Oxford Nanopore Technologies

MINION: PROTOCOL

Nanopore-only Microbial Isolate Sequencing Solution (NO-MISS) from cell cultures using SQK-RBK114 (.24 or .96)

V ISO_9205_v114_revG_09May2025

End-to-end method outlining sample extraction, library preparation, sequencing and data analysis. This protocol:

- Uses genomic DNA
- Enables multiplexing of 4-24 samples
- Takes ~60 minutes for library preparation
- Includes DNA fragmentation
- Is optimised for high output
- Is compatible with R10.4.1 flow cells

For Research Use Only

FOR RESEARCH USE ONLY

Contents

1. Overview of the protocol

Introduction to the Nanopore-only Microbial Isolate Sequencing Solution (NO-MISS) protocol

This end to end protocol describes our Nanopore-Only Microbial Isolate Sequencing Solution (NO-MISS): a flexible approach allowing sequencing of 4 to 24 microbial isolate genomes per MinION Flow Cell, generating a minimum coverage of 50x per genome.

The 50X coverage threshold is sufficient for downstream analyses including: accurate assembly and plasmid resolution, AMR profiling, core genome (cg) and whole genome (wg) multi-locus sequence typing (MLST), and cg/wgSNP typing. You can analyse your sequencing data using EPI2ME, which provides a user-friendly bioinformatics workflow.

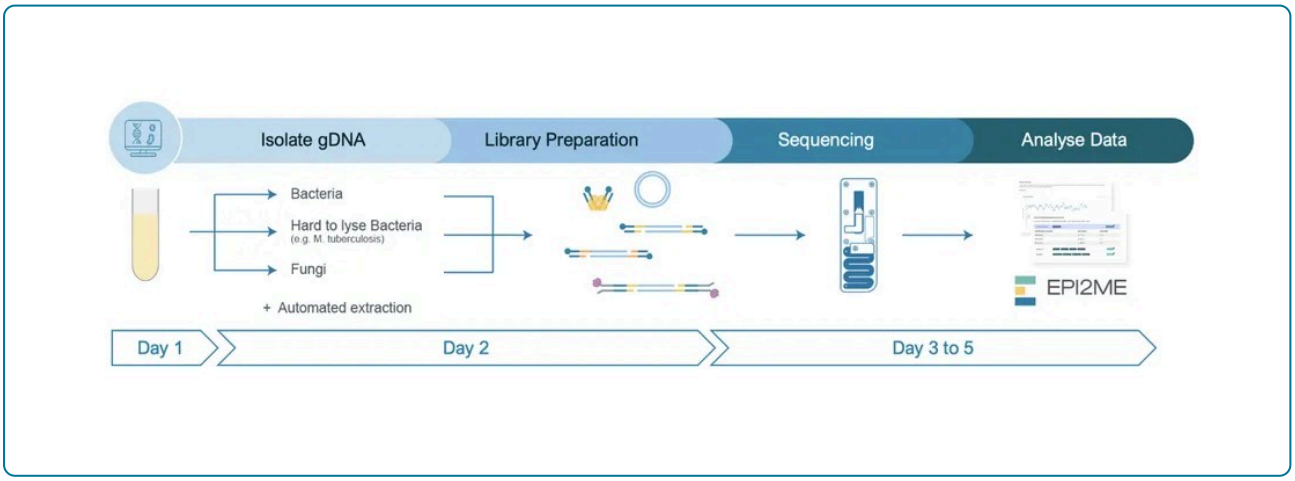
We provide multiple DNA extraction approaches, depending on requirements, and starting organism (bacteria, fungi/yeast). These are key in achieving reliable flow cell output and genome coverage. The sample specific extraction methods use NEB Monarch® Spin gDNA Extraction Kit, while the universal method uses a bead-beating method and the Maxwell® RCS PureFood Pathogen Kit.

The extracted gDNA is then tagmented and sequenced using our Rapid Barcoding Kit (SQK-RBK114.24 or SQK-RBK114.96). Up to 24 samples per sequencing experiment for bacterial isolates (up to 7 Mb genomes) and up to 8 samples for fungi/yeast isolates can be processed to achieve the 50x coverage threshold. Use a minimum of four barcodes per run to maintain performance. Detailed instructions for setting up the sequencing run on MinKNOW and downstream analysis are also included for a complete end-to-end protocol. We recommend sequencing up to 72 hours and generating at least 50x coverage per sample (approx. 0.5 Gb per barcode, assuming 5 Mb genome).

We recommend updating MinKNOW to the latest version prior to starting a sequencing run. The basecalling model v4.3 found in Dorado 0.5.0 onwards provides improved accuracy for bacterial DNA and is included in MinKNOW release v24.02 or newer.

For more information on updating MinKNOW, please refer to our MinKNOW protocol.

End-to-end workflow overview



Steps in the sequencing workflow:

Prepare for your experiment

You will need to:

- Extract your DNA, and check 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.

Sample preparation

Using the relevant gDNA extraction method, you will need to lyse your cells, extract your gDNA, and quantify the DNA:

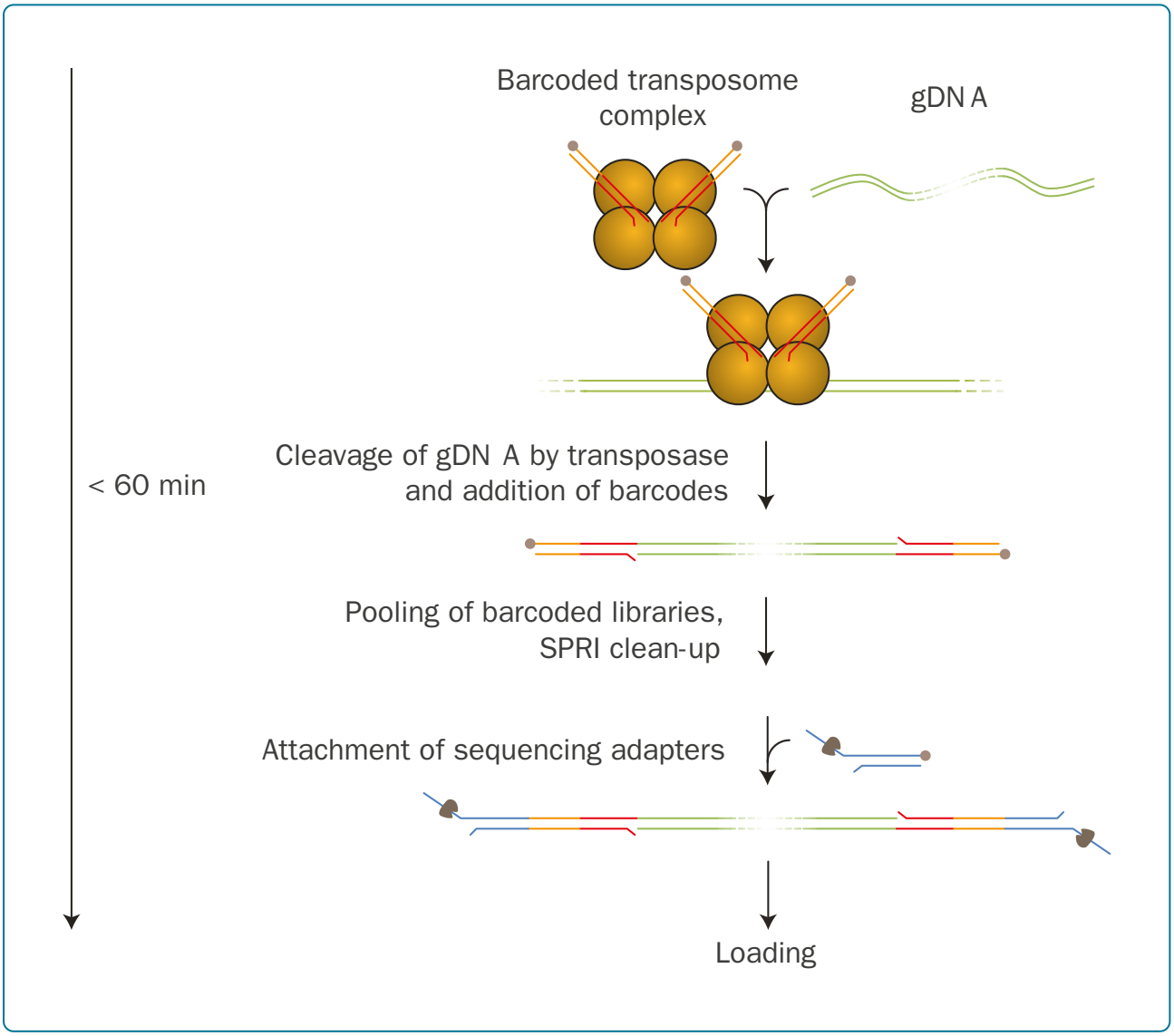
- **Universal bead-beating method:**
- Universal bead-beating gDNA extraction:
    - For high throughput requirements and universal applications.

- **Manual column-based methods:**
- Bacteria gDNA extraction:
    - For bacteria, such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens, Enterococcus faecalis,* and *Bacillus subtilis* or staphylococci such as *Staphylococcus aureus,* and *Staphylococcus epidermidis*.
- Hard to lyse organisms gDNA extraction:
    - For bacteria, such as *Mycobacterium tuberculosis*.
- Fungi gDNA extraction:
    - For fungi/yeast, such as *Candida albicans, Candida tropicalis,* and *Candida parapsilosis*.

Library preparation

The table below is an overview of the steps required in the library preparation, including timings and stopping points.

Library preparation step	Process	Time	Stop option
DNA barcoding	Tagmentation of the DNA using the Rapid Barcoding Kit V14	15 minutes	4°C overnight
Sample pooling and clean-up	Pooling of barcoded libraries and AMPure XP Bead clean-up	25 minutes	4°C overnight
Adapter ligation	Attach the sequencing adapters to the DNA ends	5 minutes	We strongly recommend sequencing your library as soon as it is adapted
Priming and loading the flow cell	Prime the flow cell and load the prepared library for sequencing	5 minutes	



Sequencing and analysis You will need to:

- Start a sequencing run using the MinKNOW software, which will collect raw data to basecall and demultiplex the barcoded reads.
- Perform downstream analysis uing the isolate mode of the wf-bacterial-genomes workflow in EPI2ME.

Compatibility of this protocol

This protocol should only be used in combination with:

- Rapid Barcoding Kit 24 V14 (SQK-RBK114.24)
- Rapid Barcoding Kit 96 V14 (SQK-RBK114.96)
- R10.4.1 flow cells (FLO-MIN114)
- Flow Cell Wash Kit (EXP-WSH004)
- Flow Cell Priming Kit V14 (EXP-FLP004)
- Sequencing Auxiliary Vials V14 (EXP-AUX003)
- Rapid Adapter Auxiliary V14 (EXP-RAA114)
- MinION Mk1B - MinION Mk1B IT requirements document
- MinION Mk1D - MinION Mk1D IT requirements document

2. Equipment and consumables

Materials

200 ng of extracted gDNA per sample
Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) OR Rapid Barcoding Kit 96 V14 (SQK-RBK114.96)

Consumables

MinION/GridION Flow Cell
Monarch® Spin gDNA Extraction Kit (NEB, T3010S/L)
Qubit™ 1x dsDNA HS Assay Kit (ThermoFisher, Q33230)
Qubit 1x dsDNA BR Assay Kit (ThermoFisher, Q33265)
PowerBead Pro tube (Qiagen, 19301)
BashingBead Buffer (Zymo, D6001-3-40)
Phosphate-buffered saline (PBS), pH 7.4 (Thermo Fisher, 10010023)
TE buffer (Sigma, 8890-100ML)
5 M sodium chloride solution (Sigma, S6546)
CTAB buffer (Promega, MC1411)
(Optional) Extra reagents for custom CTAB buffer:
CTAB (Sigma, H6269)
0.5 M EDTA (Fisher Scientific, 11568896)
Trizma® hydrochloride solution (Sigma, T2819)
Extra reagents for bacteria gDNA extraction:
Lysozyme human (Sigma, L1667)
Sodium dodecyl sulfate (SDS) at 10% v/v (Sigma, 71736)
Trizma® hydrochloride solution (Sigma, T2819)
Achromopeptidase (Sigma, A3547)
Extra reagents and consumables for hard to lyse organisms gDNA extraction:

Lysozyme human (Sigma, L1667)
Empty FastPrep® 2mL Lysing Matrix tubes (MP Biomedicals, 115076200)
Screw Cap for 2 mL Lysing Matrix tubes (MP Biomedicals, 115067005)
Glass beads, 4 mm (MP Biomedicals, 116914801)
Extra reagents for fungi gDNA extraction:
MetaPolyzyme (Sigma, MAC4L-5MG)
Agencourt AMPure XP beads (Beckman Coulter, A63881)
Extra reagents for automated bead-beating gDNA extraction:
RNase A (QIAGEN, 19101)
Proteinase K (QIAGEN, 19131)
Maxwell® RSC PureFood Pathogen kit (Promega, AS1660)
Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
Freshly prepared 80% ethanol in nuclease-free water
Nuclease-free water (e.g. ThermoFisher, AM9937)
PCR plate seals
96-well PCR plate, semi-skirted (e.g. Starlab, I1402-9800)
1.5 ml Eppendorf DNA LoBind tubes
2 ml Eppendorf DNA LoBind tubes
0.2 ml thin-walled PCR tubes or 0.2 ml 96-well PCR plate
Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

MinION or GridION device
MinION/GridION Flow Cell Light Shield
Vortex Adapter (Qiagen 13000-V1-24)
Vortex mixer
Thermal cycler
Timer
Thermomixer
Magnetic separation rack
Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific,11766427)
Eppendorf 5424 centrifuge (or equivalent)
Qubit™ fluorometer (or equivalent for QC check)
Multichannel pipette and tips
P1000 pipette and tips
P200 pipette and tips
P100 pipette and tips
P20 pipette and tips
P2 pipette and tips
Ice bucket with ice
Extra equipment for automated bead-beating gDNA extraction:
Maxwell® RSC Instrument (MPBiomedicals, AS4500)

Optional equipment

Hula mixer (gentle rotator mixer)

The above list of materials, consumables, and equipment is for all the extraction methods in the sample preparation section, as well as the library preparation section of the protocol. You will only need the consumables for the relevant extraction method for your sample input and the library preparation section.

For this protocol, the following inputs are required per sample:

Input requirements per sample for the extraction methods:

- Universal bead-beating gDNA extraction: 1 ml liquid overnight culture (~1 x 10^8 – 10^9 cfu/ml) or half of a loop of colonies from a plate
- Bacteria gDNA extraction: 200 µl liquid overnight culture (~1 x 10^8 – 10^9 cfu/ml) or 1/8 of a loop of colonies from a plate
- Hard to lyse organisms gDNA extraction: 5 – 10 mg cells from solid or liquid media
- Fungi gDNA extraction: 2 ml of ~1 x 10^7 cfu/ml overnight culture or a full 10 µl inoculating loop from a plate

For library preparation, 200 ng in 10 µl of extracted gDNA per sample is required.

Third-party reagents

Depending on the extraction protocol used, not all third-party reagents are required.

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.

Custom CTAB buffer

Custom CTAB buffer can be prepared instead of purchasing. Below are the reagents and concentrations required, with suggested examples with excess.

Reagent	Stock	Final concentration	Volume for 12 samples	Volume for 24 samples
CTAB	-	2% v/v	60 µl	120 µl
EDTA	0.5 M	40 mM	240 µl	480 µl
Sodium chloride	5 M	1.4 M	833 µl	1,666 µl
Trizma hydrochloride solution, pH 8	1 M	100 mM	300 µl	600 µl
Nuclease-free water	-	-	1567 µl	3,134 µl
Total	-	-	**3,000 µl**	**6,000 µl**

For staphylococcal inputs

Staphylococcal Lysis Buffer (SLB) is required for the bacterial gDNA extraction method for staphylococcal inputs.

Reagent	Stock	Final concentration	Volume for 12 samples with excess	Volume for 24 samples with excess
Trizma hydrochloride solution, pH 9	1 M	100 mM	150 ul	300 µl
Sodium chloride	5 M	10 mM	3 ul	6 µl
SDS	10% v/v	0.1% v/v	15 ul	30 µl
Nuclease-free water	-	-	1332 ul	2664 µl
Total volume	-	-	**1,500 ul**	**3,000 µl**

The SDS in the Staphylococcal Lysis Buffer (SLB) is essential for preventing the degradation of staphylococci DNA. Exclusion of SDS from the buffer results in a larger smear of DNA when run on a gel.

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.

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 12 weeks 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.

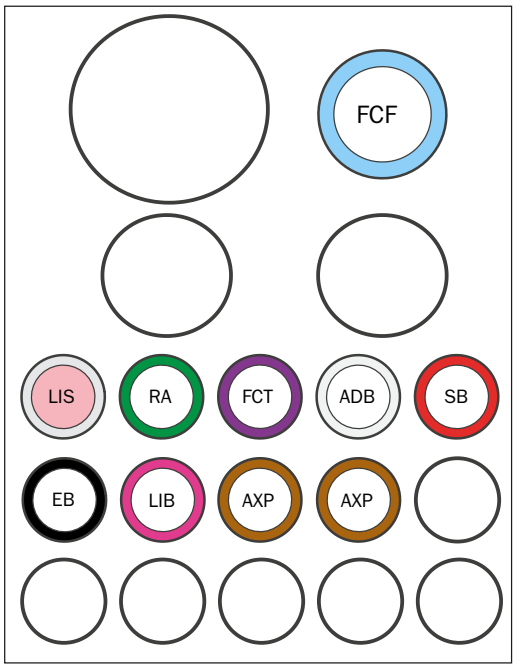
Flow cell	Minimum number of active pores covered by warranty
Flongle Flow Cell	50
MinION/GridION Flow Cell	800
PromethION Flow Cell	5000

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

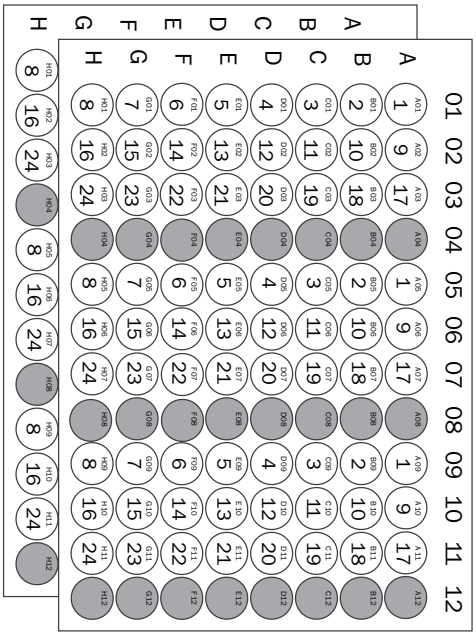
Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) contents

We are in the process of reformatting the barcodes provided in this kit into a plate format. This will reduce plastic waste and facilitates automated applications.

Plate format



RA : Rapid Adapter
SB : Sequencing Buffer
LIB : Library Beads
LIS : Library Solution
EB : Elution Buffer
AXP : AMPure XP Beads

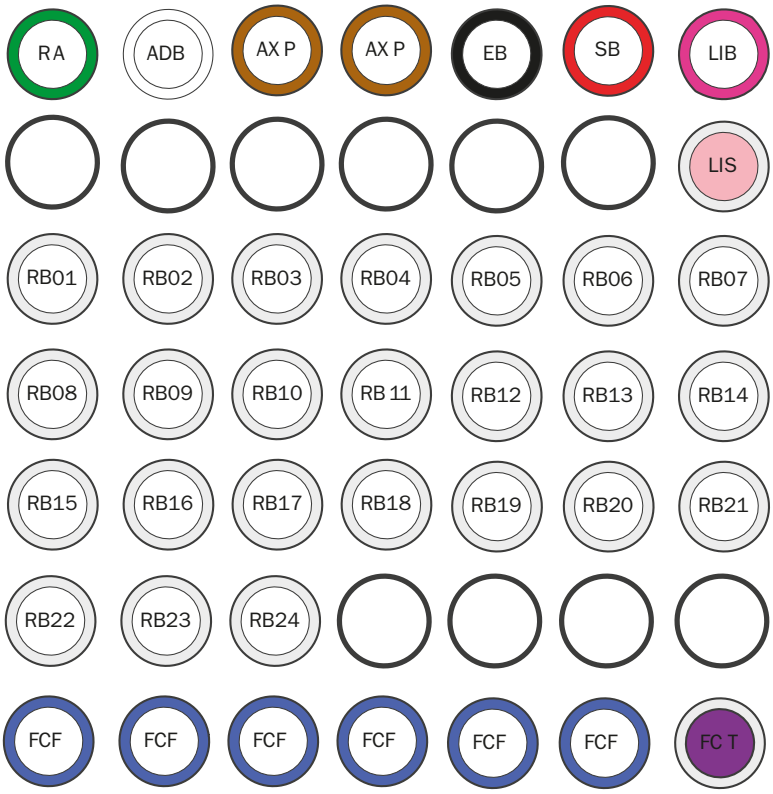


FCF : Flow Cell Flush
FCT : Flow Cell Tether
EDTA : EDTA
ADB : Adapter Buffer
1-24: Rapid barcode 01-24

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Rapid Adapter	RA	Green	1	15
Adapter Buffer	ADB	Clear	1	100
AMPure XP Beads	AXP	Amber	2	1200
Elution Buffer	EB	Black	1	500
Sequencing Buffer	SB	Red	1	700
Library Beads	LIB	Pink	1	600
Library Solution	LIS	White cap, pink label	1	600
Flow Cell Flush	FCF	Clear cap, light blue label	1	8000
Flow Cell Tether	FCT	Purple	1	200
Rapid Barcode plate	RB01-24	-	2 plates, 3 sets of barcodes per plate	5 µl per well

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.

Vial format

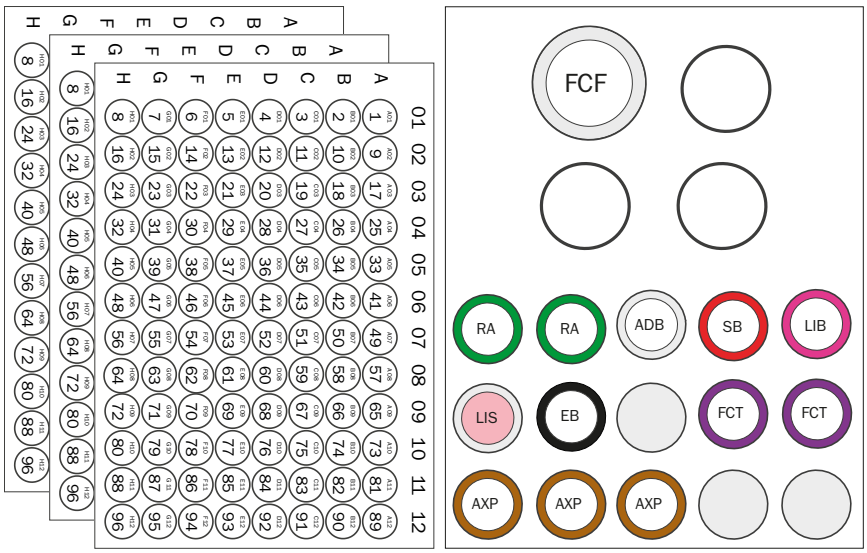


RA : Rapid Adapter
ADB : Adapter Buffer
LIB : Library Beads
LIS : Library Solution
EB : Elution Buffer
AXP : AMPure XP Beads
SB : Sequencing Buffer
FCF : Flow Cell Flush
FCT : Flow Cell Tether
RB01 : Rapid Barcode 1
RB02 : Rapid Barcode 2
RB03 : Rapid Barcode 3
RB04 : Rapid Barcode 4
RB05 : Rapid Barcode 5
RB06 : Rapid Barcode 6
RB07 : Rapid Barcode 7
RB08 : Rapid Barcode 8
RB09 : Rapid Barcode 9
RB10 : Rapid Barcode 10
RB11 : Rapid Barcode 11
RB12 : Rapid Barcode 12
RB13 : Rapid Barcode 13
RB14 : Rapid Barcode 14
RB15 : Rapid Barcode 15
RB16 : Rapid Barcode 16
RB17 : Rapid Barcode 17
RB18 : Rapid Barcode 18
RB19 : Rapid Barcode 19
RB20 : Rapid Barcode 20
RB21 : Rapid Barcode 21
RB22 : Rapid Barcode 22
RB23 : Rapid Barcode 23
RB24 : Rapid Barcode 24

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Rapid Adapter	RA	Green	1	15
Adapter Buffer	ADB	Clear	1	100
AMPure XP Beads	AXP	Amber	2	1,200
Elution Buffer	EB	Black	1	500
Sequencing Buffer	SB	Red	1	700
Library Beads	LIB	Pink	1	600
Library Solution	LIS	White cap, pink label	1	600
Flow Cell Flush	FCF	Blue	6	1,170
Flow Cell Tether	FCT	Purple	1	200
Rapid Barcodes	RB01-24	Clear	24	15

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.

Rapid Barcoding Kit 96 V14 (SQK-RBK114.96) contents



AXP : AMPure XP Beads
FCF : Flow Cell Flush
ADB : Adapter Buffer
RA : Rapid Adapter
LIB : Library Beads

EB : Elution Buffer
LIS : Library Solution
FCT : Flow Cell Tether
SB : Sequencing Buffer

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Rapid Adapter	RA	Green	2	15
Adapter Buffer	ADB	Clear	1	100
AMPure XP Beads	AXP	Amber	3	1,200
Elution Buffer	EB	Black	1	1,500
Sequencing Buffer	SB	Red	1	1,700
Library Beads	LIB	Pink	1	1,800
Library Solution	LIS	White cap, pink label	1	1,800
Flow Cell Flush	FCF	Clear	1	15,500
Flow Cell Tether	FCT	Purple	2	200
Rapid Barcodes	RB01-96	-	3 plates	8 µl per well

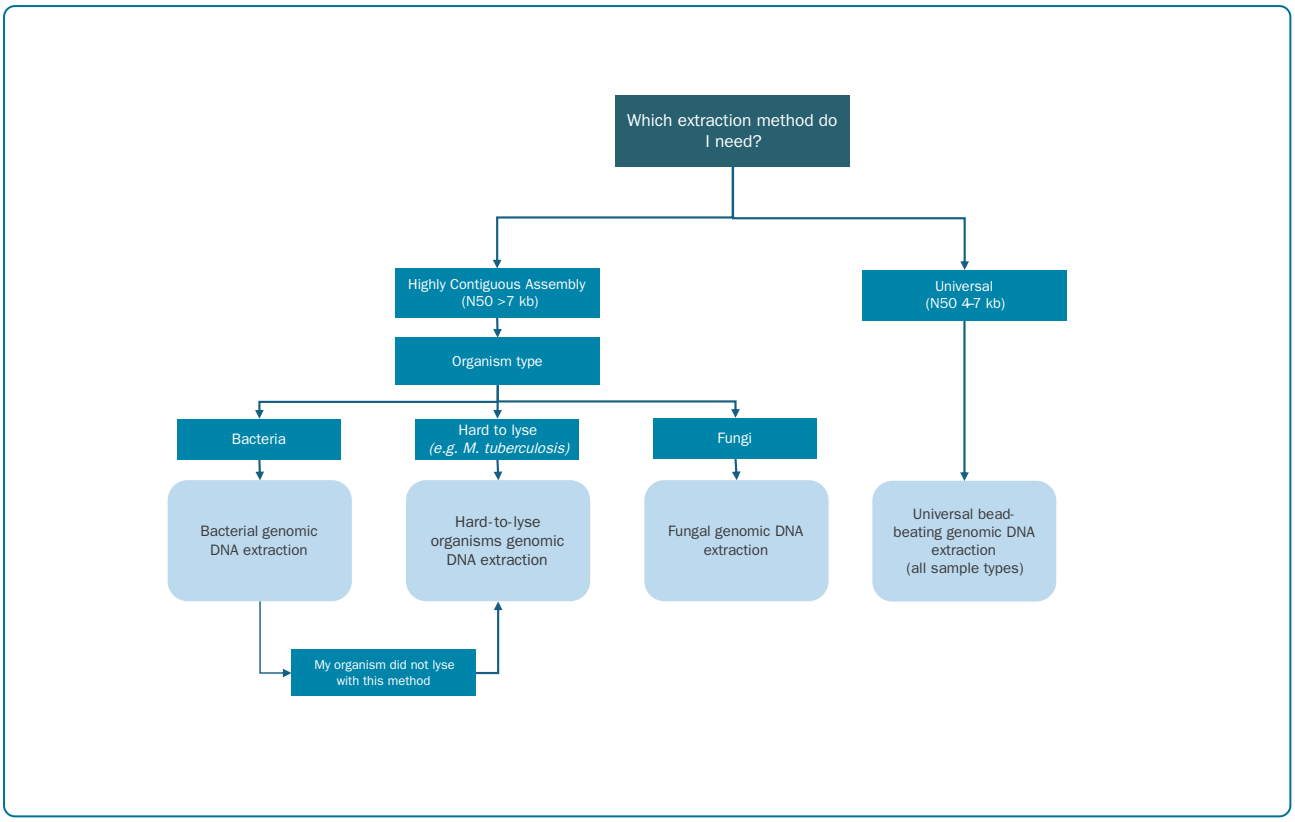
This Product Contains AMPure XP Reagent Manufactured by Beckman Coulter, Inc. and can be stored at -20°C with the kit without detriment to reagent stability.

3. Sample extraction method selection

Optimised extraction method decision

We have developed four optimised extraction methods to generate high quality genomic DNA from your cell cultures, allowing maximised sequencing output using this method.

What extraction method is right for me?


Which extraction method do I need?
Highly Contiguous Assembly (N50 >7 kb)
Universal (N50 4-7 kb)
Organism type
Bacteria
Hard to lyse (e.g. M. tuberculosis)
Fungi
Bacterial genomic DNA extraction
Hard-to-lyse organisms genomic DNA extraction
Fungal genomic DNA extraction
Universal bead-beating genomic DNA extraction (all sample types)
My organism did not lyse with this method

Sample extraction method	Sample type	Sample Input	Expected yield	Expected DNA Integrity Number (DIN)	Average sequencing read lengths	Extraction kits used
Universal bead-beating gDNA extraction	Universal applications: bacteria, fungi or yeast	1 ml liquid overnight culture (~1 x 10^8 – 10^9 cfu/ml) or half of a loop of colonies from a plate	>200 ng/µl per sample	7-9	~4-7 kb	QIAGEN PowerBead Tube and Promega Maxwell® RSC PureFood Pathogen kit
Bacteria gDNA extraction	Bacterial	200 µl liquid overnight culture (~1 x 10^8 – 10^9 cfu/ml) or 1/8 of a loop of colonies from a plate	~15-20 ng/µl per sample	9	>7 kb - Size will vary based on sample input species	NEB Monarch Spin gDNA Extraction Kit
Hard to lyse organisms gDNA extraction	*Mycobacterium tuberculosis* (or hard to extract bacterial samples)	5 – 10 mg cells from solid or liquid media	~15-40 ng/µl per sample	8	>7 kb - Size will vary based on sample input species	NEB Monarch Spin gDNA Extraction Kit
Fungi gDNA extraction	Fungi or yeast	2 ml of ~1 x 10^7 cfu/ml overnight culture or a full 10 µl inoculating loop from a plate	~40 ng/µl per sample	N/A	>7 kb - Size will vary based on sample input species	NEB Monarch Spin gDNA Extraction Kit

Note: The yield, DIN and sequencing read length of extracted DNA may vary depending on sample quality and species. Please ensure you are following the correct method and using high-quality sample inputs.

Follow the links in the table above for the extraction methods documentation.
Alternatively, these extraction methods can be found in the Extraction Protocols tab in the Documentation space on the Nanopore Community

4. Library preparation

Materials

200 ng of extracted gDNA per sample
Rapid Barcodes (RB01-24 or RB01-96)
Rapid Adapter (RA)
Adapter Buffer (ADB)
AMPure XP Beads (AXP)
Elution Buffer (EB)

Consumables

Qubit 1x dsDNA BR Assay Kit (ThermoFisher, Q33265)
Nuclease-free water (e.g. ThermoFisher, AM9937)
Freshly prepared 80% ethanol in nuclease-free water
Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, 0030129504) with heat seals
0.2 ml thin-walled PCR tubes
1.5 ml Eppendorf DNA LoBind tubes
2 ml Eppendorf DNA LoBind tubes
Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

Ice bucket with ice
Timer
Thermal cycler
Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific,11766427)
Magnetic separation rack
Hula mixer (gentle rotator mixer)
Qubit™ fluorometer (or equivalent for QC check)
P1000 pipette and tips
P200 pipette and tips
P100 pipette and tips
P20 pipette and tips
P10 pipette and tips
P2 pipette and tips
Multichannel pipette and tips

Sample throughput and Rapid Barcode use requirements with NO-MISS

This method has been developed to process 24 samples with a genome size of up to 7 Mb simultaneously.

For samples with a larger genome size (>7 Mb), we recommend lowering the number of samples to be barcoded and sequenced simultaneously to 8 samples.

For optimal output, we currently do not recommend using fewer than 4 barcodes.

Note: This method provides a standardised process for sample throughput that we have validated in-house. These settings will be applicable to the majority of use-cases and we recommend all new users to follow the recommended method. Experienced users can adjust sample throughput (4 to 48 barcoded samples) based on sample quality, genome size, and coverage requirements.

Check your flow cell.

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

See the flow cell check instructions in the MinKNOW protocol for more information.

1 Program the thermal cycler: 30°C for 2 minutes, then 80°C for 2 minutes.

2 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
Rapid Barcodes (RB01-24 or RB01-96))	Not frozen	✓	✓
Rapid Adapter (RA)	Not frozen	✓	✓
AMPure XP Beads (AXP)	✓	✓	Mix by pipetting or vortexing immediately before use
Elution Buffer (EB)	✓	✓	✓
Adapter Buffer (ADB)	✓	✓	Mix by vortexing

3 Prepare the DNA in nuclease-free water.

1. Transfer 200 ng of genomic DNA per sample into 0.2 ml thin-walled PCR tubes or an Eppendorf twin.tec® PCR plate 96 LoBind.
2. Adjust the volume of each sample to 10 µl with nuclease-free water.
3. Pipette mix the tubes thoroughly and spin down briefly in a microfuge.

4 Select a unique barcode for every sample to be run together on the same flow cell.

Note: Use one barcode per sample.

5 In the 0.2 ml thin-walled PCR tubes or an Eppendorf twin.tec® PCR plate 96 LoBind, mix the following:

Reagent	Volume per sample
Template DNA (200 ng from previous step)	10 µl
Rapid Barcodes (RB01-24 or RB01-96, one for each sample)	1.5 µl
Total	**11.5** µl

6 Ensure the components are thoroughly mixed by pipetting and spin down briefly.

7 Incubate the tubes or plate at 30°C for 2 minutes and then at 80°C for 2 minutes. Briefly put the tubes or plate on ice to cool.

8 Spin down the tubes or plate to collect the liquid at the bottom.

9 Pool all barcoded samples in a clean 2 ml Eppendorf DNA LoBind tube, noting the total volume.

.	Volume per sample	For 24 samples
Total volume	11.5 µl	276 µl

10 Resuspend the AMPure XP Beads (AXP) by vortexing.

11 Add an equal volume of resuspended AMPure XP Beads (AXP) to the entire pooled barcoded sample, and mix by flicking the tube.

.	Volume per sample	For 24 samples
Volume of AMPure XP Beads (AXP) added	11.5 µl	276 µl

12 **Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.**

13 **Prepare at least 2 ml of fresh 80% ethanol in nuclease-free water.**

14 **Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.**

15 **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.**

16 **Repeat the previous step.**

17 **Briefly 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.**

18 **Remove the tube from the magnetic rack and resuspend the pellet by pipetting in 15 µl Elution Buffer (EB). Incubate for 10 minutes at room temperature.**

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

20 **Remove and retain the full volume 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 and Qubit dsDNA BR assay.

Expect ~150 ng/µl for 24 samples, assuming 70% of DNA was retained during the wash.

21 **Transfer 11 µl of the sample into a clean 1.5 ml Eppendorf DNA LoBind tube.**

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

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

23 **Add 1 µl of the diluted Rapid Adapter (RA) to the barcoded DNA.**

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

25 **Incubate the reaction for 5 minutes at room temperature.**

Tip: While this incubation step is taking place you can proceed to the Flow Cell priming and loading section of the protocol.

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

5. Priming and loading the MinION and GridION Flow Cell

Materials Flow Cell Flush (FCF)

	Flow Cell Tether (FCT) Library Beads (LIB) Sequencing Buffer (SB)
Consumables	MinION/GridION Flow Cell Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616) 1.5 ml Eppendorf DNA LoBind tubes
Equipment	MinION or GridION device MinION/GridION Flow Cell Light Shield P1000 pipette and tips P100 pipette and tips P20 pipette and tips P10 pipette and tips

Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).

Priming and loading a flow cell

We recommend all new users watch the 'Priming and loading your flow cell' video before your first run.

1 **Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.**

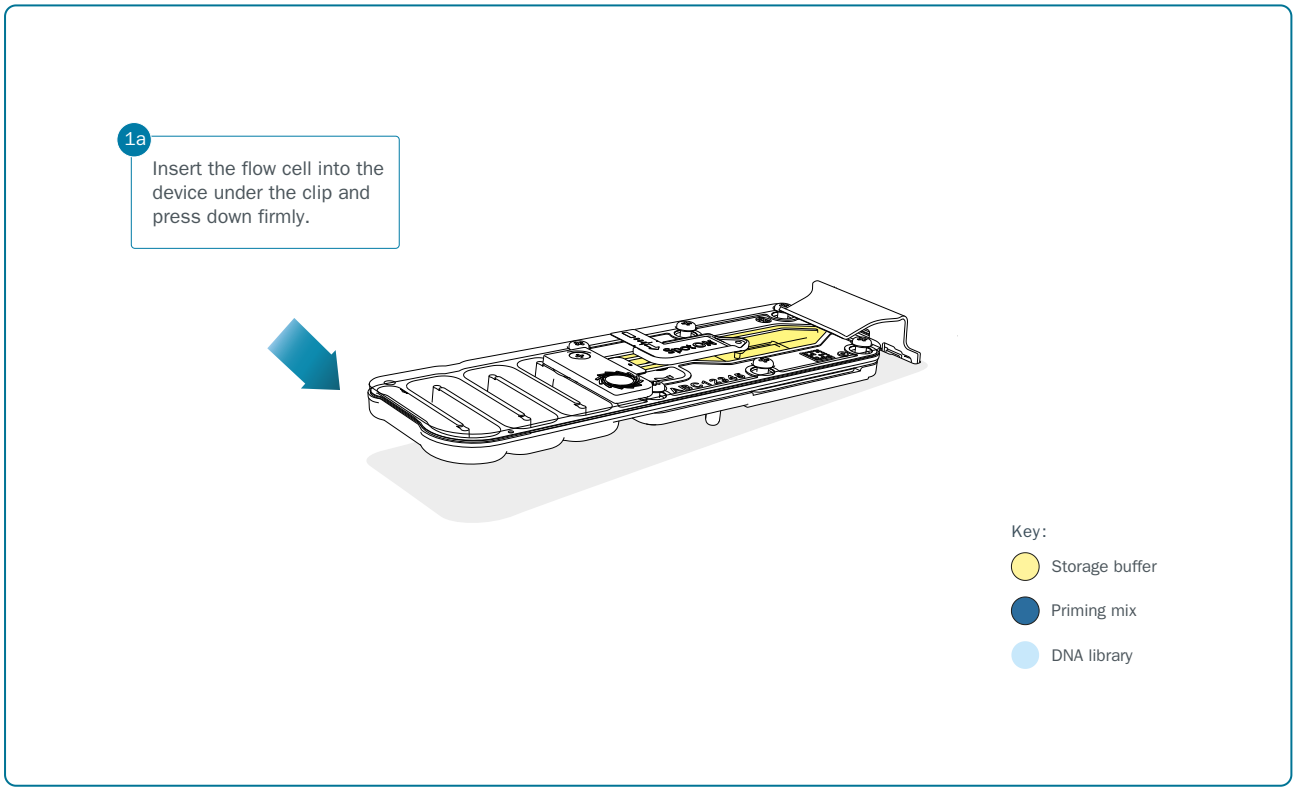
For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), we recommend adding Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.

Note: We do not recommend using any other albumin type (e.g. recombinant human serum albumin).

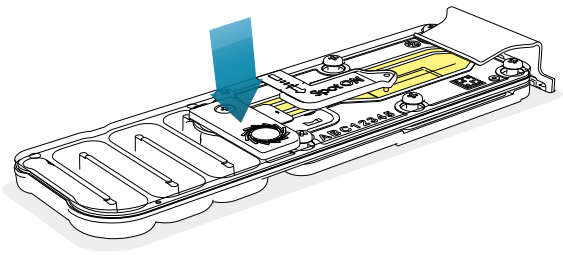
2 **To prepare the flow cell priming mix with BSA, combine the following reagents in a fresh 1.5 ml Eppendorf DNA LoBind tube. Mix by inverting the tube and pipette mix at room temperature:**

Reagents	Volume per flow cell
Flow Cell Flush (FCF)	1,170 µl
Bovine Serum Albumin (BSA) at 50 mg/ml	5 µl
Flow Cell Tether (FCT)	30 µl
Final total volume in tube	**1,205 µl**

3 **Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the priming port cover to ensure correct thermal and electrical contact.**



1b Insert the flow cell into the device under the clip and press down firmly on the Priming port cover.



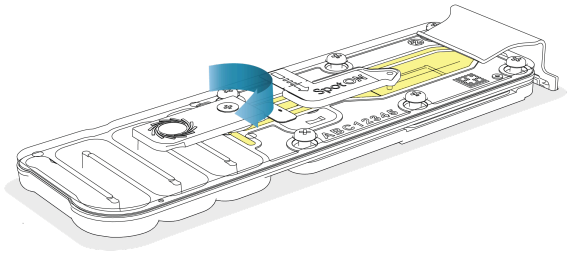
Complete a flow cell check to assess the number of pores available before loading the library.

This step can be omitted if the flow cell has been checked previously.

See the flow cell check instructions in the MinKNOW protocol for more information.

4 Slide the flow cell priming port cover clockwise to open the priming port.

2 Slide open the **Priming port** cover.

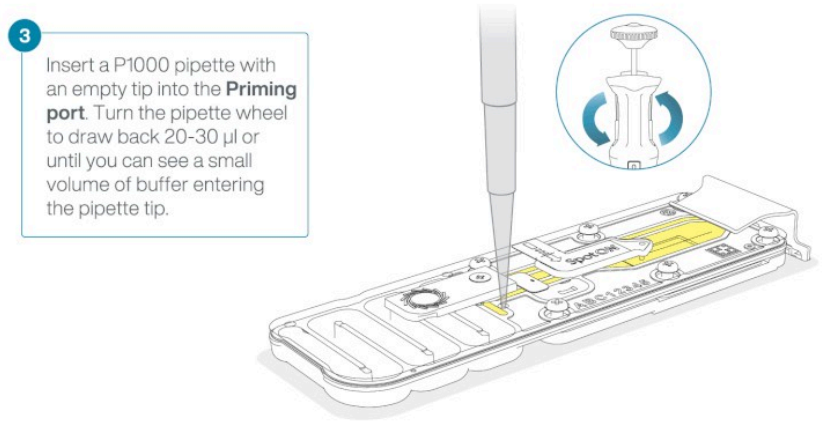


Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

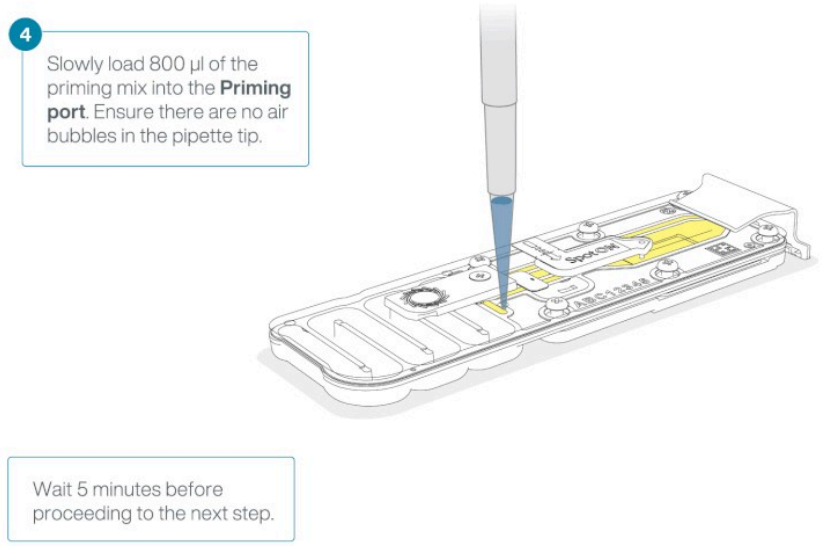
5 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:

1. Set a P1000 pipette to 200 µl
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 µl, to draw back 20-30 µl, or until you can see a small volume of buffer entering the pipette tip

Note: Visually check that there is continuous buffer from the priming port across the sensor array.



6 Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes. During this time, prepare the library for loading by following the steps below.



7 Thoroughly mix the contents of the Library Beads (LIB) by pipetting.

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

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

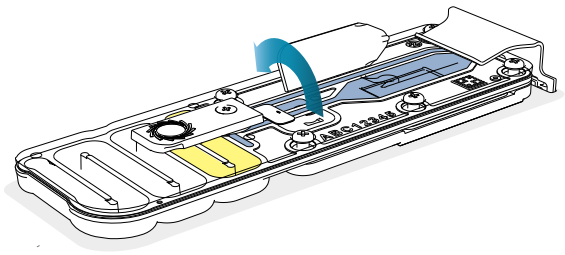
8 In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:

Reagent	Volume per flow cell
Sequencing Buffer (SB)	37.5 µl
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using	25.5 µl
DNA library	12 µl
Total	**75 µl**

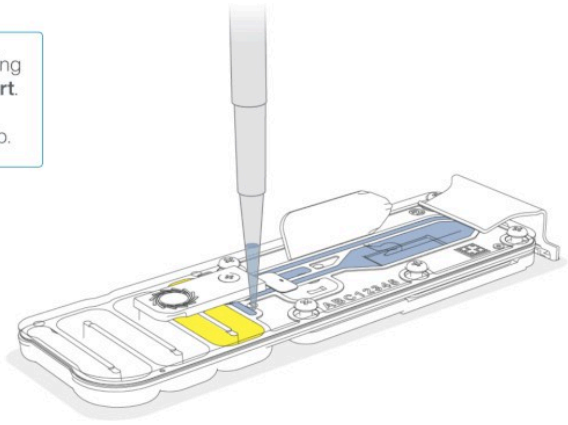
9 Complete the flow cell priming:

1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load **200 µl** of the priming mix into the flow cell priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.

5 Gently flip open the SpotON sample port cover.



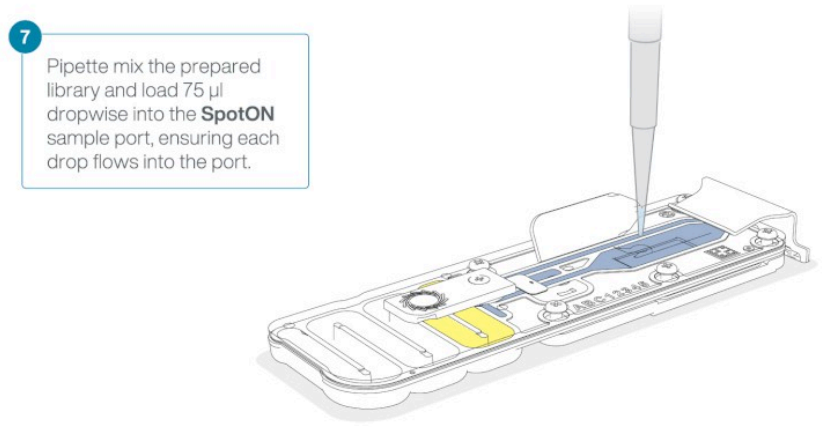
6 Load 200 µl of the priming mix into the **Priming Port**. Ensure there are no air bubbles in the pipette tip.



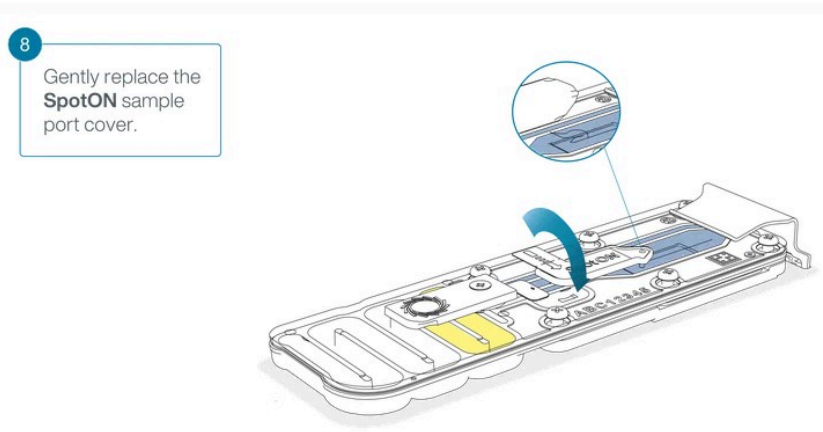
10 Mix the prepared library gently by pipetting up and down just prior to loading.

11 Add 75 µl of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding

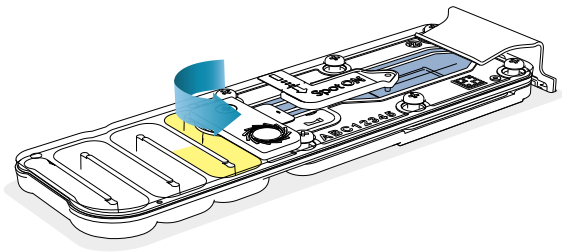
the next.



12 **Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port.**



9 Gently close the **Priming port**.

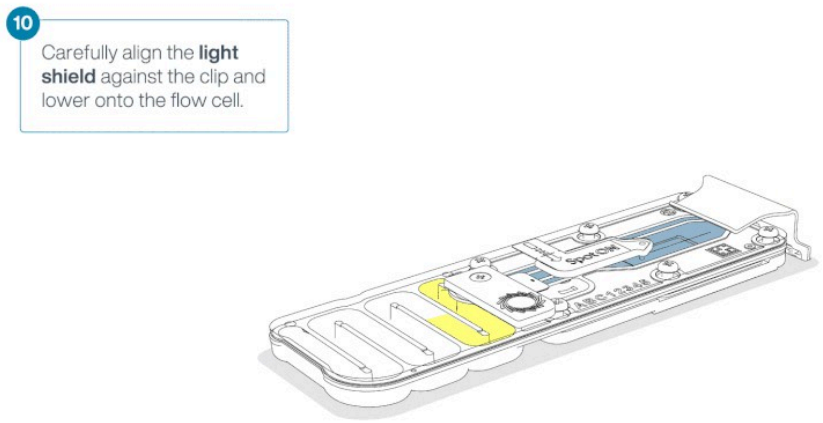


Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.

We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

13 Place the light shield onto the flow cell, as follows:

1. Carefully place the leading edge of the light shield against the clip. **Note:** Do not force the light shield underneath the clip.
2. Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.



The MinION Flow Cell Light Shield is not secured to the flow cell and careful handling is required after installation.

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

6. Data acquisition and basecalling

Ensure you are using the most recent version of MinKNOW.

We recommend updating MinKNOW to the latest version prior to starting a sequencing run. The basecalling model v4.3 found in Dorado 0.5.0 onwards provides improved accuracy for bacterial DNA and is included in MinKNOW release v24.02 or newer.

For more information on updating MinKNOW, please refer to our MinKNOW protocol.

How to start sequencing

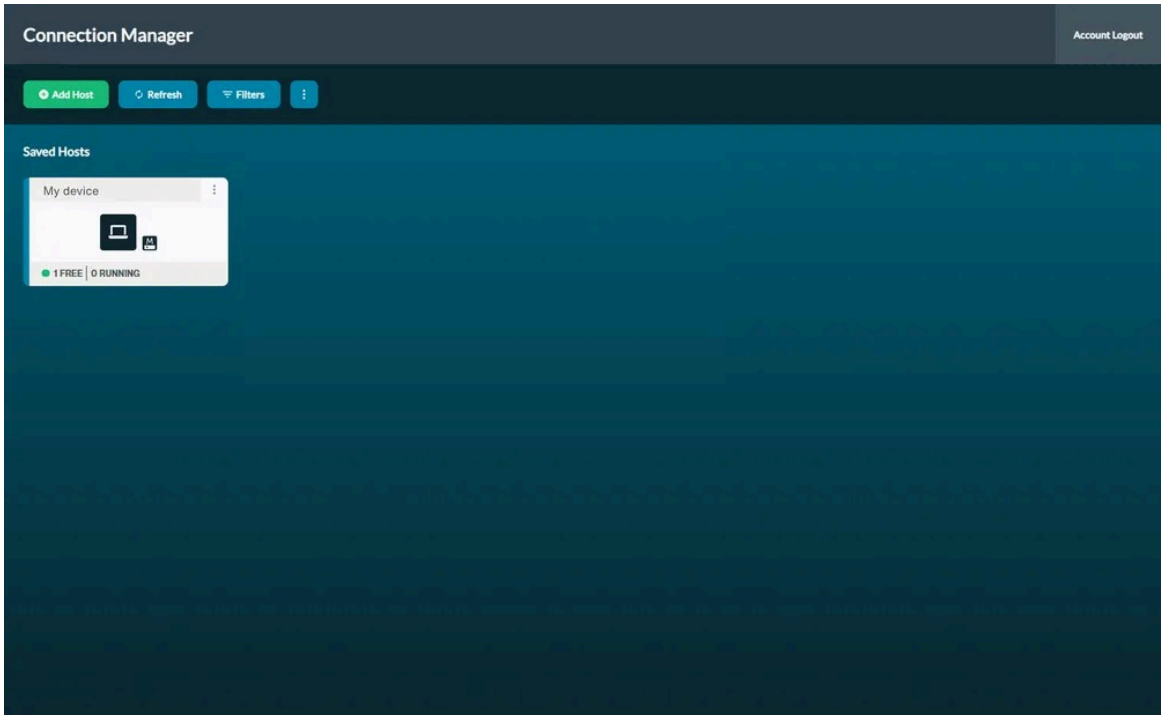
The sequencing device control, data acquisition and real-time basecalling are carried out by the MinKNOW software. Please ensure MinKNOW is installed on your computer or device. Further

instructions for setting up a sequencing run can be found in the MinKNOW protocol.

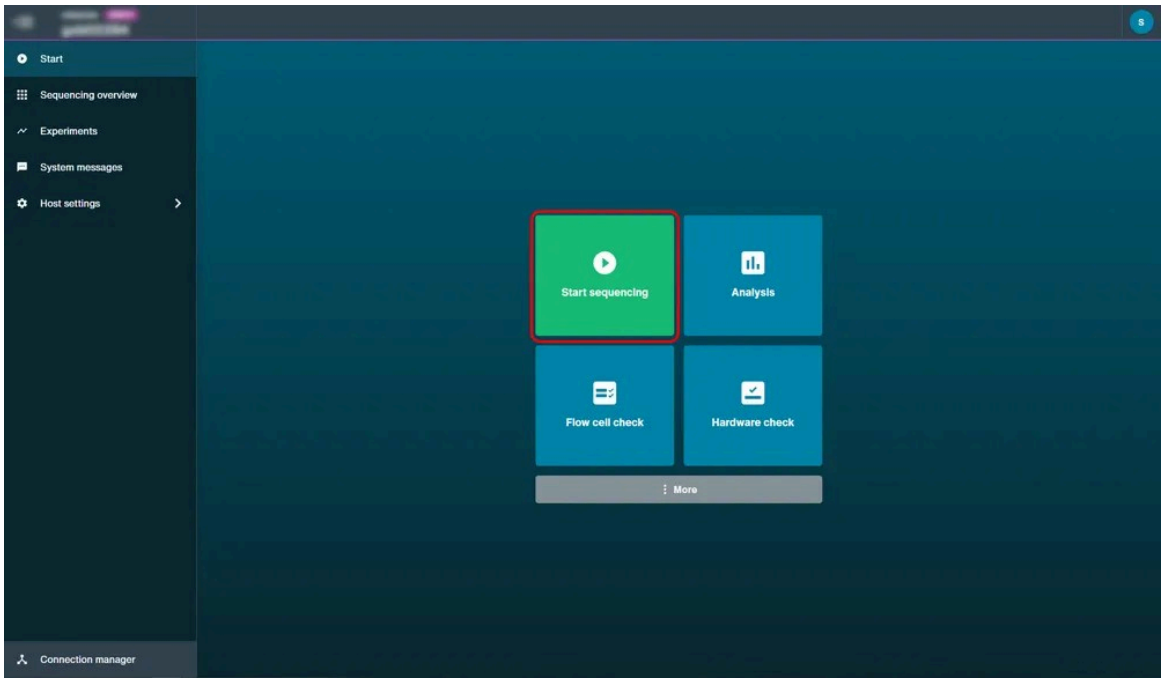
We recommend setting up a sequencing run on a MinION or GridION device using the basecalling and barcoding recommendations outlined below. All other parameters can be left to their default settings.

1 Open the MinKNOW software using the desktop shortcut and log into the MinKNOW software using your Community credentials.

2 Click on your connected device.

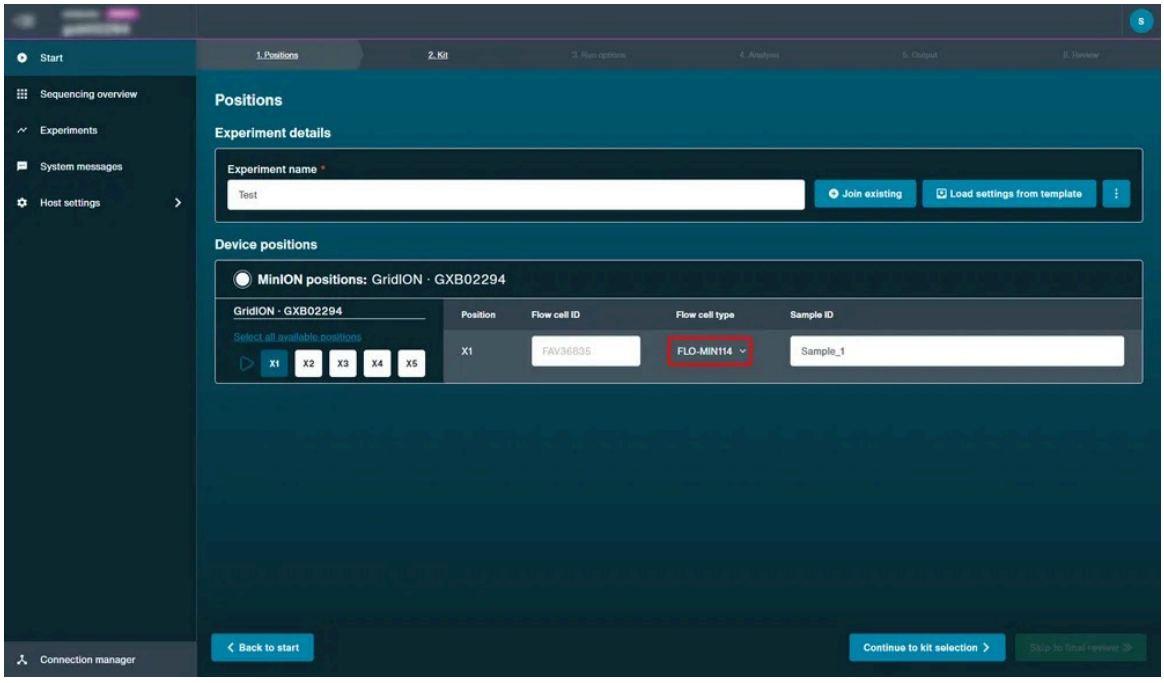


3 Set up a sequencing run by clicking Start sequencing.



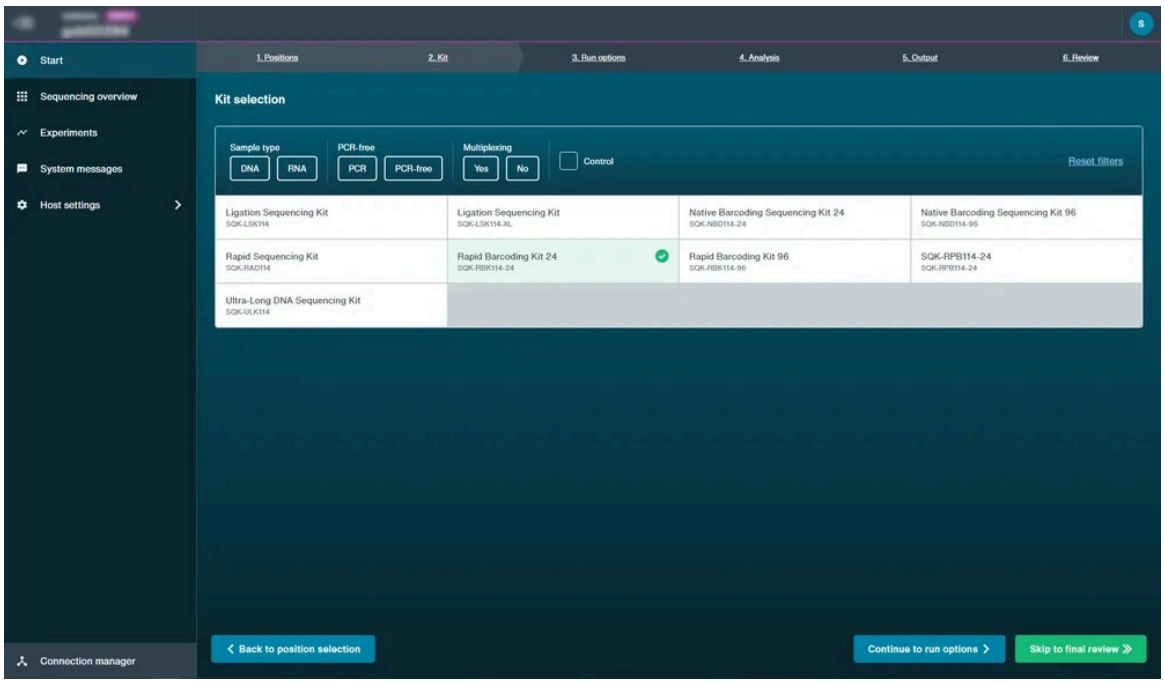
4 Type in the experiment name, select the flow cell postition and enter sample ID. Choose FLO-MIN114 flow cell type from the drop-down menu.

Click **Continue to kit selection**.



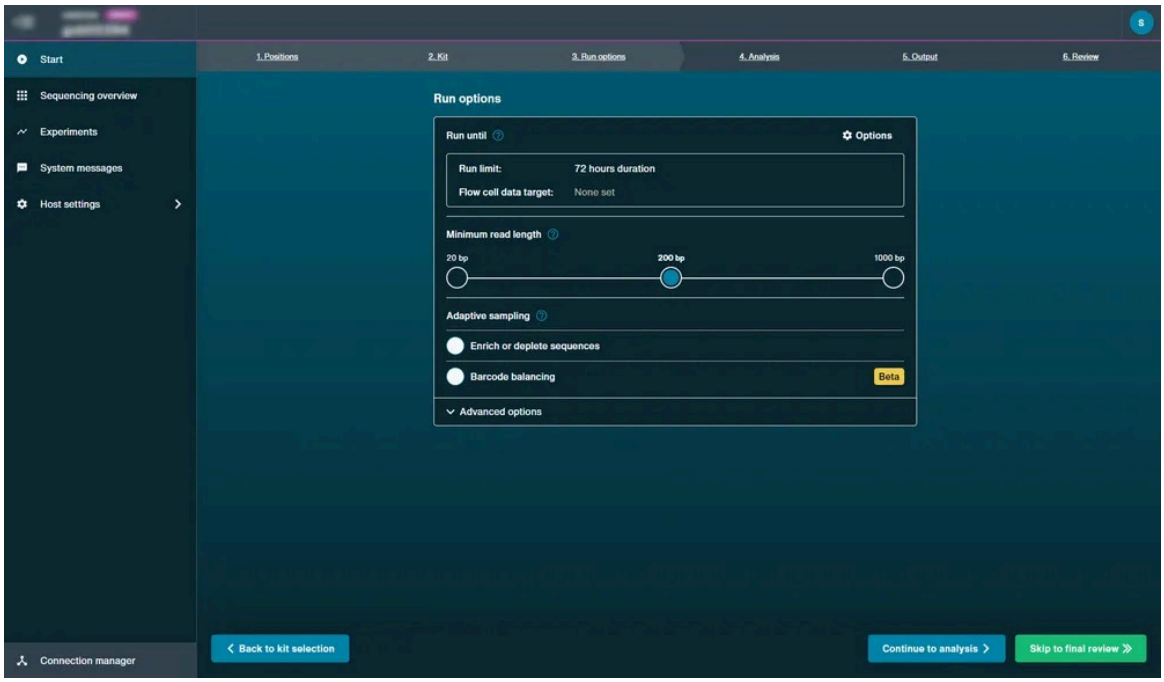
5 Select the Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) or Rapid Barcoding Kit 96 V14 (SQK-RBK114.96)

Click **Continue to Run Options** to continue.



6 Keep the run options to their default settings of 72 hour run length and 200 bp minimum read length.

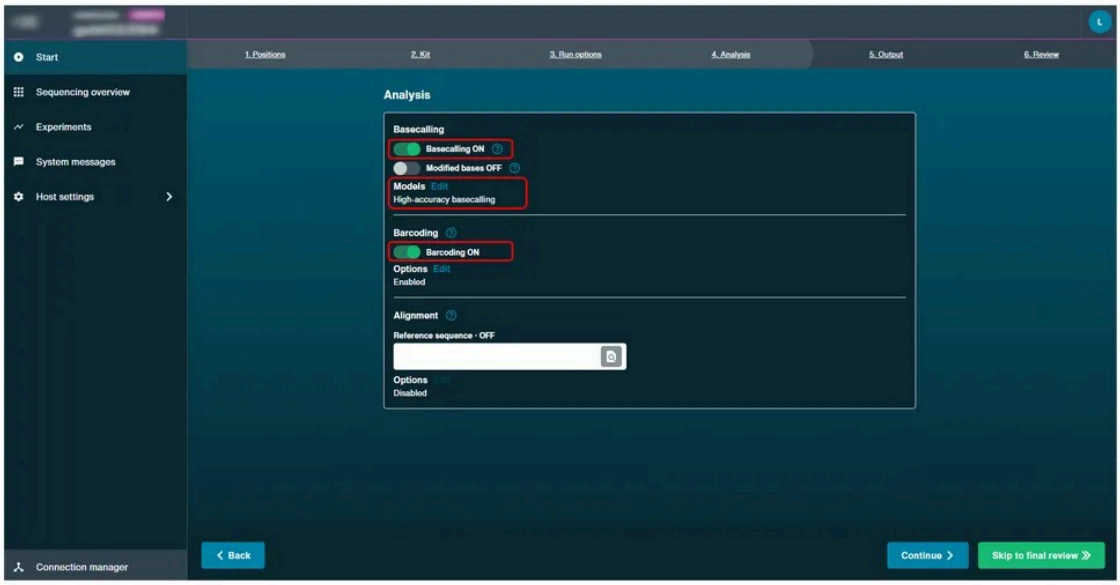
Click **Continue to basecalling** to continue.

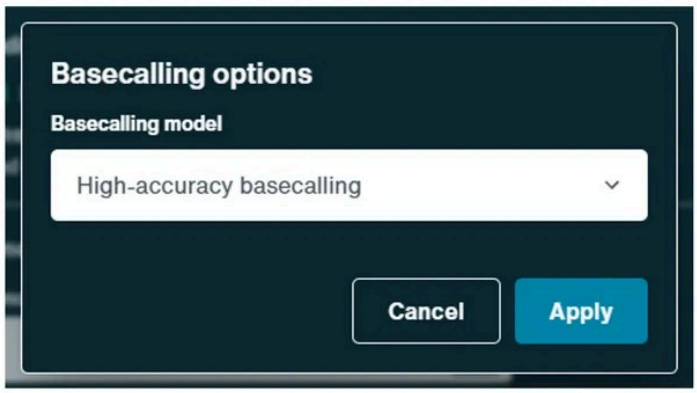


7 Set up basecalling and barcoding using the following parameters:

1. Ensure basecalling is ON.
2. Next to "Models", click **Edit options** and choose High accuracy basecaller (HAC) from the drop-down menu.
3. Ensure barcoding in ON.

Click **Continue to output** and continue.

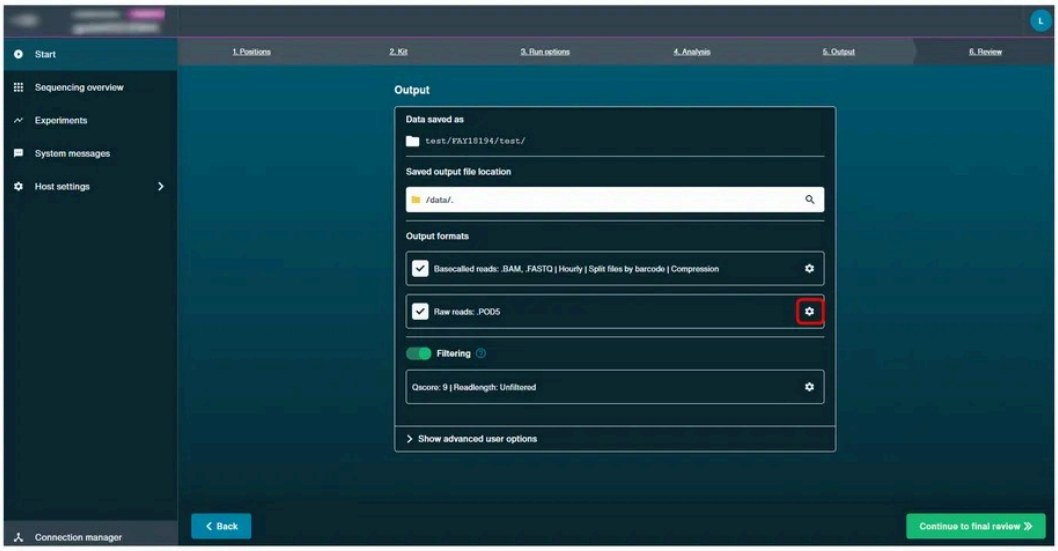


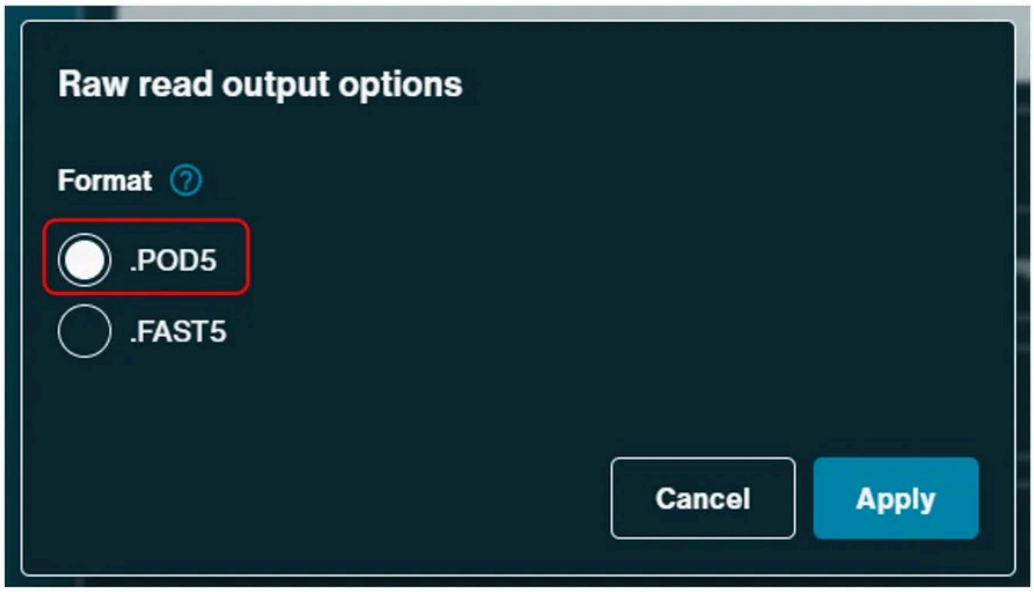


8 Set up the output format and filtering as follows:

1. Ensure **.POD5** is selected as the Raw reads output format.
2. Ensure **.FASTQ** is selected for basecalled reads.
3. Ensure filtering is ON.

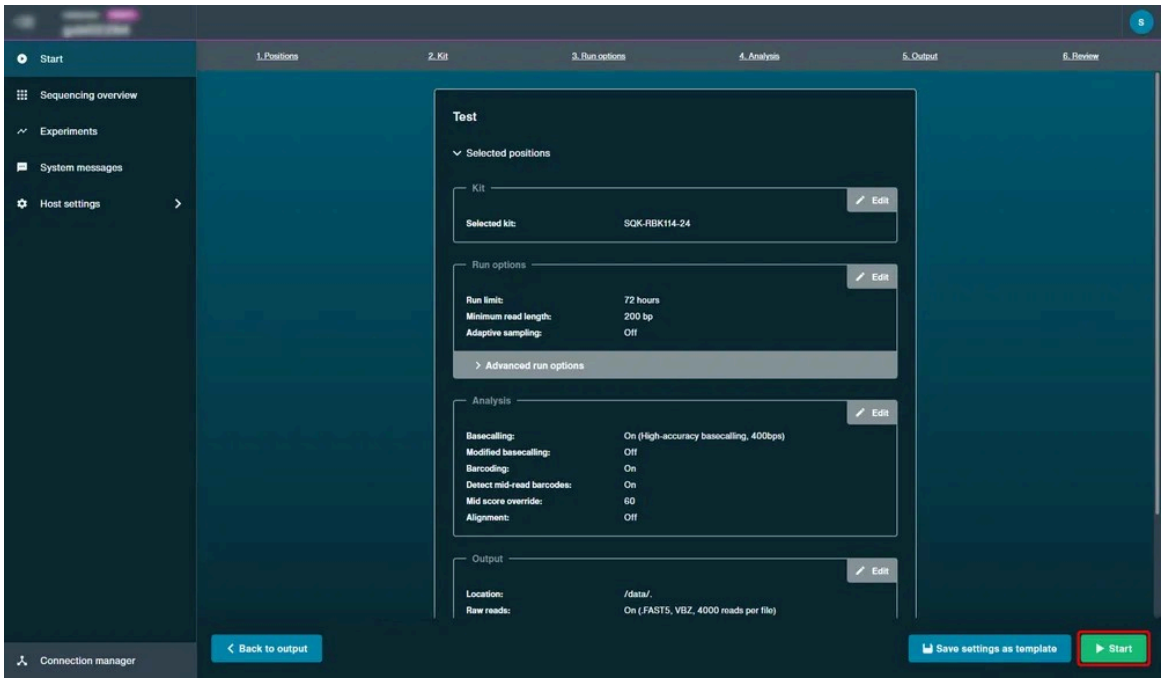
Click **Continue to final review** to continue.





9 Click "Start" to start sequencing.

You will be automatically navigated to the "Sequencing Overview" page to monitor the sequencing run.



7. Downstream analysis

Post-basecalling analysis

We recommend performing downstream analysis using EPI2ME which facilitates bioinformatic analyses by allowing users to run Nextflow workflows in a desktop application. EPI2ME maintains a collection of bioinformatic workflows which are curated and actively maintained by experts in long-read sequence analysis.

Further information about the available EPI2ME workflows are available here, along with the Quick Start Guide to start your first bioinformatic workflow.

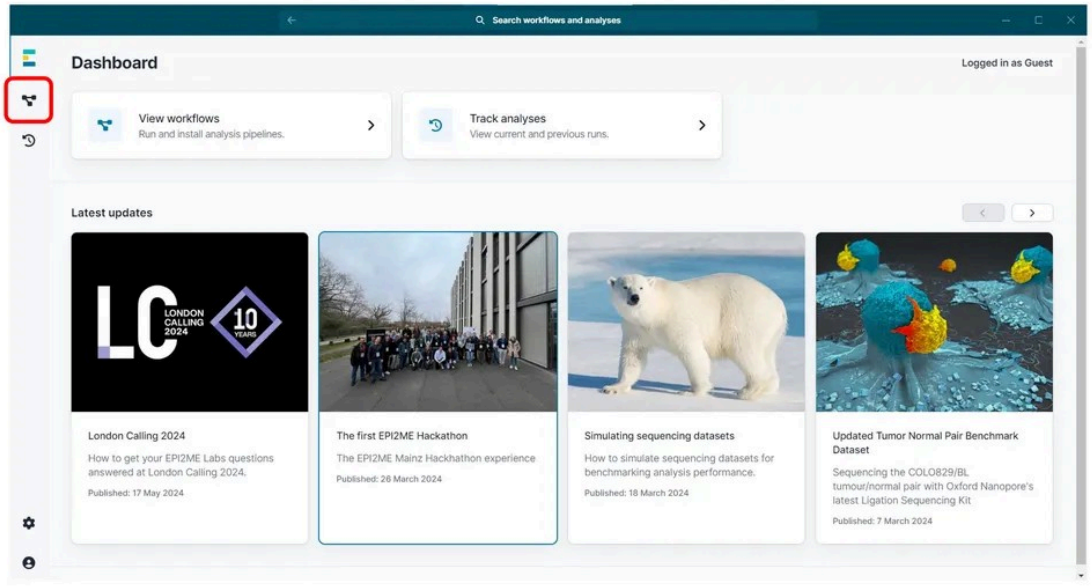
For the accurate and reliable assembly or alignment of bacterial or fungal isolate genomes generated from the included protocols, we recommend using the wf-bacterial-genomes workflow. At its core, the workflow is an efficient assembly pipeline which also polishes genomes using Medaka.

Whilst running the workflow using the default parameters will produce high quality genome assemblies, using the 'Isolates' mode will perform additional analyses designed to increase genome quality and aid genome interrogation for common pathogens in the clinical and food safety fields. 'Isolates' analyses includes MLST(7-gene), species confirmation and AMR prediction in addition to sample-specific reports.

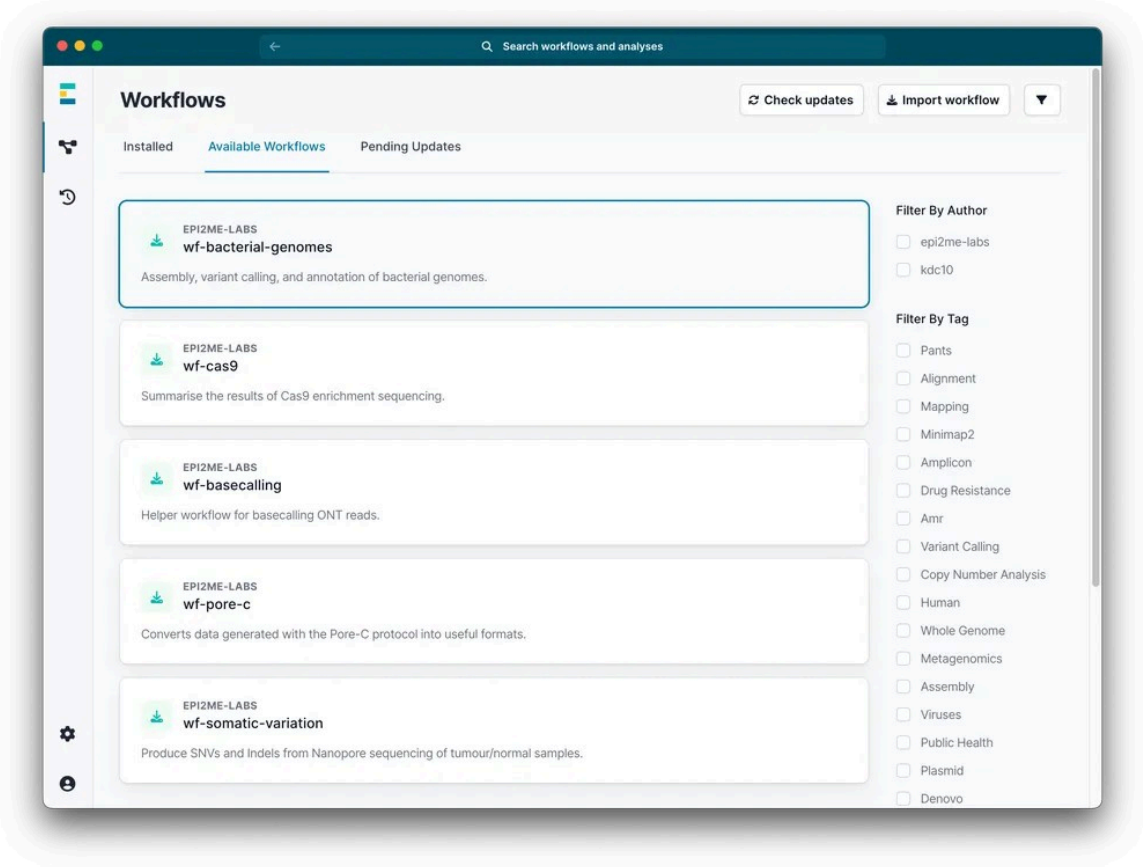
Note: You can also run this workflow through command line. However, we only recommend this option for experienced users. For more information, please visit the wf-bacterial-genomes page on GitHub.

1 Open the EPI2ME app using the desktop shortcut.

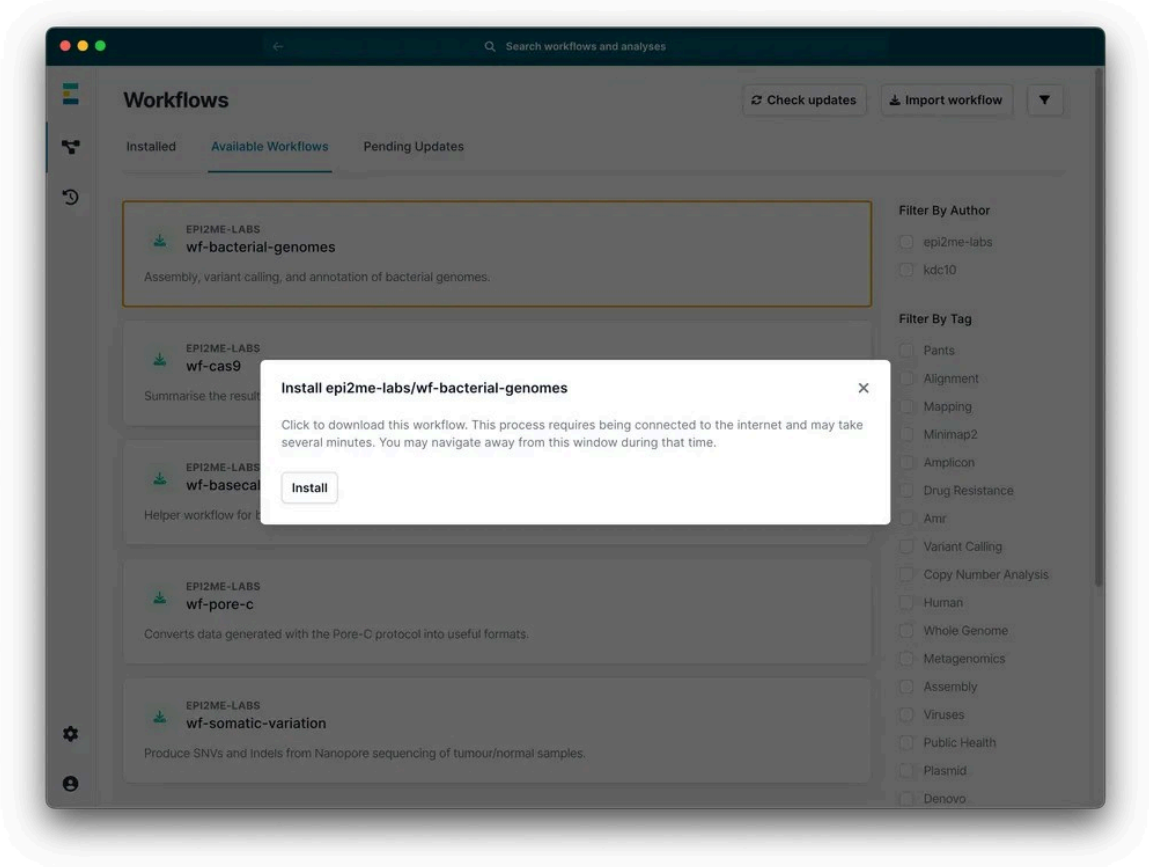
2 On the landing page, open the workflow tab on the left-hand sidebar.



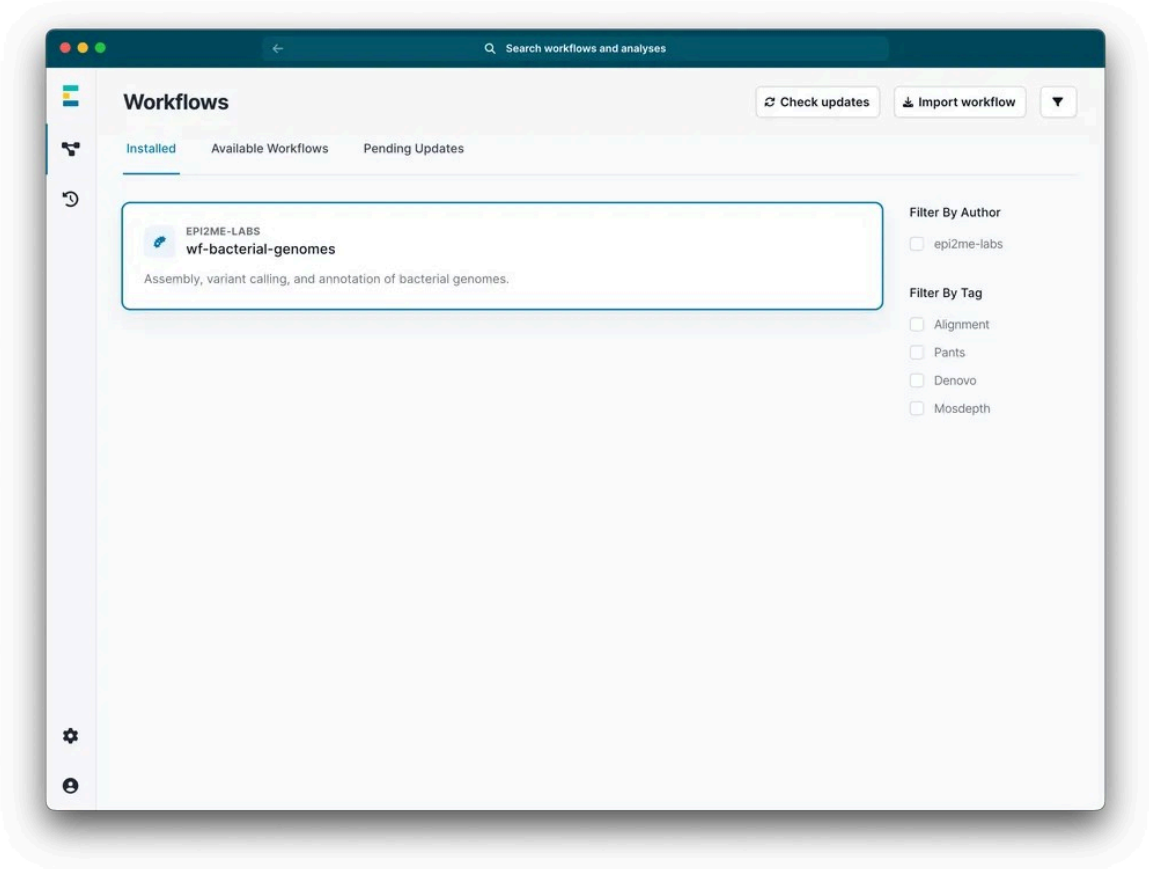
3 Navigate to the Available workflows tab and click on wf-bacterial-genomes option.



4 Click install.

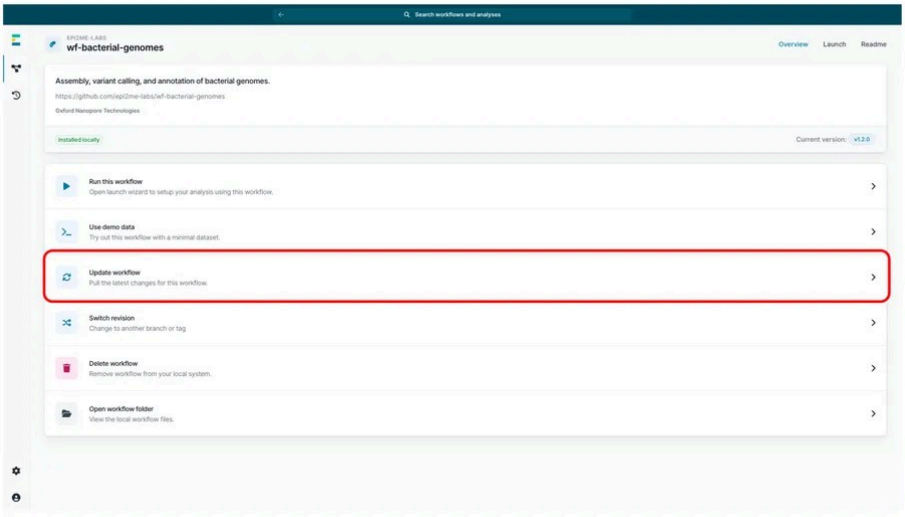


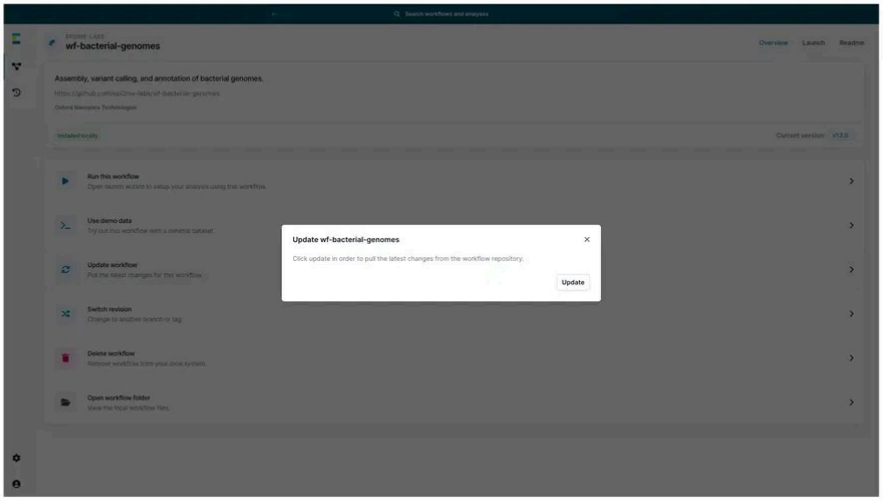
5 Navigate to the Installed tab and click on the installed wf-bacterial-genomes workflow.


Search workflows and analyses
Workflows
Check updates
Import workflow
Installed
Available Workflows
Pending Updates
EPI2ME-LABS
wf-bacterial-genomes
Assembly, variant calling, and annotation of bacterial genomes.
Filter By Author
epi2me-labs
Filter By Tag
Alignment
Pants
Denovo
Mosdepth

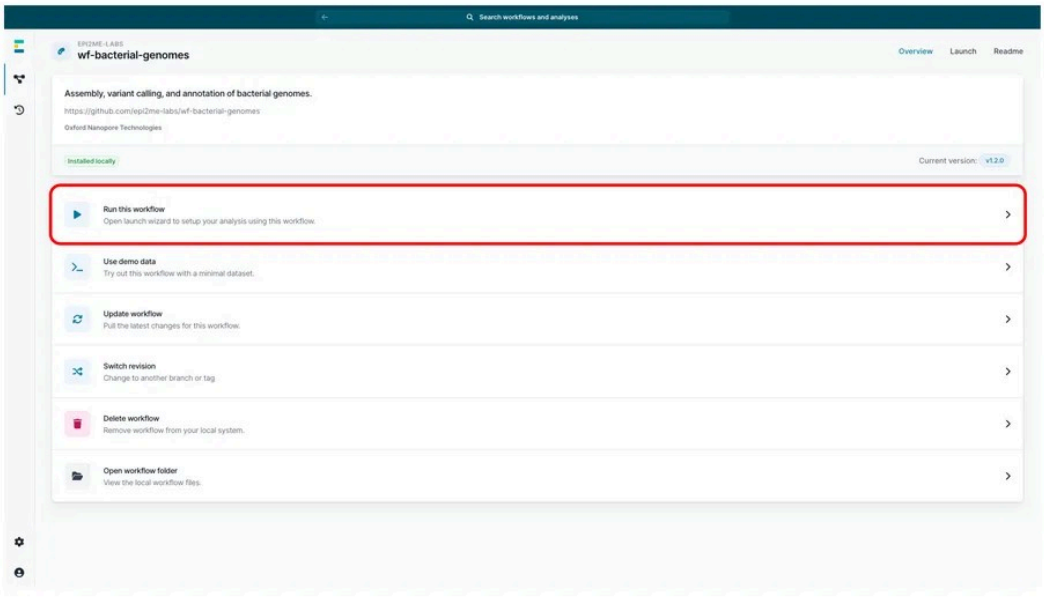
If the workflow was already installed, check for updates by clicking 'Update workflow'.

Ensure you are using v1.3.0 or newer of the workflow. We recommend running the latest version of our workflows for the best results.

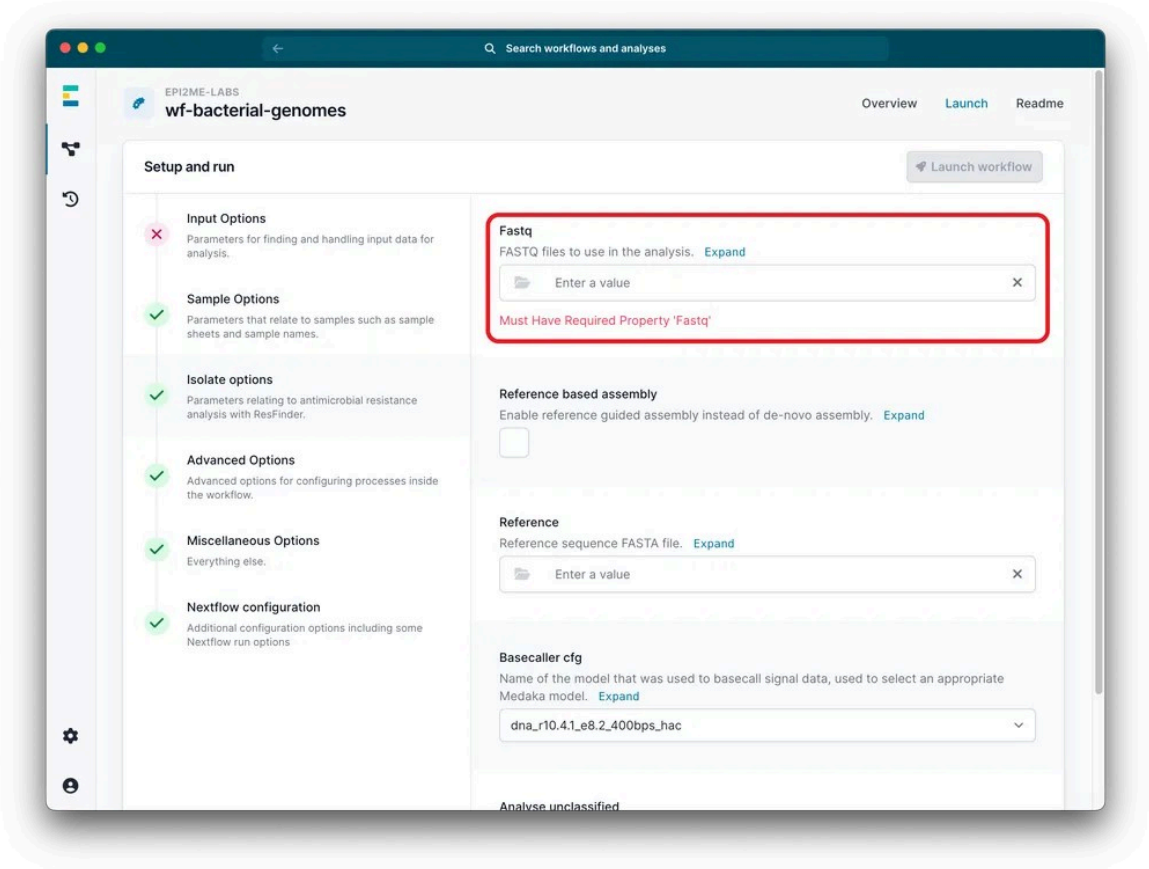




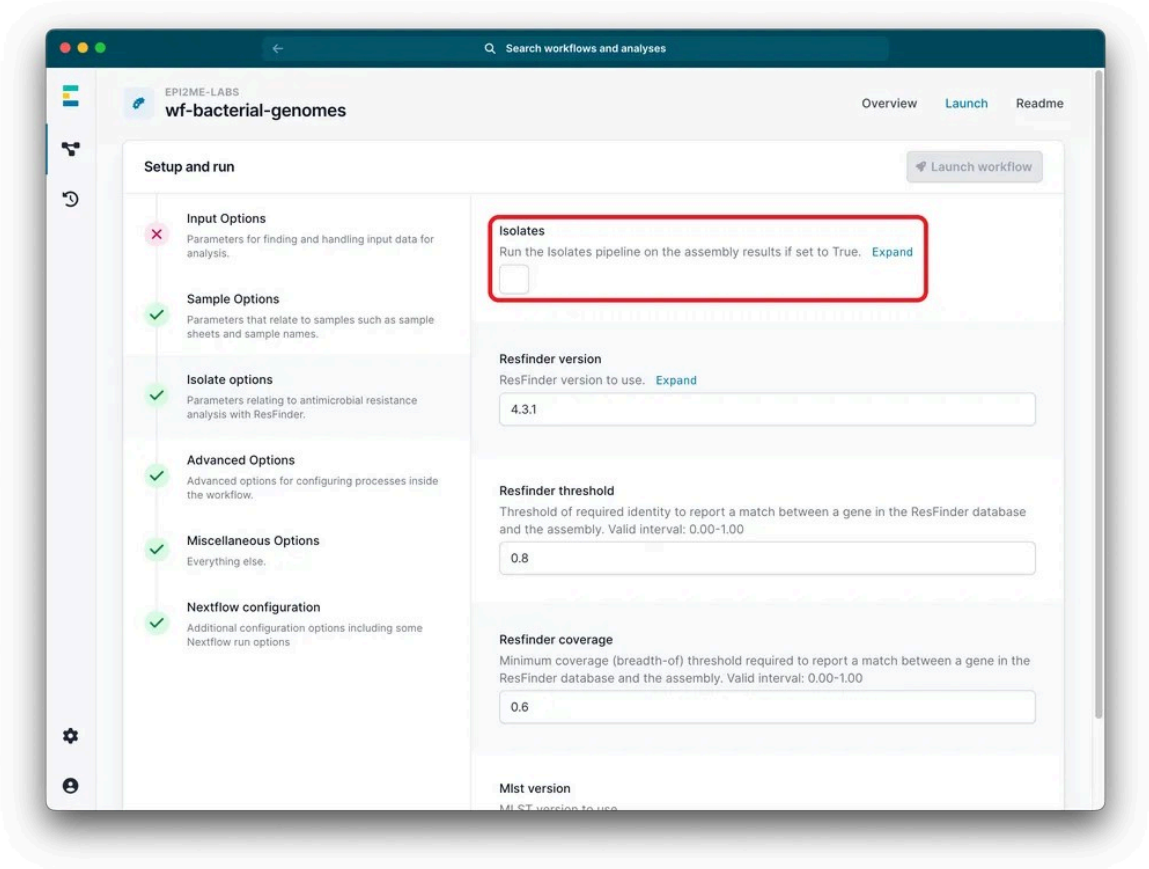
6 Click on Run this workflow to open the launch wizard.



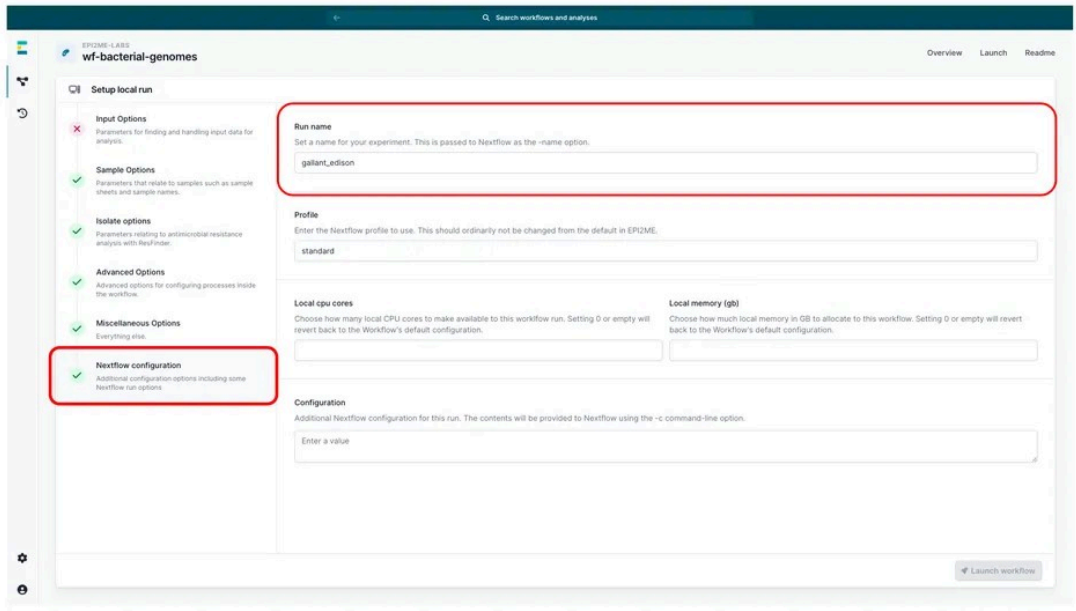
7 **Set up your run by uploading your FASTQ file in the Input Options. We recommend keeping the default settings for the other parameter options.**



8 To enable the isolates mode, tick the isolates checkbox.

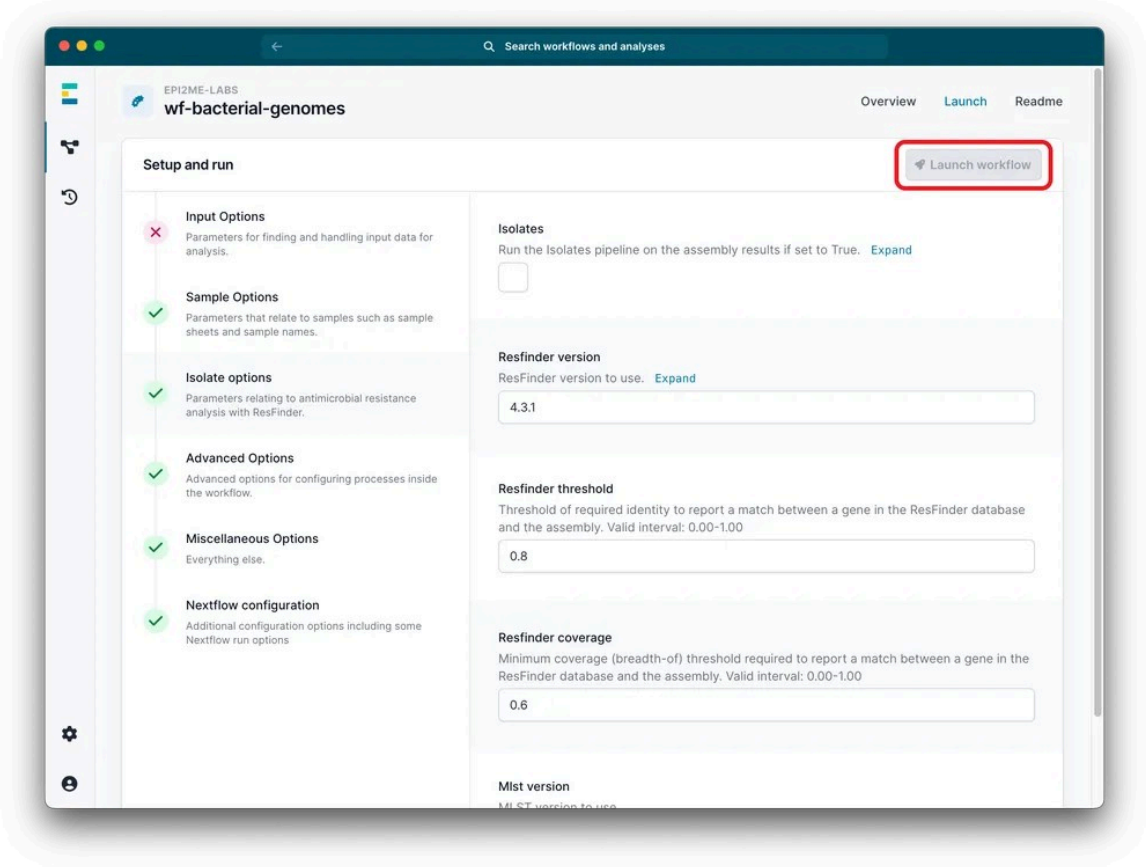


9 Navigate to the 'Nextflow configuration' tab to assign a 'Run name' to your analysis as an identifier.



10 Click Launch workflow.

Ensure all parameter options have green ticks.



11 Once the workflow finishes, a report will be produced.

8. Flow cell reuse and returns

Materials Flow Cell Wash Kit (EXP-WSH004)

1 After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Flow Cell Wash Kit protocol and store the washed flow cell at +2°C to +8°C.

The Flow Cell Wash Kit protocol is available on the Nanopore Community.

We recommend you to wash the flow cell as soon as possible after you stop the run. However, if this is not possible, leave the flow cell on the device and wash it the next day.

2 **Alternatively, follow the returns procedure to send the flow cell back to Oxford Nanopore.**

Instructions for returning flow cells can be found here.

If you encounter issues or have questions about your sequencing experiment, please refer to the Troubleshooting Guide that can be found in the online version of this protocol.

9. Issues during DNA extraction and library preparation

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

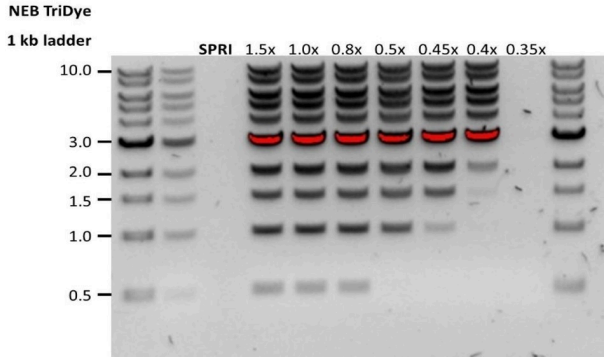
We also have an FAQ section available on the Nanopore Community Support section.

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
Inefficient lysis	Enzyme activity has degraded in the solution or the isolate species is hard to lyse.	- Make a fresh enzyme solution. - Follow the hard to lyse gDNA extraction method. - Increase the enzyme incubation for longer than 10 minutes.
Low DNA concentration	Low input into the extraction method	- Check the cell input used - Add more input and perform the extraction again - Elute in less Elution Buffer - Concentrate the DNA with a 0.4X AMPure XP Bead wash.
Low DNA integrity number (DIN)	Low quality or concentration of sample input	- Repeat the extraction with freshly made enzyme solution - Concentrate the DNA input with a 0.4X wash
Low sequencing yield	Low sample concentration	- Concentrate the DNA with a 0.4X AMPure XP wash step to remove potential inhibitors. - Check the DNA concentration and quality. RNA presence may affect quantification of total DNA.
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 DNA recovery after AMPure bead clean-up

Observation	Possible cause	Comments and actions
Low recovery	DNA loss due to a lower than intended AMPure beads-to-sample ratio	1. AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample. 2. When the AMPure beads-to-sample ratio is lower than 0.4:1, DNA fragments of any size will be lost during the clean-up.
Low recovery	DNA fragments are shorter than expected	The lower the AMPure beads-to-sample ratio, the more stringent the selection against short fragments. Please always determine the input DNA length on an agarose gel (or other gel electrophoresis methods) and then calculate the appropriate amount of AMPure beads to use. 
Low recovery after end-prep	The wash step used ethanol <70%	DNA will be eluted from the beads when using ethanol <70%. Make sure to use the correct percentage.

10. 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.

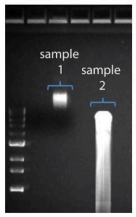
MinKNOW script failed

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 available, we recommend storing the flow cell and the loaded library at 4°C and contact Technical Support for further storage guidance.

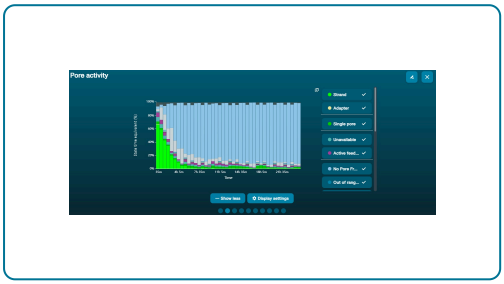
Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	Ensure the correct concentration of good quality library is loaded on to a MinION/GridION flow cell. To check the concentration, please refer to the library preparation protocol. 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 Sequencing Kit V14/Rapid 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 adding 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 link for more information on MinION temperature control.