

Introduction to the protocol

Version: RBK_9054_v2_revV_14Aug2019

Overview of the protocol

Rapid Barcoding Kit features

This kit is recommended for users who:

- wish to multiplex samples to reduce price per sample
- need a PCR-free method of multiplexing to preserve additional information such as base modifications
- require a short preparation time
- have limited access to laboratory equipment

Introduction to the Rapid Barcoding Sequencing Kit

This protocol describes how to carry out rapid barcoding of genomic DNA using the Rapid Barcoding Sequencing Kit (SQK-RBK004).

Steps in the sequencing workflow:

Prepare for your experiment

You will need to:

- Extract your DNA, and check its length, quantity and purity.

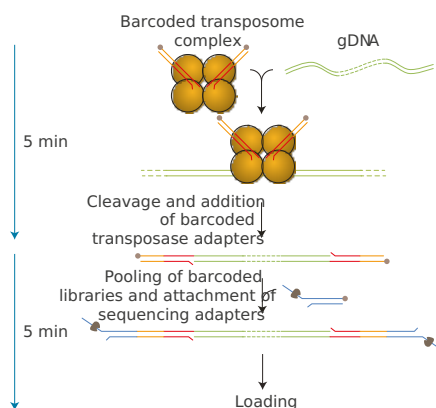
The quality checks performed during the protocol are essential in ensuring experimental success.

- 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:

- Tagment your DNA using the Fragmentation Mix RB in the kit; this simultaneously attaches a pair of barcodes to the fragments
- Pool the barcoded samples
- 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:

Equipment and consumables

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- 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 barcoding

Rapid Adapter Top Up Kit

The Rapid Adapter Top Up Kit (EXP-RAP001) supplies additional tubes of Rapid Adapter, Sequencing Buffer, Sequencing Tether, and Loading Beads. The Rapid Barcoding Sequencing Kit contains sufficient reagents for six pooled libraries using all 12 barcodes. The Top Up kit can be used for cases where several experiments are run with fewer than 12 barcodes and the Rapid Barcoding kit no longer has sufficient Rapid Adapter and Sequencing Buffer.

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Rapid Barcoding Sequencing Kit SQK-RBK004
- Flongle Sequencing Expansion (EXP-FSE001)
- Flongle Flow Cell R9.4.1 (FLO-FLG001)
- Flongle Flow Cell R10.3 (FLO-FLG111)
- EPI2ME FASTQ Barcoding workflow, or
- Barcode demultiplexing in Guppy

Equipment and consumables

Materials

- 200 ng high molecular weight genomic DNA
- Rapid Barcoding Sequencing Kit (SQK-RBK004)
- Flongle Sequencing Expansion (EXP-FSE001)
- Flow Cell Priming Kit (EXP-FLP002)

Consumables

- Flongle device - flow cell and adapter
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Agencourt AMPure XP beads
- Freshly prepared 70% ethanol in nuclease-free water
- 10 mM Tris-HCl pH 8.0 with 50 mM NaCl

Equipment

- Ice bucket with ice
- Microfuge
- Timer
- Thermal cycler or heat block at 30°C and 80°C
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips

Rapid Barcoding Sequencing (SQK-RBK004)

Equipment and consumables

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- P20 pipette and tips
- P2 pipette and tips

Optional Equipment

- Standard gel electrophoresis equipment
- Agilent Bioanalyzer (or equivalent)
- Qubit fluorometer (or equivalent for QC check)
- Eppendorf 5424 centrifuge (or equivalent)
- Magnetic rack
- Hula mixer (gentle rotator mixer)

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-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.

Equipment and consumables

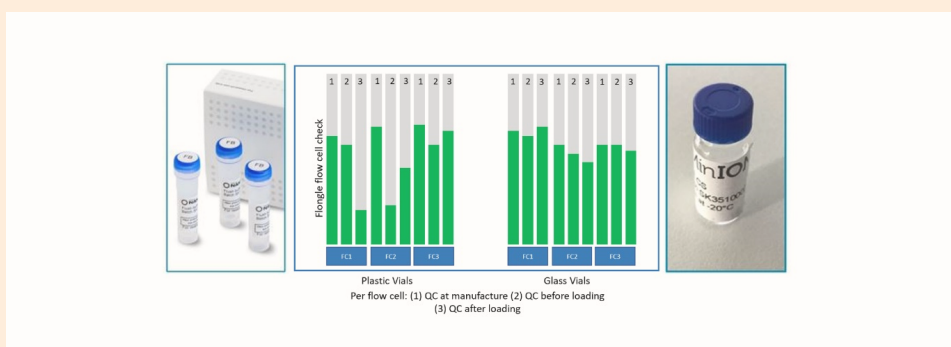
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IMPORTANT

Flongle Sequencing Expansion

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 Sequencing Expansion 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-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)

For this protocol, you will need 200 ng high molecular weight genomic 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](#).

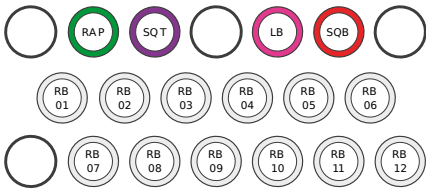
Chemical contaminants

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.

Rapid Barcoding Kit (SQK-RBK004) contents

Equipment and consumables

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- RAP : Rapid adapter

SQT : Sequencing tether

LB : Loading beads

SQB : Sequencing buffer

RB01 : Fragmentation Mix RB 1

RB02 : Fragmentation Mix RB 2

RB03 : Fragmentation Mix RB 3

RB04 : Fragmentation Mix RB 4
- RB05 : Fragmentation Mix RB 5

RB06 : Fragmentation Mix RB 6

RB07 : Fragmentation Mix RB 7

RB08 : Fragmentation Mix RB 8

RB09 : Fragmentation Mix RB 9

RB10 : Fragmentation Mix RB 10

RB11 : Fragmentation Mix RB 11

RB12 : Fragmentation Mix RB 12

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Fragmentation Mix	RB01-RB12	Clear	12, each vial with a unique barcode	20
Rapid Adapter	RAP	Green	1	10
Sequencing Tether	SQT	Purple	1	10
Loading Beads	LB	Pink	1	360
Sequencing Buffer	SQB	Red	1	300

Rapid Barcoding Kit barcode sequences

Component	Sequence
RB01	AAGAAAGTTGTCGGTGTCTTTGTG
RB02	TCGATTCCGTTTGTAGTCGTCTGT
RB03	GAGTCTTGTGTCCCAGTTACCAGG
RB04	TTCGGATTCTATCGTGTTCCTA
RB05	CTTGTCCAGGGTTTGTGTAACCTT
RB06	TTCTCGCAAAGGCAGAAAGTAGTC
RB07	GTGTTACCGTGGGAATGAATCCTT
RB08	TTCAGGGAACAAACCAAGTTACGT
RB09	AACTAGGCACAGCGAGTCTTGTT
RB10	AAGCGTTGAAACCTTTGTCCTCTC

Flongle Sequencing Expansion contents (EXP-FSE001)

Equipment and consumables

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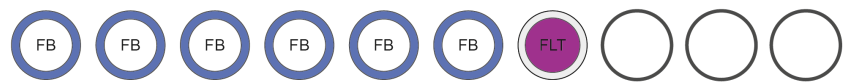
SBII : Sequencing Buffer II
LBII : Loading Beads II
LS : Loading Solution
FB : Flush Buffer

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

IMPORTANT

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

Flow Cell Priming Kit contents (EXP-FLP002)



FLB : Flush buffer
FLT : Flush tether

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

SFB Expansion contents (EXP-SFB001)

Computer requirements and software

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SFB : S fragment buffer

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Short Fragment Buffer	SFB	Grey	4	1,800

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](#).

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](#).

Library preparation

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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

Library preparation

~25 minutes

Materials

- 200 ng high molecular weight genomic DNA
- Fragmentation Mix RB01-12
- Rapid Adapter (RAP)

Consumables

- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Agencourt AMPure XP beads
- Freshly prepared 70% ethanol in nuclease-free water
- 10 mM Tris-HCl pH 8.0 with 50 mM NaCl

Equipment

- Thermal cycler or heat block at 30°C and 80°C
- Microfuge
- P10 pipette and tips

Optional Equipment

- Magnetic rack
- Hula mixer (gentle rotator mixer)

Library preparation

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Loading recommendations

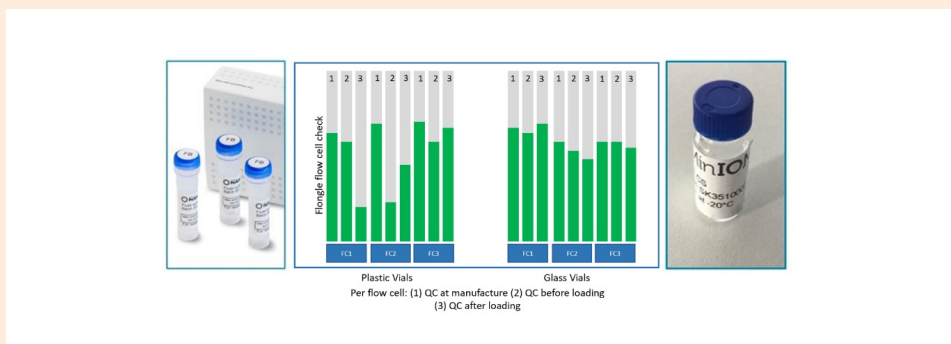
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To rapidly deploy this to Flongle users, we have produced a Flongle Sequencing Expansion with these three components in glass vials, which can perform 12 Flongle flow cell loads in total.

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

Flongle Sequencing Expansion (EXP-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)

Library preparation

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1 Thaw kit components at room temperature, spin down briefly using a microfuge and mix by pipetting as indicated by the table below:

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
Fragmentation Mix RB01-12	Not frozen	✓	✓
Rapid Adapter (RAP)	Not frozen	✓	✓
Sequencing Buffer (SQB)	✓	✓	✓*
Loading Beads (LB)	✓	✓	Mix by pipetting or vortexing immediately before use
Sequencing Tether (SQT)	✓	✓	✓

*Vortexing, followed by a brief spin in a microfuge, is recommended for Sequencing Buffer (SQB).

2 Prepare the DNA in nuclease-free water

- Transfer ~200 ng genomic DNA into a 1.5 ml Eppendorf DNA LoBind tube
- Adjust the volume to 3.75 µl with nuclease-free water
- Mix by flicking the tube to avoid unwanted shearing
- Spin down briefly in a microfuge

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

Reagent	Volume
200 ng template DNA	3.75 µl
Fragmentation Mix RB01-12 (one for each sample)	1.25 µl
Total	5 µl

4 Mix gently by flicking the tube, and spin down.

5 Incubate the tube at 30°C for 1 minute and then at 80°C for 1 minute. Briefly put the tube on ice to cool it down.

6 Pool all barcoded samples in your desired ratio, noting the total volume.

7 Resuspend the AMPure XP beads by vortexing.

8 To the entire pooled barcoded sample from Step 6, add an equal volume of resuspended AMPure XP beads, and mix by flicking the tube.

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

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

Loading the Flongle flow cell

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- 11 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.**
- 12 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.**
- 13 Repeat the previous step.**
- 14 Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry.**
- 15 Remove the tube from the magnetic rack and resuspend pellet in 5 µl of 10 mM Tris-HCl pH 7.5-8.0 with 50 mM NaCl. Incubate for 2 minutes at room temperature.**
- 16 Pellet the beads on a magnet until the eluate is clear and colourless.**
- 17 Remove and retain 5 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**
 - Remove and retain the eluate which contains the DNA in a clean 1.5 ml Eppendorf DNA LoBind tube
 - Dispose of the pelleted beads
- 18 Add 0.5 µl of RAP to the tube.**
- 19 Mix gently by flicking the tube, and spin down.**
- 20 Incubate the reaction for 5 minutes at room temperature.**

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.

END OF STEP

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

Loading the Flongle flow cell

Materials

- Flongle Sequencing Expansion (EXP-FSE001)
- Flush Tether (FLT)

Loading the Flongle flow cell

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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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Loading the Flongle flow cell

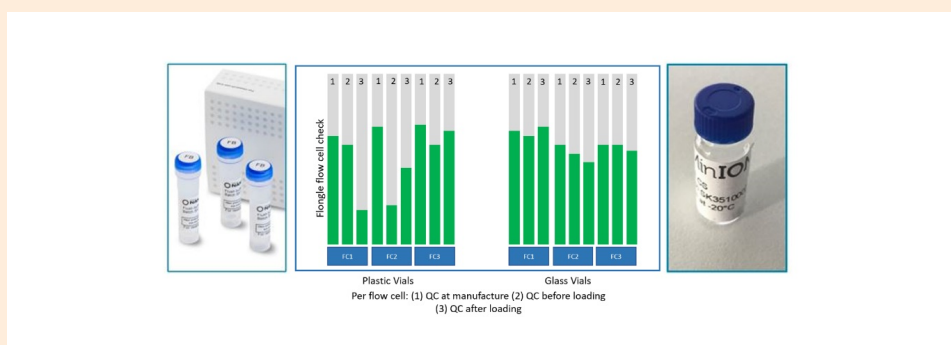
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Flongle Sequencing Expansion (EXP-FSE001) components

- 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)

IMPORTANT

Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.

Loading the Flongle flow cell

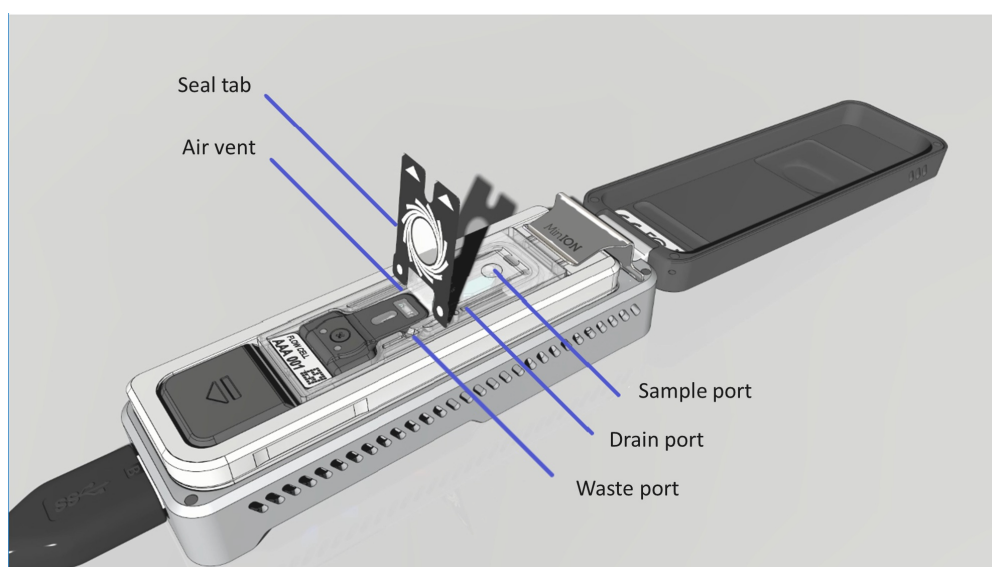
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IMPORTANT

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 your sequencing kit at room temperature.**

Loading the Flongle flow cell

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

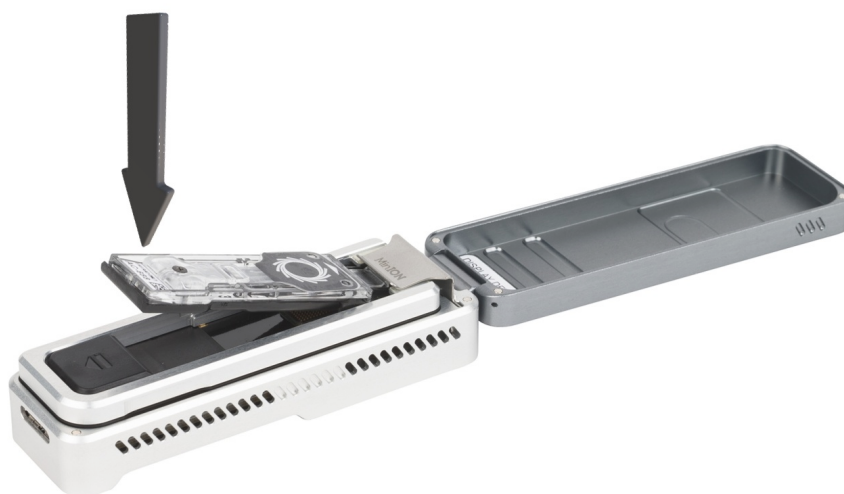
IMPORTANT

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.



Loading the Flongle flow cell

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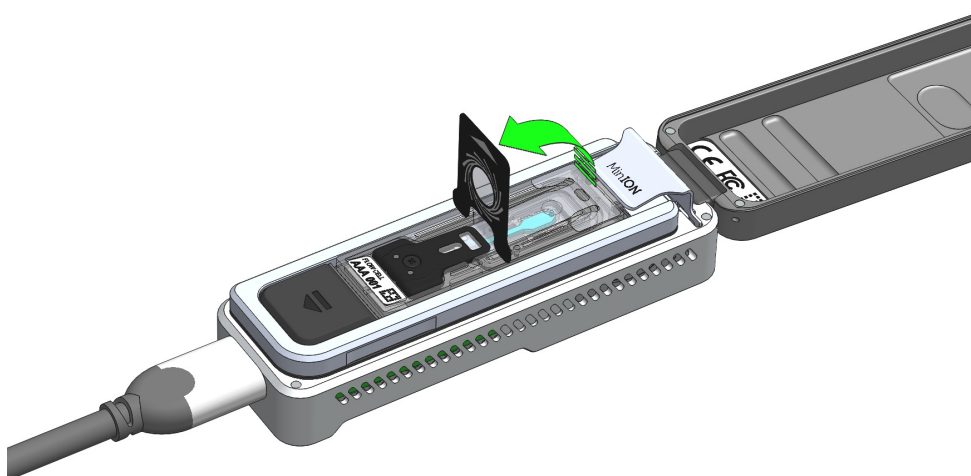
IMPORTANT

How to prime and load a Flongle flow cell

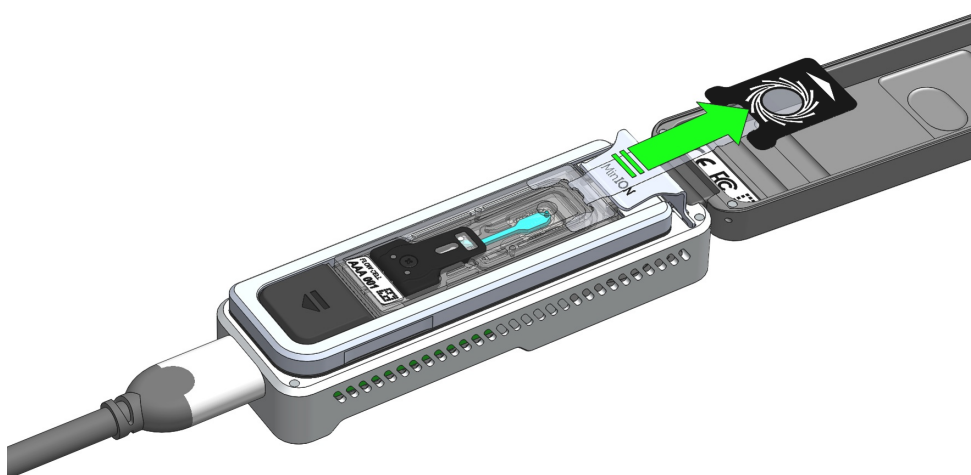
5 In a fresh 1.5 ml Eppendorf DNA LoBind tube, mix 117 μ l of Flush Buffer (FB) with 3 μ l of Flush Tether (FLT) and mix by pipetting.

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

Lift up the seal tab:



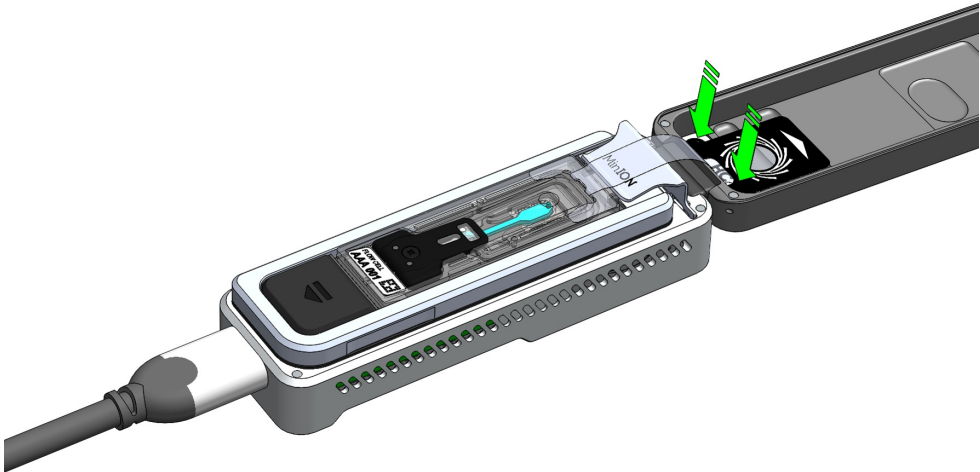
Pull the seal tab to open access to the sample port:



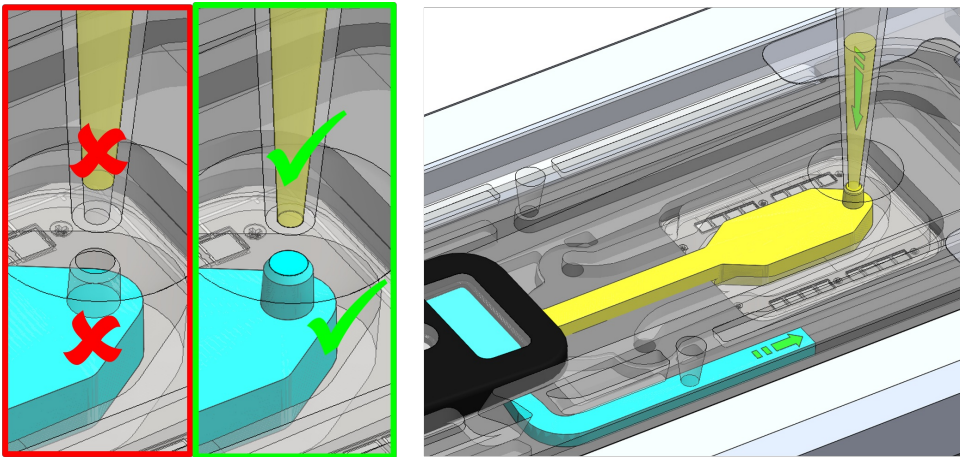
Hold the seal tab open by using adhesive on the tab to stick to the MinION Mk 1B lid:

Loading the Flongle flow cell

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- 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. To avoid flushing the flow cell too vigorously, load the priming mix by twisting the pipette plunger down.



- 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:

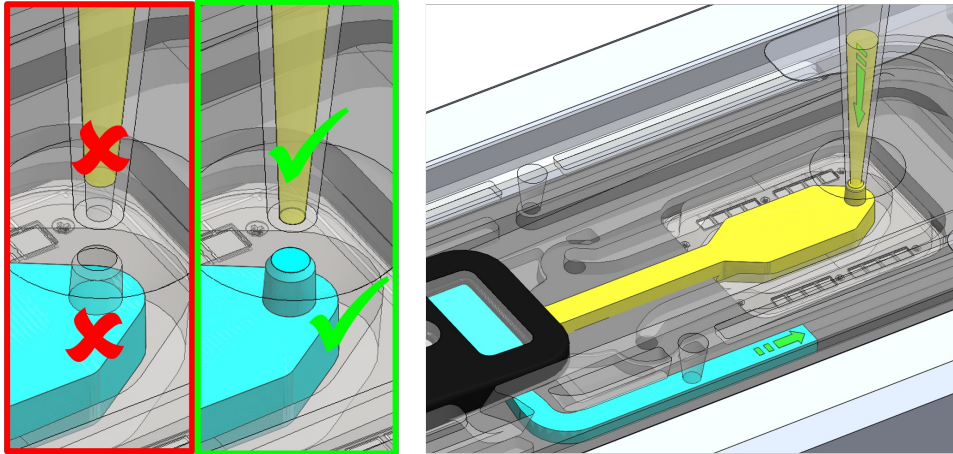
Reagents	Volume
Sequencing Buffer II (SBI)	15 μ l
Loading Beads II (LBII) mixed immediately before use, or Loading Solution (LS), if using. LS can be used instead of LBII when preparing libraries with the Ligation Sequencing Kit (SQK-LSK110)	10 μ l
DNA library	5 μ l
Total	30 μl

Rapid Barcoding Sequencing (SQK-RBK004)

Loading the Flongle flow cell

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- 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 twisting the pipette plunger down.

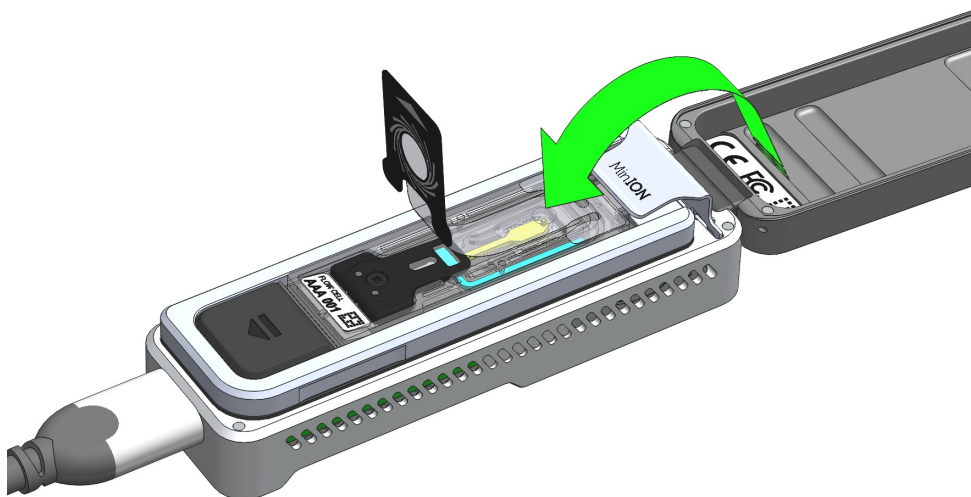


Sequencing and data analysis

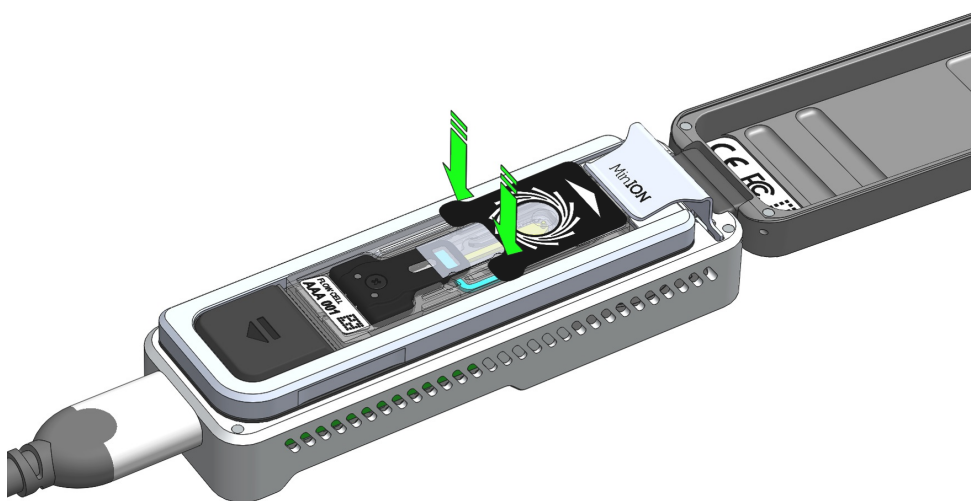
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10 Seal the Flongle flow cell using the adhesive on the seal tab.

Stick the transparent adhesive tape to the sample port.



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.

Downstream analysis

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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 MinION Mk1C device

Follow the instructions in the [MinION Mk1C protocol](#).

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

Follow the instructions in the [MinIT protocol](#).

4. Data acquisition using MinKNOW on a computer and basecalling at a later time using 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 [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

Troubleshooting

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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.

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

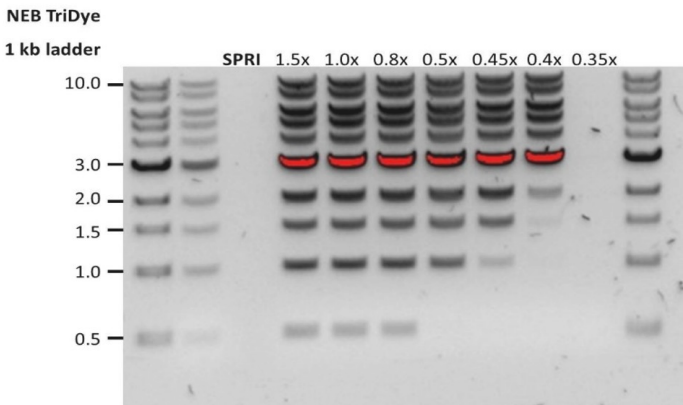
Observation	Possible cause	Comments and actions
Low DNA purity (Nanodrop reading for DNA OD 260/280 is <1.8 and OD 260/230 is <2.0–2.2)	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the Contaminants Know-how piece. Please try an alternative extraction 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.

Troubleshooting

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Observation	Possible cause	Comments and actions
Low recovery	DNA fragments are shorter than expected	<p>The lower the AMPure beads-to-sample ratio, the more stringent the selection against short fragments. Please always determine the input DNA length on an agarose gel (or other gel electrophoresis methods) and then calculate the appropriate amount of AMPure beads to use.</p> 
Low recovery after end-prep	The wash step used ethanol <70%	DNA will be eluted from the beads when using ethanol <70%. Make sure to use the correct percentage.

The VoITRAX 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 VoITRAX USB-C cable to the computer	Insufficient power supply to the VoITRAX	The green LED signals that 3 A are being supplied to the device. This is the requirement for the full capabilities of the VoITRAX V2 device. Please use computers that meet the requirements listed on the VoITRAX V2 protocol .

The VoITRAX software shows an inaccurate amount of reagents loaded

Observation	Possible cause	Comments and actions
The VoITRAX software shows an inaccurate amount of reagents loaded	Pipette tips do not fit the VoITRAX 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 VoITRAX cartridge. Rainin 20 µl is the most suitable.

Issues during the sequencing run

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Observation	Possible cause	Comments and actions
The VoITRAX 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 VoITRAX software before loading.

Issues during the sequencing run

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

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

Issues during the sequencing run

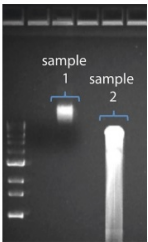
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Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support.

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-LSK109 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	<p>Read length reflects input DNA fragment length. Input DNA can be fragmented during extraction and library prep.</p> <ol style="list-style-type: none"> 1. Please review the Extraction Methods in the Nanopore Community for best practice for extraction. 2. Visualise the input DNA fragment length distribution on an agarose gel before proceeding to the library prep.  <p>In the image above, Sample 1 is of high molecular weight, whereas Sample 2 has been fragmented.</p> <ol style="list-style-type: none"> 3. During library prep, avoid pipetting and vortexing when mixing reagents. Flicking or inverting the tube is sufficient.

Large proportion of recovering pores

Issues during the sequencing run

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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	<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">1. A nuclease flush can be performed, or2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems. <div><p>Duty Time Summary of channel states over time</p></div> <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
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	<p>Known compounds, include polysaccharides, typically associate with plant genomic DNA.</p> <ol style="list-style-type: none">1. Please refer to the Plant leaf DNA extraction method.2. Clean-up using the QIAGEN PowerClean Pro kit.3. Perform a whole genome amplification with the original gDNA sample using the QIAGEN REPLI-g kit.
Large proportion of inactive 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

Issues during the sequencing run

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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	<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.
No input .fast5 was found or basecalled	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
No Pass or Fail folders were generated after basecalling	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
Unusually slow processing on a GPU computer	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.

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

Issues during the sequencing run

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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 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	<p>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:</p>  <p>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.</p>
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 the following AWS IP ranges . Access to the following IP addresses is also needed: 178.79.175.200 96.126.99.215
The MinIT software cannot be updated	The device already has the latest version of the software	Occassionally, 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. Bitwise or Putty, as described in the MinIT protocol) on a Windows computer or the terminal window on a Mac, run the command, <code>dpkg -f grep minit</code> , to find out the version of the MinIT software and <code>sudo apt update</code> if an update is needed. If the issue still persists, please contact Technical Services with details of the error.