Overview of the protocol

IMPORTANT

This is an Early Access product

For more information about our Early Access programmes, please seethis article on product release phases

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

Rapid PCR Barcoding Kit 24 V14 features

This kit is recommended for users who:

- Wish to multiplex samples to reduce price per sample
- Have a low starting amount of DNA
- Require a simple library preparation procedure

Introduction to the Rapid PCR Barcoding 24 V14 protocol

This protocol describes how to carry out rapid low input PCR barcoding of genomic DNA using the Rapid PCR Barcoding Kit 24 V14 (SQK-RPB114.24). There are 24 unique barcodes, allowing the user to pool up to 24 different samples in one sequencing experiment.

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.

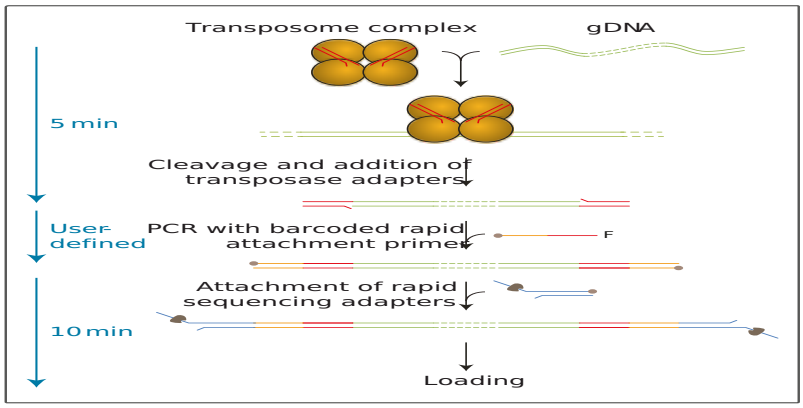
- Ensure you have your sequencing kit, the correct equipment and third-party reagents
- Download the software for acquiring and analysing your data
- Check your flow cell to ensure it has enough pores for a good sequencing run

Library preparation

You will need to:

- Tagment your DNA using the Fragmentation Mix in the kit
- PCR using the barcoded primer supplied in the kit
- Attach sequencing adapters supplied in the kit to the DNA ends
- Prime the flow cell, and load your DNA library into the flow cell

Note that after the PCR, the average length of DNA fragments should be <5 kb.



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 the barcoding workflow

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Rapid PCR Barcoding Kit 24 V14 (SQK-RPB114.24)
- R10.4.1 flow cells (FLO-FLG114)
- Flow Cell Wash Kit (EXP-WSH004)
- Flongle Sequencing Expansion (EXP-FSE002)

Equipment and consumables

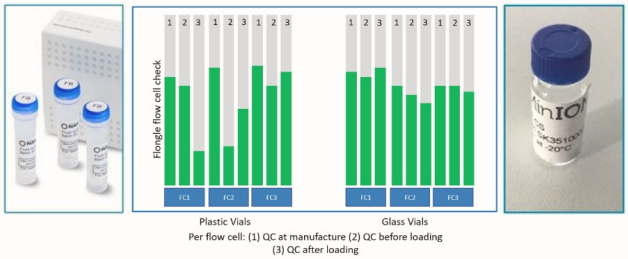
Materials	• 1–5 ng high molecular weight genomic DNA • Rapid PCR Barcoding Kit 24 V14 (SQK-RPB114.24) • Flongle Sequencing Expansion (EXP-FSE002)
Consumables	• Flongle device - flow cell and adapter • 1.5 ml Eppendorf DNA LoBind tubes • 0.2 ml thin-walled PCR tubes • Nuclease-free water (e.g. ThermoFisher, AM9937) • Freshly prepared 80% ethanol in nuclease-free water • LongAmp Taq 2X Master Mix (e.g. NEB, cat # M0287) • Qubit dsDNA HS Assay Kit (Invitrogen, Q32851) • Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment	• Ice bucket with ice • Microfuge • Timer • Thermal cycler • Magnetic rack • Hula mixer (gentle rotator mixer) • P1000 pipette and tips • P200 pipette and tips • P100 pipette and tips • P20 pipette and tips • P2 pipette and tips • Multichannel pipette • Qubit fluorometer (or equivalent for QC check)
Optional Equipment	• Agilent Bioanalyzer (or equivalent)

IMPORTANT

Flongle Sequencing Expansion

There are three buffers that come into direct contact with a flow cell at the point of loading (Sequencing Buffer, Flush Buffer/Flow Cell Flush, and Loading Beads/Library Beads or Loading Solution/Library Solution). We have found that there is a very low level of contaminants seeping out of the plastic vials that contain these buffers, and this impacts the robustness of the Flongle flow cell system (MinION and PromethION are not impacted by this).



To rapidly deploy this to Flongle users, we have produced a Flongle Sequencing Expansion (EXP-FSE001 and EXP-FSE002), which can perform 12 Flongle flow cell loads in total. The buffers in the Flongle Sequencing Expansion are shipped in glass vials to avoid the contaminant seeping issue. It is important to use these buffers when using Flongle Flow Cells.

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)

Flongle Sequencing Expansion (EXP-FSE002) components for Kit 14

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

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

For this protocol, you will need 1-5 ng high molecular weight genomic DNA.

Note: Your input DNA must be at least 4 kb in length to ensure correct tagmentation and PCR amplification.

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.

Third-party reagents

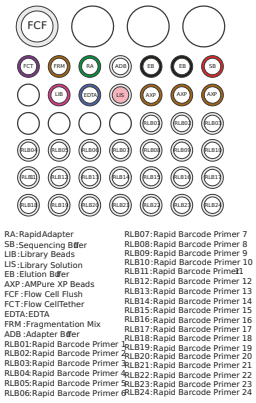
We have validated and recommend the use of all the third-party reagents used in this protocol. Alternatives have not been tested by Oxford Nanopore Technologies.

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

IMPORTANT

The Rapid Adapter (RA) used in this kit and protocol is not interchangeable with other sequencing adapters.

Rapid PCR Barcoding Kit 24 V14 (SQK-RPB114.24) contents



Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Fragmentation Mix	FRM	Brown	1	160
Rapid Adapter	RA	Green	1	15
Adapter Buffer	ADB	Clear	1	100
AMPure XP Beads	AXP	Amber	3	1,200
Elution Buffer	EB	Black	2	500
EDTA	EDTA	Blue	1	700
Sequencing Buffer	SB	Red	1	700

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Library Beads	LIB	Pink	1	600
Library Solution	LS	White cap, pink label	1	600
Flow Cell Flush	FCF	Clear	1	8,000
Flow Cell Tether	FCT	Purple	1	200
Rapid Barcode Primer 01-24	RLB01-24	Clear	24 (one per barcode)	15

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.

To maximise the use of the Rapid Barcoding Kits, the Rapid Adapter Auxiliary V14 (EXP-RAA114) and the Sequencing Auxiliary Vials V14 (EXP-AUX003) expansion packs are available.

These expansions provide additional library preparation and flow cell priming reagents to allow users to utilise any unused barcodes for those running in smaller subsets.

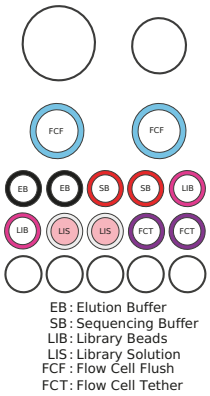
Both expansion packs used together will provide enough reagents for 6 library preparations.

Rapid Adapter Auxiliary V14 (EXP-RAA114) contents:

RA : Rapid Adapter
ADB : Adapter Buffer

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Rapid Adapter	RA	1	Green	15
Adapter Buffer	ADB	1	Clear	100

Sequencing Auxiliary Vials V14 (EXP-AUX003) contents:



Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Elution Buffer	EB	Black	2	500
Sequencing Buffer	SB	Red	2	700
Library Solution	LIS	White cap, pink label	2	600
Library Beads	LIB	Pink	2	600
Flow Cell Flush	FCF	Light blue label	2	8,000
Flow Cell Tether	FCT	Purple	2	200

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.

Rapid barcode primers

Component	Sequence
RLB01	AAGAAAGTTGTCGGTGTCTTTGTG
RLB02	TCGATTCCGTTTGTAGTCGTCTGT
RLB03	GAGTCTTGTGTCCCAGTTACCAGG
RLB04	TTCGGATTCTATCGTGTTTCCCTA
RLB05	CTTGTCCAGGGTTTGTGTAACCTT
RLB06	TTCTCGCAAAGGCAGAAAGTAGTC
RLB07	GTGTTACCGTGGGAATGAATCCTT
RLB08	TTCAGGGAACAAACCAAGTTACGT
RLB09	AACTAGGCACAGCGAGTCTTGGTT
RLB10	AAGCGTTGAAACCTTTGTCCTCTC
RLB11	GTTTCATCTATCGGAGGGAATGGA
RLB12	GTTGAGTTACAAAGCACCGATCAG
RLB13	AGAACGACTTCCATACTCGTGTGA
RLB14	AACGAGTCTCTTGGGACCCATAGA
RLB15	AGGTCTACCTCGCTAACACCACTG
RLB16	CGTCAACTGACAGTGGTTCGTACT
RLB17	ACCCTCCAGGAAAGTACCTCTGAT
RLB18	CCAAACCCAACAACCTAGATAGGC
RLB19	GTTCCTCGTGCAGTGTCAAGAGAT
RLB20	TTGCGTCCTGTTACGAGAACTCAT
RLB21	GAGCCTCTCATTGTCCGTTCTCTA
RLB22	ACCACTGCCATGTATCAAAGTACG
RLB23	CTTACTACCCAGTGAACCTCCTCG
RLB24	GCATAGTTCTGCATGATGGGTTAG

Computer requirements and software

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.

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 and basecalls in real time. You will be using MinKNOW for every sequencing experiment to sequence, basecall and demultiplex if your samples were barcoded.

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

For instructions on how to create an EPI2ME account and install the EPI2ME Desktop Agent, please refer to the EPI2ME Platform 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 or within four weeks of purchasing 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

~170 minutes

Materials

- 1–5 ng high molecular weight genomic DNA
- Fragmentation Mix (FRM)
- Rapid Barcode Primers (RLB01-24, at 10 µM)
- EDTA (EDTA)
- AMPure XP Beads (AXP)

- Elution Buffer (EB)
- Rapid Adapter (RA)
- Adapter Buffer (ADB)

Consumables

- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- LongAmp Taq 2X Master Mix (e.g. NEB, cat # M0287)
- Freshly prepared 80% ethanol in nuclease-free water
- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

- Ice bucket with ice
- Thermal cycler
- Hula mixer (gentle rotator mixer)
- Magnetic rack
- Microfuge
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips

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 or vortexing
Rapid Barcode Primers (RLB01-24)	Not frozen	✓	Pipette
Fragmentation Mix (FRM)	Not frozen	✓	Pipette
Rapid Adapter (RA)	Not frozen	✓	Pipette
Adapter Buffer (ADB)	✓	✓	Vortex or Pipette
AMPure XP Beads (AXP)	✓	✓	Mix by pipetting or vortexing immediately before use
Elution Buffer (EB)	✓	✓	Vortex or Pipette
EDTA (EDTA)	✓	✓	Vortex or Pipette

Note: Once thawed, keep all kit components on ice.

IMPORTANT

Your input DNA must be at least 4 kb in length to ensure correct tagmentation and PCR amplification.

2 **Prepare the DNA in nuclease-free water.**

- Transfer 1–5 ng of each genomic DNA sample into a 1.5 ml Eppendorf DNA LoBind tube
- Adjust the volume to 3 µl with nuclease-free water
- Mix thoroughly by flicking avoiding unwanted shearing
- Spin down briefly in a microfuge

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

Reagent	Volume
1-5 ng template DNA	3 µl
Fragmentation Mix (FRM)	1 µl
Total	**4 µl**

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

5 **In a thermal cycler, incubate the tube at 30°C for 2 minutes and then at 80°C for 2 minutes. Briefly put the tube on ice to cool it down.**

6 **For each sample, set up a PCR reaction as follows in a 0.2 ml thin-walled PCR tube:**

Reagent	Volume
Nuclease-free water	20 µl
Tagmented DNA	4 µl
RLB (01-24, at 10 µM)	1 µl
LongAmp Taq 2X master mix	25 µl
Total	**50 µl**

If the amount of input material is altered, the number of PCR cycles may need to be adjusted to produce the same yield.

7 **Mix gently by flicking the tube, and spin down.**

8 **Amplify using the following cycling conditions:**

Cycle step	Temperature	Time	No. of cycles
Initial denaturation	95°C	3 mins	1
Denaturation	95°C	15 sec	
Annealing	56°C	15 sec	14
Extension	65°C	6 min	
Final extension	65°C	6 min	1
Hold	4°C	∞	

9 **Add 4 µl of EDTA to each barcoded sample, mix thorougly by pipetting and spin down briefly.**

> **TIP**
>
> **EDTA is added at this step to stop the reaction.**

10 **Incubate for 5 minutes at room temperature.**

11 **Quantify 1 µl of each barcoded sample using a Qubit fluorometer (or equivalent for QC check).**

> **TIP**
>
> **We recommend pooling samples in an equimolar ratio to a final combined concentration of 200–400 fmol (~400–800 ng) for optimum barcode balance during sequencing.**
>
> If users want to to perform multiple flow cell loads from their library preparation, we recommend pooling in the higher concentration range.

12 **Pool all barcoded samples in equimolar ratios to a combined final concentration of 200–400 fmol (~400–800 ng) in a 1.5 ml Eppendorf DNA LoBind tube.**

For a 200 fmol final pool:

Number of barcoded samples pooled	2 samples	6 samples	12 samples	24 samples
Concentration of each barcoded sample added to the pool	100 fmol (~200 ng)	33.3 fmol (~66.7 ng)	16.7 fmol (~33.3 ng)	8.3 fmol (~16.7 ng)
Final pool concentration	**200 fmol** (~400 ng)	**200 fmol** (~400 ng)	**200 fmol** (~400 ng)	**200 fmol** (~400 ng)

For a 300 fmol final pool:

Number of barcoded samples pooled	2 samples	6 samples	12 samples	24 samples
Concentration of each barcoded sample added to the pool	150 fmol (~300 ng)	50 fmol (~100 ng)	25 fmol (~50 ng)	12.5 fmol (~25 ng)
Final pool concentration	**300 fmol** (~600 ng)	**300 fmol** (~600 ng)	**300 fmol** (~600 ng)	**300 fmol** (~600 ng)

For a 400 fmol final pool:

Number of barcoded samples pooled	2 samples	6 samples	12 samples	24 samples
Concentration of each barcoded sample added to the pool	200 fmol (~400 ng)	66.7 fmol (~133.3 ng)	33.3 fmol (~66.7 ng)	16.7 fmol (~33.3 ng)
Final pool concentration	**400 fmol** (~800 ng)	**400 fmol** (~800 ng)	**400 fmol** (~800 ng)	**400 fmol** (~800 ng)

Note: Please ensure you have quantified your samples prior to this step and take forward an equimolar concentration of each of the samples for optimal barcode balancing.
Samples may vary in concentration following the barcoded PCR, therefore the volume of each barcoded sample added to the pool will be different.

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

14 **To the pool of barcoded samples, add a 0.6X volume ratio of resuspended AMPure XP Beads (AXP) and mix by pipetting:**

Volume of barcoded sample pool	37.5 µl	75 µl	150 µl	300 µl	600 µl
Volume of AMPure XP Beads (AXP)	22.5 µl	45 µl	90 µl	180 µl	360 µl

Note: Table contains example volumes for reference. Please adjust the volume of AMPure XP Beads (AXP) added for the volume of your barcoded sample pool to ensure a 0.6X volume ratio.

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

16 **Prepare 2 ml of fresh 80% ethanol in nuclease-free water.**

17 **Briefly spin down the sample and pellet on a magnetic rack until supernatant is clear and colourless. Keep the tube on the magnetic rack, and pipette off the supernatant.**

18 **Keep the tube on the magnet and wash the beads with 1 ml of freshly-prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

19 **Repeat the previous step.**

20 **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.**

21 **Remove the tube from the magnetic rack and resuspend the pellet by pipetting in 15 µl Elution Buffer (EB). Spin down and incubate for 5 minutes at room temperature.**

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

23 **Remove and retain 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

- Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube
- Dispose of the pelleted beads

Quantify 1 µl of eluted sample using a Qubit fluorometer.

24 **Transfer 5–25 fmol of your eluted samples into a clean 1.5 ml Eppendorf DNA LoBind tube. Make up the volume to 5.5 µl with Elution Buffer (EB).**

25 **In a fresh 1.5 ml Eppendorf DNA LoBind tube, dilute the Rapid Adapter (RA) as follows and pipette mix:**

Reagent	Volume
Rapid Adapted (RA)	1.5 µl
Adapter Buffer (ADB)	3.5 µl
Total	**5 µl**

26 **Add 0.5 µl of diluted Rapid Adapter (RA) to the barcoded DNA.**

27 **Mix gently by flicking the tube, and spin down.**

28 Incubate the reaction for 5 minutes at room temperature.

END OF STEP

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

Loading the Flongle flow cell

~5 minutes

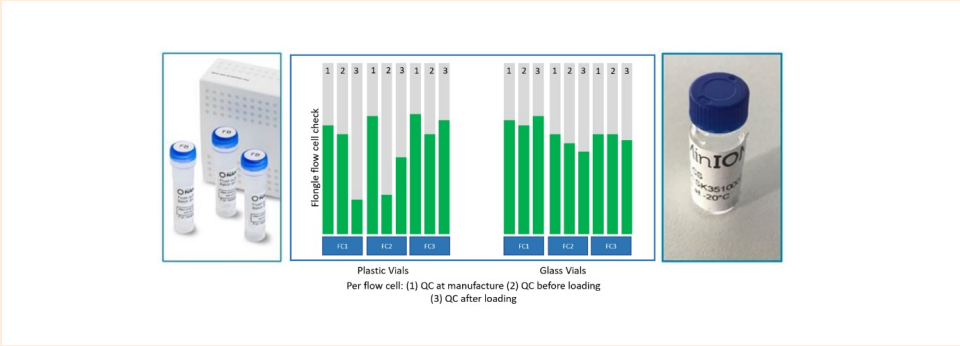
Materials	• Flongle Sequencing Expansion (EXP-FSE002) • Flow Cell Tether (FCT)
Consumables	• 1.5 ml Eppendorf DNA LoBind tubes • Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • Flongle flow cell
Equipment	• Flongle adapter • MinION or GridION • P200 pipette and tips • P10 pipette and tips

IMPORTANT

Flongle Sequencing Expansion (EXP-FSE002)

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

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



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

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

Flongle Sequencing Expansion (EXP-FSE002) components
- Sequencing Buffer (SB)
- Flow Cell Flush (FCF)
- Library Beads (LIB) or Library Solution (LIS)

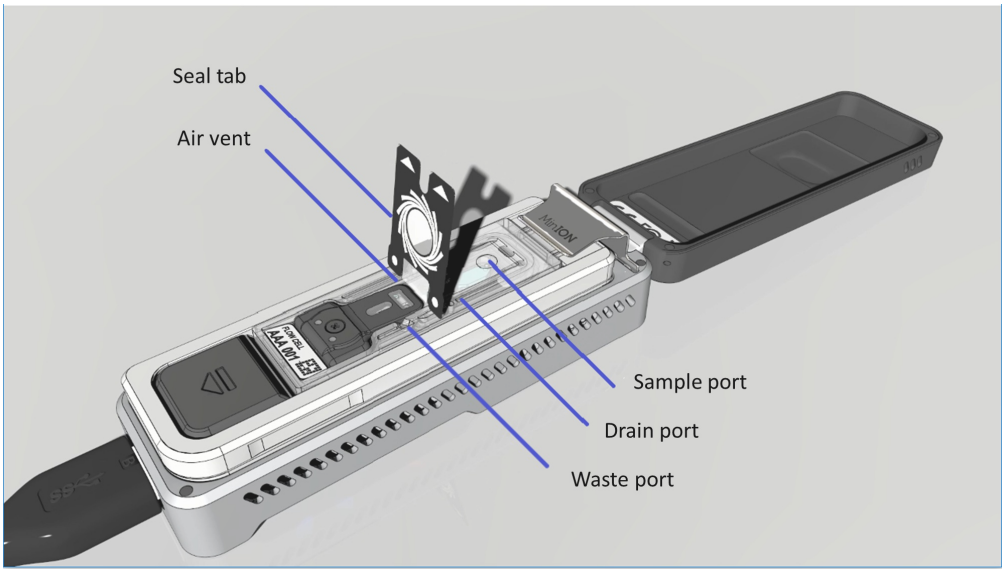
Sequencing Kit components
- Flow Cell Tether (FCT)

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

IMPORTANT

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 (LIB).

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 before mixing by vortexing. Then spin down and store on ice.**

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

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

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

IMPORTANT

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

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

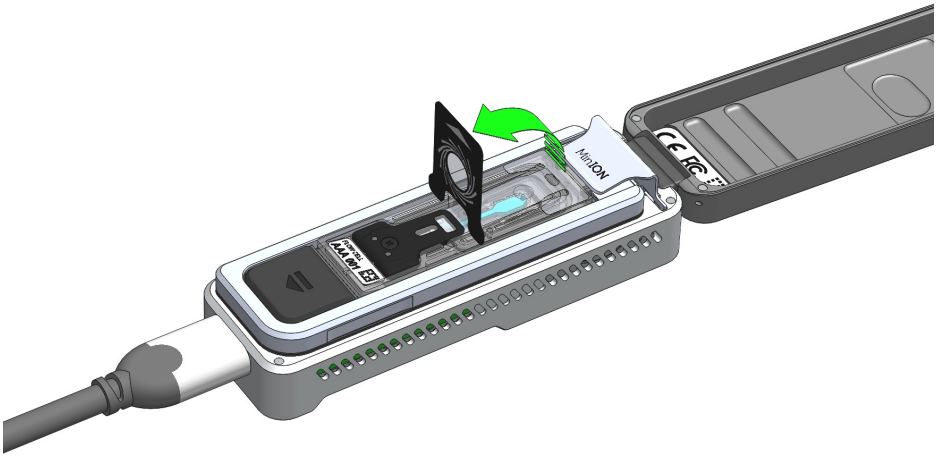
The flow cell should sit evenly and flat inside the adapter, to avoid any bubbles forming inside the fluidic compartments.

IMPORTANT

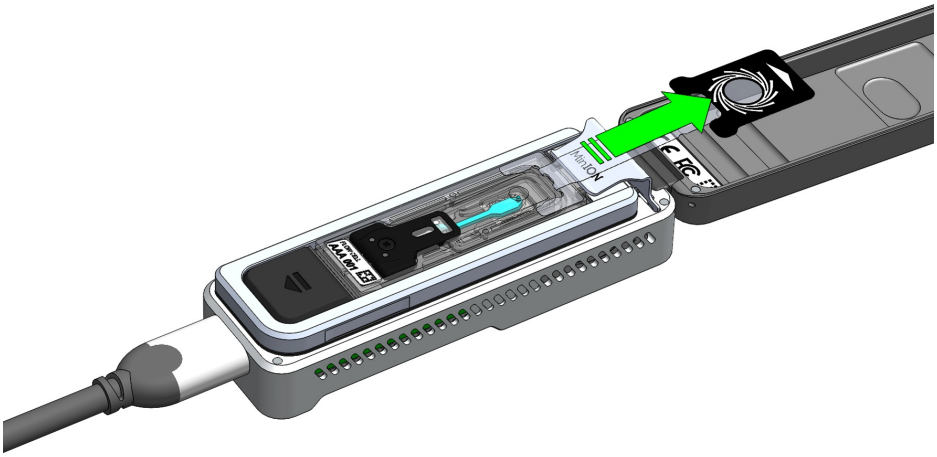
How to prime and load a Flongle flow cell

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

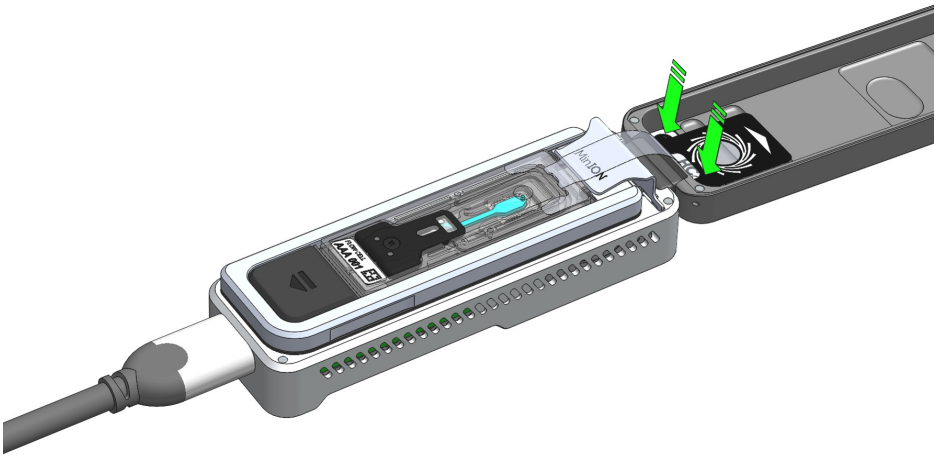
1. Lift up the seal tab:



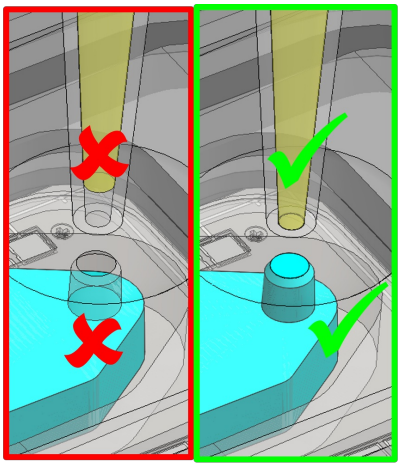
2. Pull the seal tab to open access to the sample port:

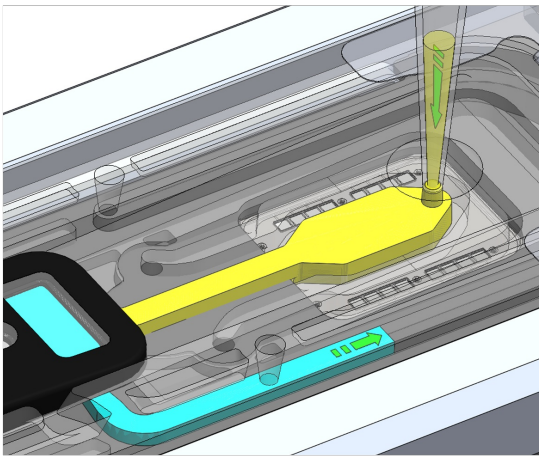


3. Hold the seal tab open by using adhesive on the tab to stick to the MinION Mk 1B lid:



6 **To prime your flow cell with the mix of Flow Cell Flush (FCF) and Flow Cell Tether (FCT) that was prepared earlier, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 pipette tip inside the sample port and slowly dispense the 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.**

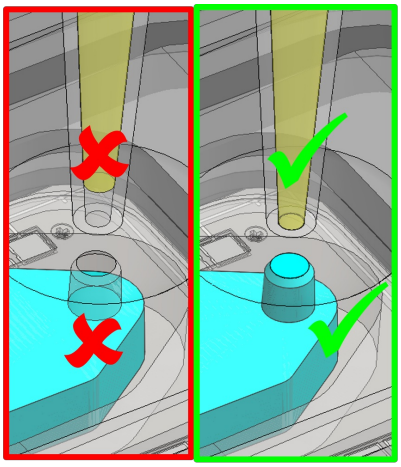


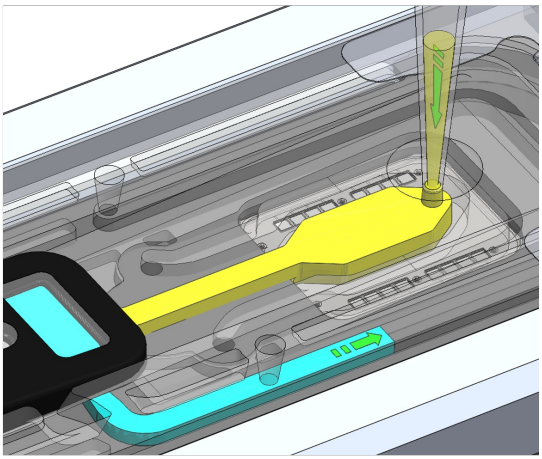


7 **Vortex the vial of Library Beads (LIB). Note that the beads settle quickly, so immediately prepare the Sequencing Mix in a fresh 1.5 ml Eppendorf DNA LoBind tube for loading the Flongle, as follows:**

Reagents	Volume
Sequencing Buffer (SB)	15 µl
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 µl
DNA library	5 µl
Total	**30 µl**

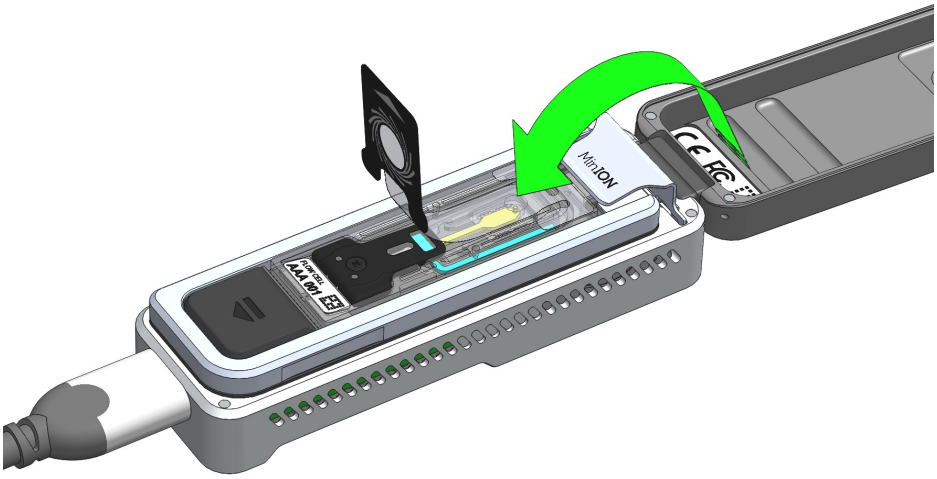
8 **To add the Sequencing Mix to the flow cell, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 tip inside the sample port and slowly dispense the Sequencing Mix into the flow cell by slowly pipetting down. We also recommend twisting the pipette plunger down to avoid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



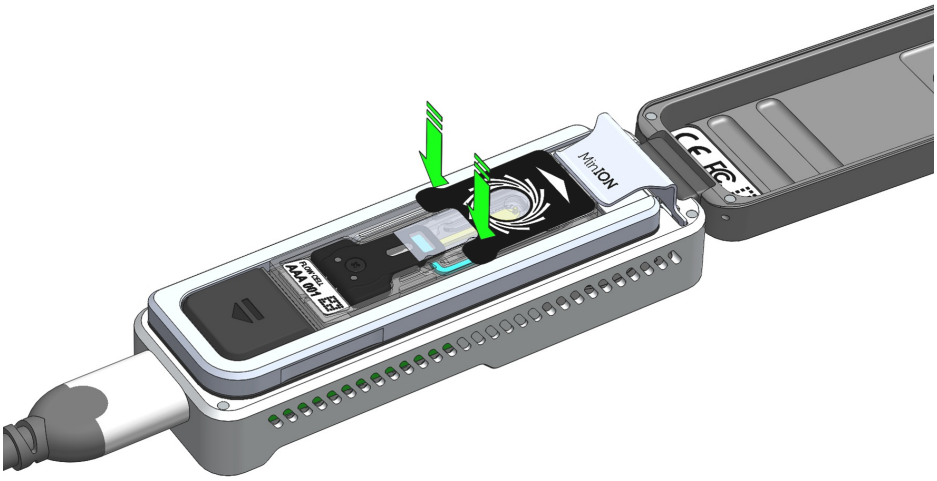


9 Seal the Flongle flow cell using the adhesive on the seal tab, as follows:

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



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



10 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. There are multiple 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.

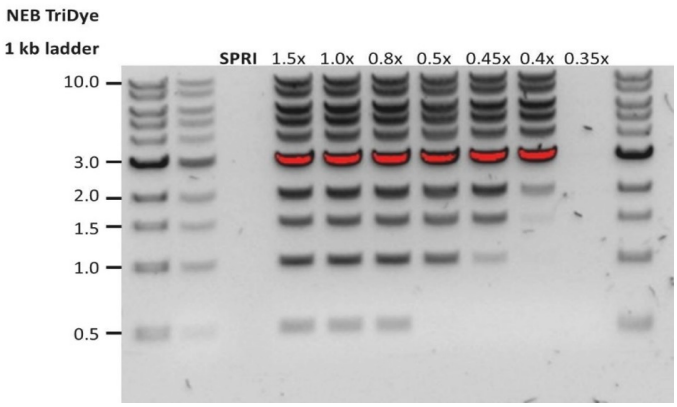
If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via LiveChat in the Nanopore Community.

Low sample quality

Observation	Possible cause	Comments and actions

Observation	Possible cause	Comments and actions
Low DNA purity (Nanodrop reading for DNA OD 260/280 is <1.8 and OD 260/230 is <2.0–2.2)	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the Contaminants document. Please try an alternative extraction method that does not result in contaminant carryover. Consider performing an additional SPRI clean-up step.
Low RNA integrity (RNA integrity number <9.5 RIN, or the rRNA band is shown as a smear on the gel)	The RNA degraded during extraction	Try a different RNA extraction method. For more info on RIN, please see the RNA Integrity Number document. Further information can be found in the DNA/RNA Handling page.
RNA has a shorter than expected fragment length	The RNA degraded during extraction	Try a different RNA extraction method. For more info on RIN, please see the RNA Integrity Number document. Further information can be found in the DNA/RNA Handling page. We recommend working in an RNase-free environment, and to keep your lab equipment RNase-free when working with RNA.

Low DNA recovery after AMPure bead clean-up

Observation	Possible cause	Comments and actions
Low recovery	DNA loss due to a lower than intended AMPure beads-to-sample ratio	1. AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample. 2. When the AMPure beads-to-sample ratio is lower than 0.4:1, DNA fragments of any size will be lost during the clean-up.
Low recovery	DNA fragments are shorter than expected	The lower the AMPure beads-to-sample ratio, the more stringent the selection against short fragments. Please always determine the input DNA length on an agarose gel (or other gel electrophoresis methods) and then calculate the appropriate amount of AMPure beads to use. 
Low recovery after end-prep	The wash step used ethanol <80%	DNA will be eluted from the beads when using ethanol <80%. Make sure to use the correct percentage.

Issues during the sequencing run using a Rapid-

based sequencing kit

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.

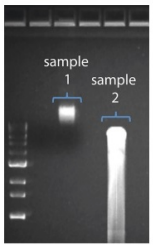
MinKNOW script failed

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. If you do not have another sequencing device availalbe, we recommend storing the flow cell and the loaded library at 4°C and contact Technical Support for further storage guidance.

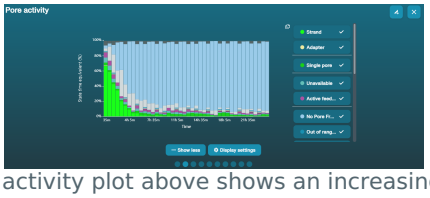
Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	10–50 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 Rapid PCR Barcoding Kit V14 was used, and sequencing adapters did not attach to the DNA	Make sure to closely follow the protocol and use the correct volumes and incubation temperatures. A Lambda control library can be prepared to test the integrity of reagents.
Pore occupancy close to 0	No tether on the flow cell	Tethers are added during flow cell priming (FCT tube). Make sure FCT was added to FCF before priming.

Shorter than expected read length

Observation	Possible cause	Comments and actions
Shorter than expected read length	Unwanted fragmentation of DNA sample	Read length reflects input DNA fragment length. Input DNA can be fragmented during extraction and library prep. 1. Please review the Extraction Methods in the Nanopore Community for best practice for extraction. 2. Visualise the input DNA fragment length distribution on an agarose gel before proceeding to the library prep.  In the image above, Sample 1 is of high molecular weight, whereas Sample 2 has been fragmented. 3. During library prep, avoid pipetting and vortexing when mixing reagents. Flicking or inverting the tube is sufficient.

Large proportion of unavailable pores

Observation	Possible cause	Comments and actions
Large proportion of unavailable pores (shown as blue in the channels panel and pore activity plot)  The pore activity plot above shows an increasing proportion of "unavailable" pores over time.	Contaminants are present in the sample	Some contaminants can be cleared from the pores by the unblocking function built into MinKNOW. If this is successful, the pore status will change to "sequencing pore". If the portion of unavailable pores stays large or increases: 1. A nuclease flush using the Flow Cell Wash Kit (EXP-WSH004) can be performed, or 2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.

Large proportion of inactive pores

Observation	Possible cause	Comments and actions
Large proportion of inactive/unavailable pores (shown as light blue in the channels panel and pore activity plot. Pores or membranes are irreversibly damaged)	Air bubbles have been introduced into the flow cell	Air bubbles introduced through flow cell priming and library loading can irreversibly damage the pores. Watch the Priming and loading your flow cell video for best practice
Large proportion of inactive/unavailable pores	Certain compounds co-purified with DNA	Known compounds, include polysaccharides, typically associate with plant genomic DNA. 1. Please refer to the Plant leaf DNA extraction method. 2. Clean-up using the QIAGEN PowerClean Pro kit. 3. Perform a whole genome amplification with the original gDNA sample using the QIAGEN REPLI-g kit.
Large proportion of inactive/unavailable pores	Contaminants are present in the sample	The effects of contaminants are shown in the Contaminants Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover.

Temperature fluctuation

Observation	Possible cause	Comments and actions
Temperature fluctuation	The flow cell has lost contact with the device	Check that there is a heat pad covering the metal plate on the back of the flow cell. Re-insert the flow cell and press it down to make sure the connector pins are firmly in contact with the device. If the problem persists, please contact Technical Services.

Failed to reach target temperature

Observation	Possible cause	Comments and actions
MinKNOW shows "Failed to reach target temperature"	The instrument was placed in a location that is colder than normal room temperature, or a location with poor ventilation (which leads to the flow cells overheating)	MinKNOW has a default timeframe for the flow cell to reach the target temperature. Once the timeframe is exceeded, an error message will appear and the sequencing experiment will continue. However, sequencing at an incorrect temperature may lead to a decrease in throughput and lower q-scores. Please adjust the location of the sequencing device to ensure that it is placed at room temperature with good ventilation, then re-start the process in MinKNOW. Please refer to this 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
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.