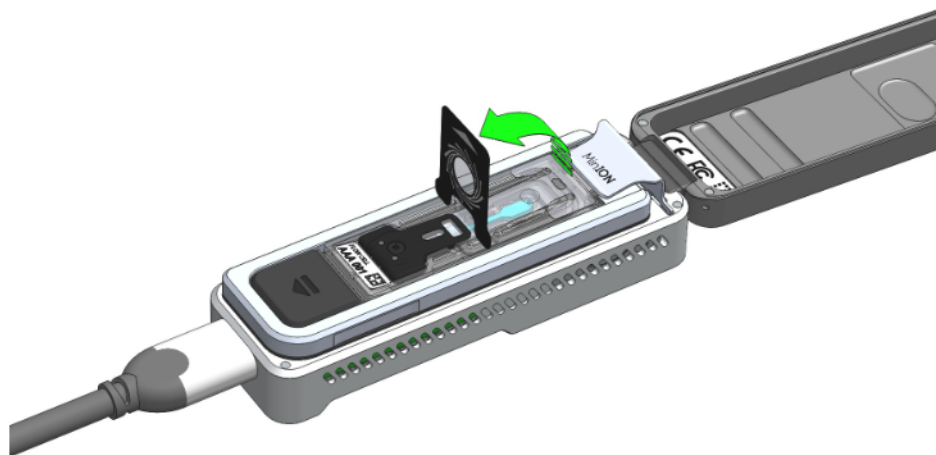


**IMPORTANT**

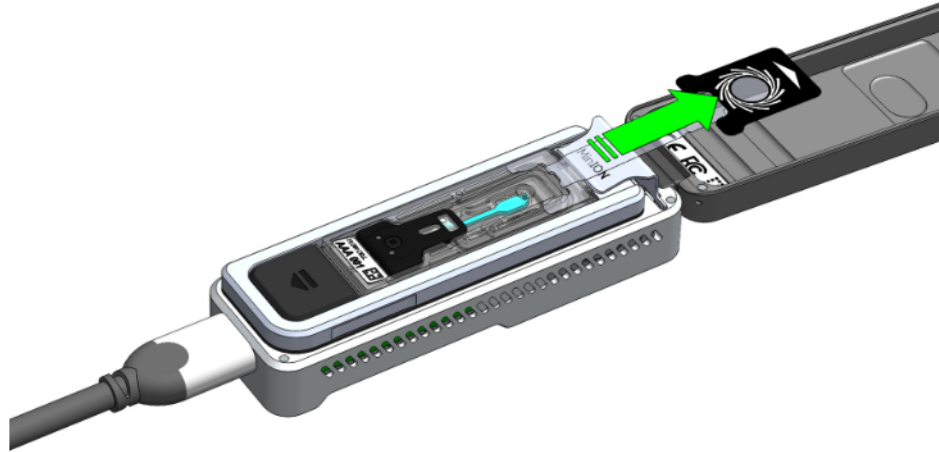
**How to prime and load a Flongle flow cell**

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

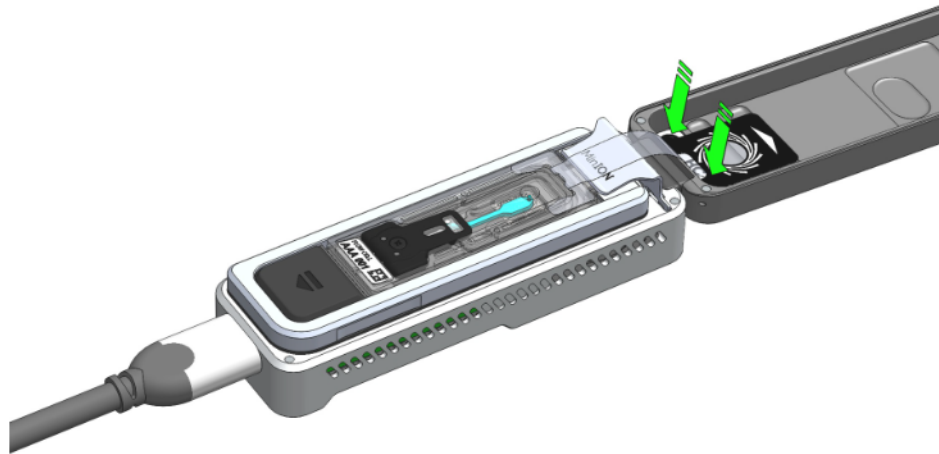
1. Lift up the seal tab:



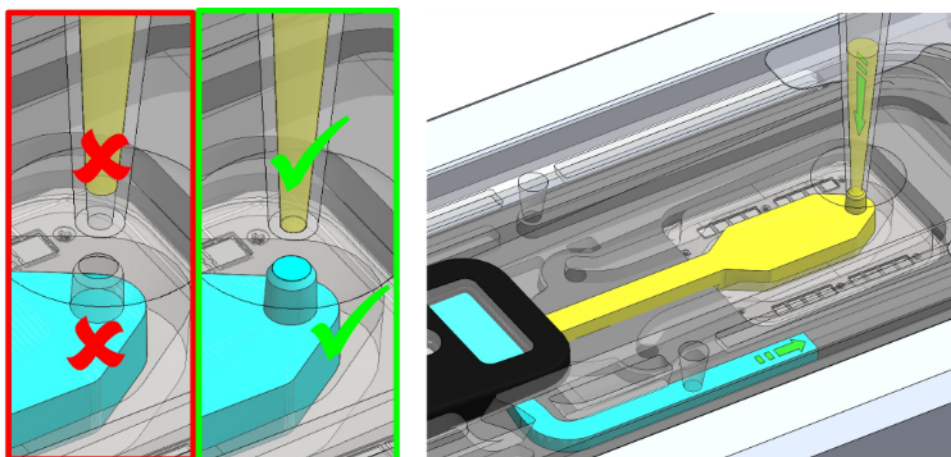
2. Pull the seal tab to open access to the sample port:



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



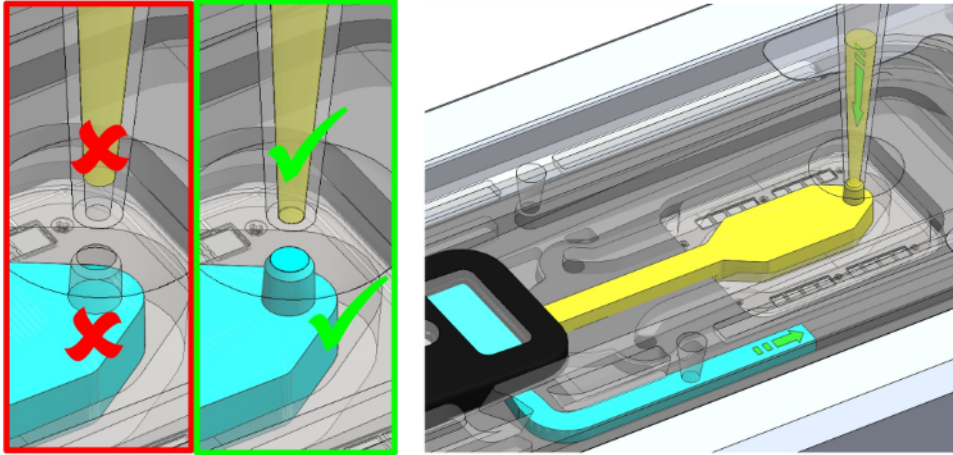
- 6 To prime your flow cell with the mix of Flow Cell Flush (FCF) and Flow Cell Tether (FCT) that was prepared earlier, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 pipette tip inside the sample port and slowly dispense the priming fluid into the Flongle flow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



- 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:

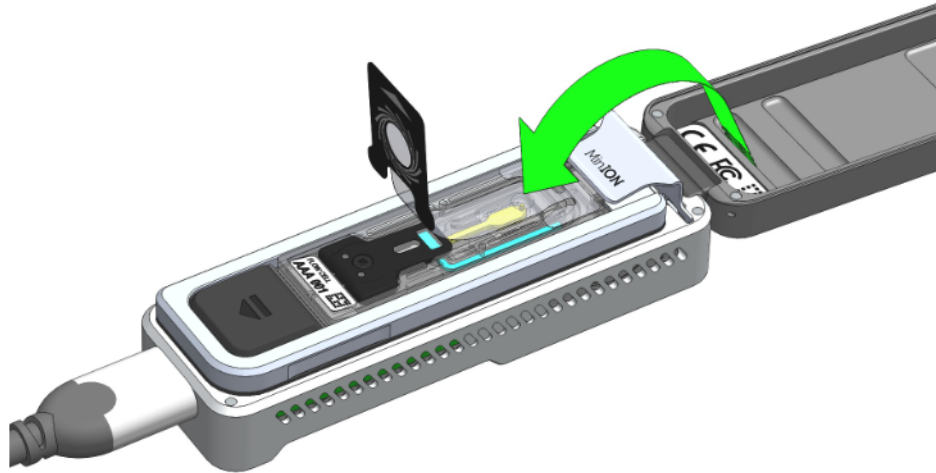
Reagents	Volume
Sequencing Buffer (SB)	15 $\mu$ l
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using. LIS can be used instead of LIB when preparing libraries with the Ligation Sequencing Kit V14 (SQK-LSK114)	10 $\mu$ l
DNA library	5 $\mu$ l
<b>Total</b>	<b>30 <math>\mu</math>l</b>

- 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.

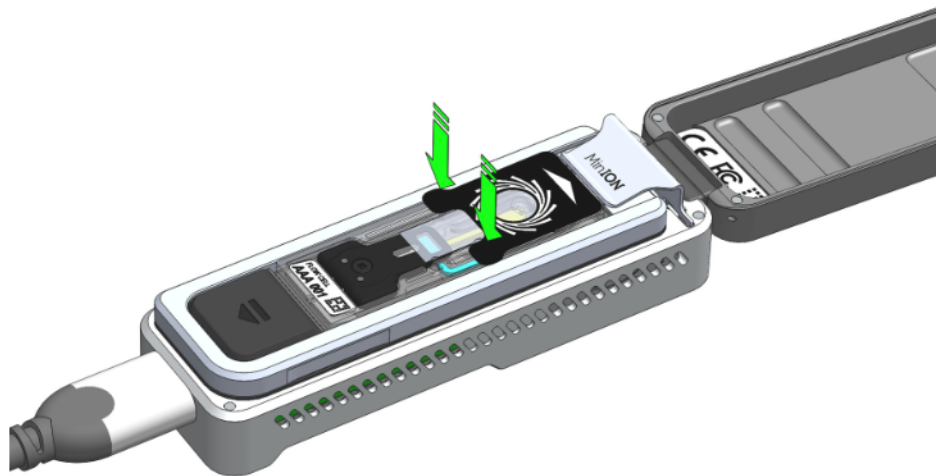


**9 Seal the Flongle flow cell using the adhesive on the seal tab, as follows:**

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.



**10 Replace the sequencing platform lid.**

## Data acquisition and basecalling

## IMPORTANT

### Kit 14 sequencing and duplex basecalling info sheet

The Kit 14 chemistry is a new development from Oxford Nanopore Technologies with improved duplex basecalling, which requires a different set of tools. For more information, please see the [Kit 14 sequencing and duplex basecalling info sheet](#). We strongly recommend that you read it before proceeding with Kit 14 chemistry sequencing experiments and basecalling duplex data.

## 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.. 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 PromethION device

Follow the instructions in the [PromethION user manual](#) or the [PromethION 2 Solo user manual](#).

### 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.

# Issues during DNA/RNA extraction and library preparation

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

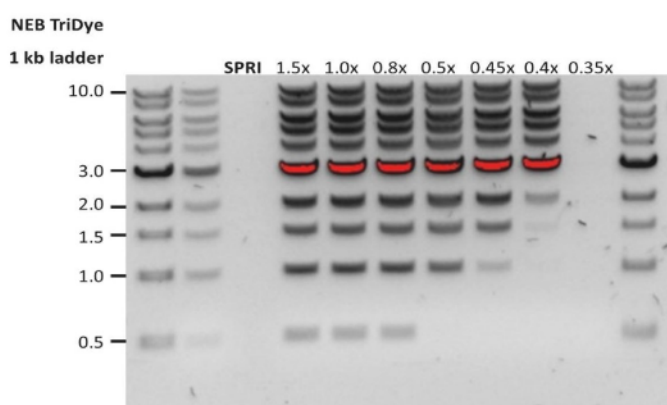
We also have an FAQ section available on the [Nanopore Community Support](#) section.

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

### Low sample quality

Observation	Possible cause	Comments and actions
<b>Low DNA purity (Nanodrop reading for DNA OD 260/280 is &lt;1.8 and OD 260/230 is &lt;2.0-2.2)</b>	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the <a href="#">Contaminants Know-how</a> piece. Please try an alternative <a href="#">extraction method</a> that does not result in contaminant carryover.  Consider performing an additional SPRI clean-up step.
<b>Low RNA integrity (RNA integrity number &lt;9.5 RIN, or the rRNA band is shown as a smear on the gel)</b>	The RNA degraded during extraction	Try a different <a href="#">RNA extraction method</a> . For more info on RIN, please see the <a href="#">RNA Integrity Number Know-how piece</a> .
<b>RNA has a shorter than expected fragment length</b>	The RNA degraded during extraction	Try a different <a href="#">RNA extraction method</a> . For more info on RIN, please see the <a href="#">RNA Integrity Number Know-how piece</a> .  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
<b>Low recovery</b>	DNA loss due to a lower than intended AMPure beads-to-sample ratio	<ol style="list-style-type: none"> <li>AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample.</li> <li>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.</li> </ol>
<b>Low recovery</b>	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> 

Observation	Possible cause	Comments and actions
<b>Low recovery after end-prep</b>	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](mailto: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
<b>MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check</b>	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 <a href="#">this video</a> .
<b>MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check</b>	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).

Observation	Possible cause	Comments and actions
<b>MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check</b>	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 <a href="#">Contaminants Know-how piece</a> . Please try an alternative <a href="#">extraction method</a> that does not result in contaminant carryover.

#### MinKNOW script failed

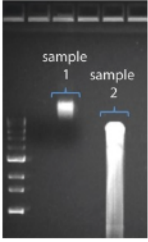
Observation	Possible cause	Comments and actions
<b>MinKNOW shows "Script failed"</b>		Restart the computer and then restart MinKNOW. If the issue persists, please collect the <a href="#">MinKNOW log files</a> and contact Technical Support.

#### Pore occupancy below 40%

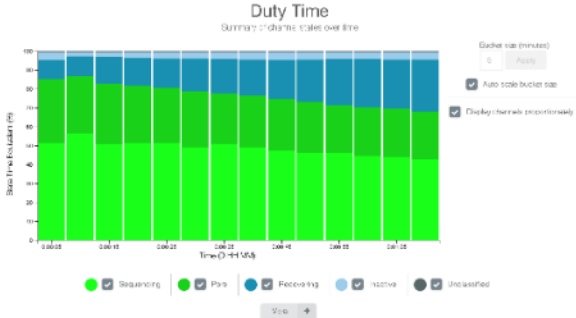
Observation	Possible cause	Comments and actions
<b>Pore occupancy &lt;40%</b>	Not enough library was loaded on the flow cell	5–50 fmol of good quality library can be loaded on to a MinION Mk1B/GridION flow cell. Please quantify the library before loading and calculate mols using tools like the <a href="#">Promega Biomath Calculator</a> , choosing "dsDNA: µg to pmol"
<b>Pore occupancy close to 0</b>	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.
<b>Pore occupancy close to 0</b>	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.
<b>Pore occupancy close to 0</b>	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
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Observation	Possible cause	Comments and actions
<b>Shorter than expected read length</b>	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"> <li>1. Please review the <a href="#">Extraction Methods</a> in the Nanopore Community for best practice for extraction.</li> <li>2. Visualise the input DNA fragment length distribution on an agarose gel before proceeding to the library prep.</li> </ol>  <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"> <li>3. During library prep, avoid pipetting and vortexing when mixing reagents. Flicking or inverting the tube is sufficient.</li> </ol>

### Large proportion of recovering pores

Observation	Possible cause	Comments and actions
<b>Large proportion of recovering pores (shown as dark blue in the channels panel and duty time plot)</b>	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 "single pores". If the portion of recovering pores (unavailable pores in the extended view) stays large or increases:</p> <ol style="list-style-type: none"> <li>1. A <a href="#">nuclease flush</a> can be performed, or</li> <li>2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.</li> </ol>  <p>The duty time plot above shows an increasing proportion of "recovering" pores over the course of a sequencing experiment</p>

### Large proportion of inactive pores

Observation	Possible cause	Comments and actions
<b>Large proportion of inactive pores (shown as light blue in the channels panel and duty time plot. Pores or membranes are irreversibly damaged)</b>	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 <a href="#">Priming and loading your flow cell</a> video for best practice
<b>Large proportion of inactive pores</b>	Certain compounds co-purified with DNA	Known compounds, include polysaccharides, typically associate with plant genomic DNA.  1. Please refer to the <a href="#">Plant leaf DNA extraction method</a> . 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.
<b>Large proportion of inactive pores</b>	Contaminants are present in the sample	The effects of contaminants are shown in the <a href="#">Contaminants</a> 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
<b>Reduction in sequencing speed and q-score later into the run</b>	Fast fuel consumption is typically seen when the flow cell is overloaded with library (~5-10 fmol of library is recommended).	Add more fuel to the flow cell by following the instructions in the <a href="#">MinKNOW protocol</a> . In future experiments, load lower amounts of library to the flow cell.

#### Temperature fluctuation

Observation	Possible cause	Comments and actions
<b>Temperature fluctuation</b>	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
<b>MinKNOW shows "Failed to reach target temperature"</b>	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 <a href="#">this FAQ</a> for more information on MinION Mk 1B temperature control.

#### Guppy - no input .fast5 was found or basecalled

Observation	Possible cause	Comments and actions
<b>No input .fast5 was found or basecalled</b>	<i>input_path</i> did not point to the .fast5 file location	The <i>--input_path</i> 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.
<b>No input .fast5 was found or basecalled</b>	The .fast5 files were in a subfolder at the <i>input_path</i> location	To allow Guppy to look into subfolders, add the <i>--recursive</i> flag to the command

#### Guppy - no Pass or Fail folders were generated after basecalling

Observation	Possible cause	Comments and actions
<b>No Pass or Fail folders were generated after basecalling</b>	The <i>--qscore_filtering</i> flag was not included in the command	The <i>--qscore_filtering</i> 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
<b>Unusually slow processing on a GPU computer</b>	The <i>--device</i> flag wasn't included in the command	The <i>--device</i> 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 <i>--device cuda:0 cuda:1</i> , when 2 GPUs are specified to use by the Guppy command.