

Overview of the protocol

IMPORTANT

This is an Early Access product

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

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

Rapid Barcoding Kit features

This kit is recommended for users who:

- Wish to multiplex samples to reduce price per sample
- Need a PCR-free method of multiplexing to preserve additional information such as base modifications
- Require a short preparation time
- Have limited access to laboratory equipment

Introduction to plasmid sequencing using the Rapid Barcoding Kit 24 or 96 V14

This protocol describes how to carry out rapid barcoding of plasmid DNA using the Rapid Barcoding Kit 24 or 96 V14 (SQK-RBK114.24 or SQK-RBK114.96) to sequence up to 96 plasmid samples. This method can be utilised for routine verification of plasmid constructs in molecular biology research, quality control of plasmid DNA samples in biotechnology applications, and analysis of engineered plasmids for gene therapy development. During library preparation, the plasmid DNA is fragmented with the Rapid Barcodes before the samples are pooled and cleaned up. Rapid sequencing adapters are attached to the DNA ends before sequencing on a flow cell.

We recommend new users to sequence for 12 hours, although a shorter run-time may be sufficient. After sequencing, perform downstream analysis using the EPI2ME Labs Clone Validation (wf-clone-validation) workflow. A report is generated with a consensus sequence from each plasmid. Detailed instructions for setting up MinNOW and the EPI2ME Labs workflow are included.

Steps in the sequencing workflow:

Prepare for your experiment

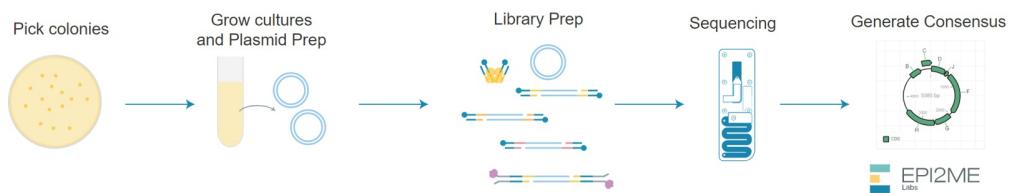
You will need to:

- 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:

- Fragment your DNA using the Rapid Barcodes; this simultaneously attaches a pair of barcodes to the fragments.
- Pool the barcoded samples.
- Attach the rapid sequencing adapters to the DNA ends.
- Prime the flow cell, and load your DNA library into the flow cell.



Sequencing and analysis

You will need to:

- Start a sequencing run using the MinKNOW software, which will collect raw data from the device into basecalled reads and will perform barcode demultiplexing.
- Start the EPI2ME software and use the Clone Validation workflow for analysis.

IMPORTANT

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)

Equipment and consumables

Materials

- 50 ng high molecular weight plasmid DNA per sample
- Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) OR Rapid Barcoding Kit 96 V14 (SQK-RBK114.96)

Consumables

- 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
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- Freshly prepared 80% ethanol in nuclease-free water
- (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)

Equipment	<ul style="list-style-type: none">• MinION or GridION device• Ice bucket with ice• Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, cat # 11766427)• Timer• Thermal cycler or heat blocks• 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	<ul style="list-style-type: none">• Standard gel electrophoresis equipment• Agilent Bioanalyzer (or equivalent)

For this protocol, you will need 50 ng high molecular weight plasmid DNA per sample.

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.

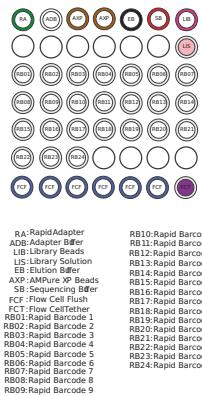
For instructions on how to perform quality control of your DNA sample, please read [the Input DNA/RNA QC protocol](#).

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.

IMPORTANT

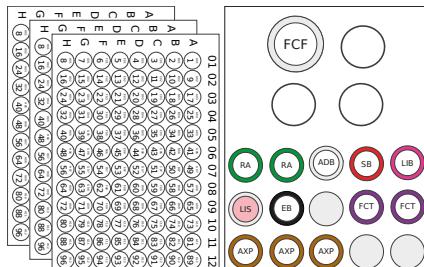
The Rapid Adapter (RA) used in this kit and protocol is not interchangeable with other sequencing adapters.

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

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (μ l)
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 CellTether
 SB: Sequencing Buffer

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (μ l)
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 μ l 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.

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.

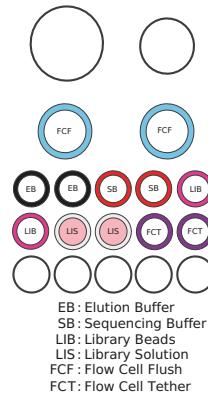
Rapid Adapter Auxiliary V14 (EXP-RAA114) contents:



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:



EB : Elution Buffer
 SB : Sequencing Buffer
 LIB : Library Beads
 LIS : Library Solution
 FCF : Flow Cell Flush
 FCT : Flow Cell Tether

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
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

Rapid barcode sequences

Component	Sequence
RB01	AAGAAAGTTGTCGGTGTCTTG

Component	Sequence
RB02	TCGATTCCGTTGTAGCGTCTGT
RB03	GAGTCTTGTGCCAGTTACCAAGG
RB04	TTCGGATTCTATCGTGTTCCTA
RB05	CTTGTCCAGGGTTGTGAACCTT
RB06	TTCTCGCAAAGGCAGAAAGTAGTC
RB07	GTGTTACCGTGGGAATGAATCCTT
RB08	TTCAGGGAACAAACCAAGTTACGT
RB09	AACTAGGCACAGCGAGTCTGGTT
RB10	AAGCGTTGAAACCTTGTCCCTCTC
RB11	GTTCATCTATCGGAGGGAAATGGA
RB12	CAGGTAGAAAGAACAGCAGAACATCGGA
RB13	AGAACGACTTCCATACTCGTGTGA
RB14	AACGAGTCTTGGGACCCATAGA
RB15	AGGTCTACCTCGCTAACACCCTG
RB16	CGTCAACTGACAGTGGTCGTACT
RB17	ACCCTCCAGGAAAGTACCTCTGAT
RB18	CCAAACCCAACAACCTAGATAGGC
RB19	GTTCCTCGTGCAGTGTCAAGAGAT
RB20	TTGCGTCCTGTTACGAGAACTCAT
RB21	GAGCCTCTCATTGCCGTTCTCTA
RB22	ACCACTGCCATGTATCAAAGTACG
RB23	CTTACTACCCAGTGAACCTCCTCG
RB24	GCATAGTTCTGCATGATGGGTTAG
RB25	GTAAGTTGGGTATGCAACGCAATG
RB26	CATACAGCGACTACGCATTCTCAT
RB27	CGACGGTTAGATTCACCTCTTACA
RB28	TGAAACCTAAGAAGGCACCGTATC
RB29	CTAGACACCTGGGTTGACAGACC
RB30	TCAGTGAGGATCTACTTCGACCCA
RB31	TGCGTACAGCAATCAGTTACATTG
RB32	CCAGTAGAAGTCCGACAACGTCAT
RB33	CAGACTTGGTACGGTTGGGTAAC

Component	Sequence
RB34	GGACGAAGAACTCAAGTCAAAGGC
RB35	CTACTTACGAAGCTGAGGGACTGC
RB36	ATGTCCAGTTAGAGGAGGAAACA
RB37	GCTTGCATTGATGCTTAGTATCA
RB38	ACCACAGGAGGACGATAACAGAGAA
RB39	CCACAGTGTCAACTAGAGCCTCTC
RB40	TAGTTGGATGACCAAGGATAGCC
RB41	GGAGTCGTCCAGAGAAGTACACG
RB42	CTACGTGTAAGGCATACTGCCAG
RB43	CTTCGTTGTTGACTCGACGGTAG
RB44	AGTAGAAAGGGTCCCTCCACTC
RB45	GATCCAACAGAGATGCCTTCAGTG
RB46	GCTGTGTTCCACTTCATTCTCTG
RB47	GTGCAACTTCCCACAGGTAGTTC
RB48	CATCTGGAACGTGGTACACCTGTA
RB49	ACTGGTGCAGCTTGAACATCTAG
RB50	ATGGACTTTGGTAACCCCTGCCTG
RB51	GTTGAATGAGCCTACTGGGTCCCTC
RB52	TGAGAGACAAGATTGTTCGTGGAC
RB53	AGATTCAAGACCGTCTCATGCAAAG
RB54	CAAGAGCTTGACTAAGGAGCATG
RB55	TGGAAGATGAGACCCCTGATCTACG
RB56	TCACTACTAACAGGTGGCATGAA
RB57	GCTAGGTCAATCTCCCGTGAAGT
RB58	CAGGTTACTCCTCCGTGAGTCTGA
RB59	TCAATCAAGAAGGGAAAGCAAGGT
RB60	CATGTTCAACCAAGGCTTCTATGG
RB61	AGAGGGTACTATGTGCCTCAGCAC
RB62	CACCCACACTTACTTCAGGACGTA
RB63	TTCTGAAGTTCCCTGGGTCTTGAAC
RB64	GACAGACACCGTTCATGACTTTC
RB65	TTCTCAGTCTCCTCCAGACAAGG

Component	Sequence
RB66	CCGATCCTGTGGCTCTAACTTC
RB67	GTTTGTCACTCGTGTGCTCACCC
RB68	GAATCTAAGCAAACACGAAGGTGG
RB69	TACAGTCCGAGCCTCATGTGATCT
RB70	ACCGAGATCCTACGAATGGAGTGT
RB71	CCTGGGAGCATCAGGTAGTAACAG
RB72	TAGCTGACTGTCTCCATACCGAC
RB73	AAGAACACAGGATGACAGAACCCCTC
RB74	TACAAGCATCCCAACACTTCCACT
RB75	GACCATTGTGATGAACCTGTTGT
RB76	ATGCTTGTACATCAACCCCTGGAC
RB77	CGACCTGTTCTCAGGGATACAAC
RB78	AACAACCGAACCTTGAAATCAGAA
RB79	TCTCGGAGATAGTTCTCACTGCTG
RB80	CGGATGAACATAGGATAGCGATT
RB81	CCTCATTTGTGAAGTTGTTCGG
RB82	ACGGTATGTCGAGTTCCAGGACTA
RB83	TGGCTTGATCTAGGTAAGGTCGAA
RB84	GTAGTGGACCTAGAACCTGTGCCA
RB85	AACGGAGGAGTTAGTTGGATGATC
RB86	AGGTGATCCCAACAAGCGTAAGTA
RB87	TACATGCTCCTGTTAGGGAGG
RB88	TCTTCTACTACCGATCCGAAGCAG
RB89	ACAGCATCAATGTTGGCTAGTTG
RB90	GATGTAGAGGGTACGGTTGAGGC
RB91	GGCTCCATAGGAACTCACGCTACT
RB92	TTGTGAGTGGAAAGATAACAGGACC
RB93	AGTTTCCATCACTTCAGACTTGGG
RB94	GATTGTCCCTCAAAC TGCCACCTAC
RB95	CCTGTCTGGAAGAAGAATGGACTT
RB96	CTGAACGGTCATAGAGTCCACCAT

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

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 MinION Flow Cell prior to starting a sequencing experiment. This should be done within three months of purchasing the 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](#).

The minimum number of active pores in a MinION Flow Cell that is covered by warranty is 800 pores.

Library preparation

~60 minutes

Materials

- 50 ng of high molecular weight plasmid DNA per sample
 - Rapid Barcodes (RB01-24) or Rapid Barcode Plate (RB01-96)
 - Rapid Adapter (RA)
 - Adapter Buffer (ADB)
 - AMPure XP Beads (AXP)
 - Elution Buffer (EB)
-

Consumables

- 0.2 ml thin-walled PCR tubes or 0.2 ml 96-well PCR plate
 - 1.5 ml Eppendorf DNA LoBind tubes
 - 2 ml Eppendorf DNA LoBind tubes
 - Nuclease-free water (e.g. ThermoFisher, AM9937)
 - Freshly prepared 80% ethanol in nuclease-free water
 - Qubit™ Assay Tubes (Invitrogen, Q32856)
 - Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
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Equipment

- Ice bucket with ice
- Timer
- Thermal cycler
- Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, cat # 11766427)
- Magnetic rack
- Hula mixer (gentle rotator mixer)
- 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

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 Rapid Barcode Plate (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, as follows. Approximately 50 ng of plasmid DNA is required in 9 µl of volume for each sample for barcoding.

- Dilute your plasmid DNA samples with nuclease-free water to approximately 50 ng. See the table below for dilutions:

Starting Conc.	Volume of DNA	Volume of nuclease-free water	Total volume
100 ng/µl	2 µl	34 µl	36 µl
90 ng/µl	2 µl	31 µl	33 µl
80 ng/µl	2 µl	27 µl	29 µl
70 ng/µl	3 µl	35 µl	38 µl
60 ng/µl	2 µl	20 µl	22 µl
50 ng/µl	2 µl	16 µl	18 µl
40 ng/µl	5 µl	31 µl	36 µl
30 ng/µl	5 µl	22 µl	27 µl
20 ng/µl	5 µl	13 µl	18 µl
10 ng/µl	10 µl	8 µl	18 µl
<5.56 ng/µl	9 µl	0 µl	9 µl

- Pipette mix the dilutions, and spin down briefly.
- Add 9 µl of volume for each sample into a 0.2 ml PCR tube or plate.

4 Select a unique barcode for every sample to be run together on the same flow cell. Up to 96 samples can be barcoded and combined in one experiment.

Please note: Only use one barcode per sample.

5 In 0.2 ml thin-walled PCR tubes or plate, mix the following reagents. The Rapid Barcodes can be transferred using a multichannel pipette:

Reagent	Volume
50 ng template DNA	9 µl
Rapid Barcodes (RB01-96, one for each sample)	1 µl
Total	10 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 the barcoded samples into a clean 1.5 ml Eppendorf DNA LoBind tube, noting the total volume.

	Volume per sample	For 12 samples	For 24 samples	For 48 samples	For 96 samples
Total volume	10 µl	120 µl	240 µl	480 µl	960 µl

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

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

	Volume per sample	For 12 samples	For 24 samples	For 48 samples	For 96 samples
Volume of AXP	10 µl	120 µl	240 µl	480 µl	960 µl

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

13 Prepare at least 3 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 1.5 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 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 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.

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

Note: We recommend transferring a maximum of 800 ng of the DNA library.

If necessary, take forward only the necessary volume for 800 ng of DNA library and make up the rest of the volume to 11 µl using Elution Buffer (EB).

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.

END OF STEP

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

Priming and loading the SpotON flow cell

~10 minutes

Materials

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

Consumables

- 1.5 ml Eppendorf DNA LoBind tubes
- SpotON Flow Cell
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)

Equipment

- MinION or GridION device
- MinION Flow Cell Light Shield
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

IMPORTANT

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

TIP

Priming and loading a flow cell

We recommend all new users watch the [Priming and loading your flow cell](#) video before your first run.

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.

IMPORTANT

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).

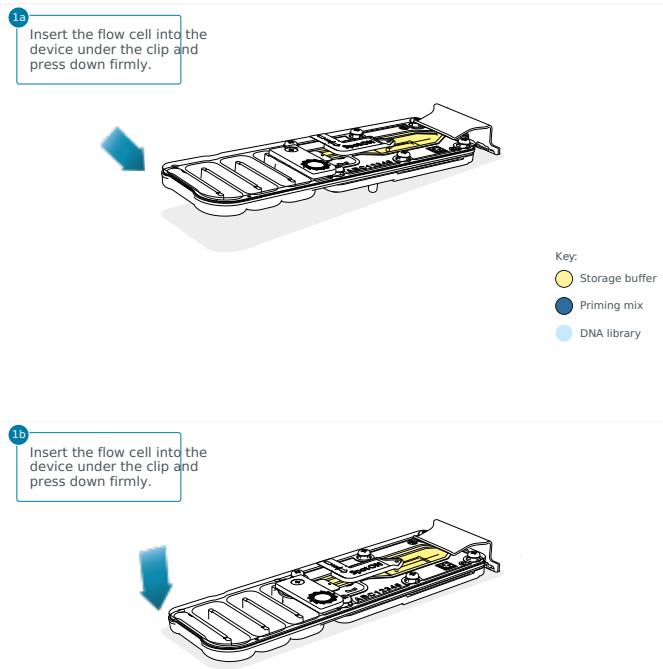
2 To prepare the flow cell priming mix with BSA, combine the following reagents and pipette mix at room temperature:

Note: The vials of Flow Cell Flush (FCF) in kit **SQK-RBK114.24** and **SQK-RBK114.96** have different formats. Please ensure you are using the correct volume when preparing your flow cell priming mix.

- If using **SQK-RBK114.24**: The reagents can be added directly to the single-use tube of Flow Cell Flush (FCF).
- If using **SQK-RBK114.96**: Prepare the reagents in a suitable tube.

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
Total volume	1,205 µl

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



Optional Action

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.

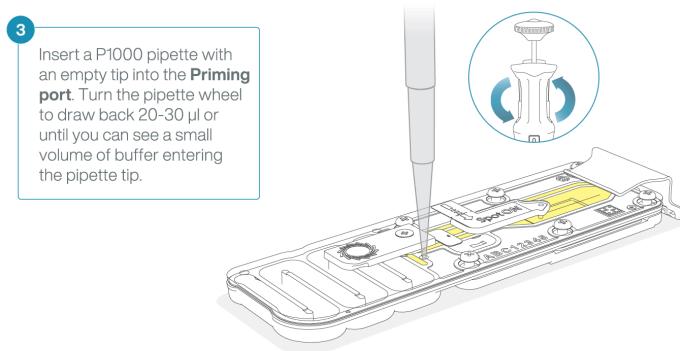
IMPORTANT

Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 μ l, 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.

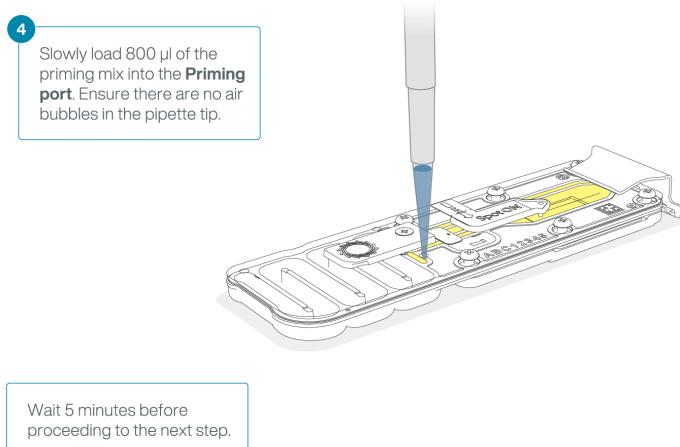
5 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:

1. Set a P1000 pipette to 200 μ l
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 μ l, to draw back 20-30 μ l, 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.

IMPORTANT

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.

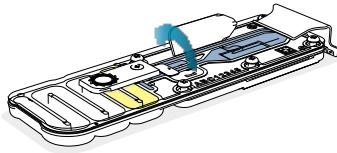
8 In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:

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

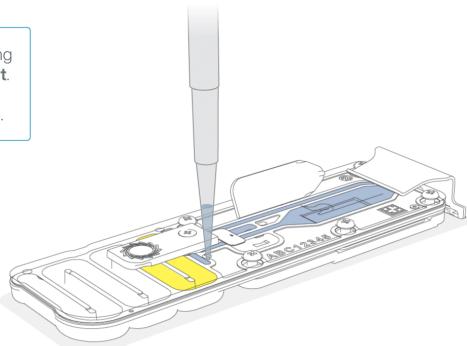
9 Complete the flow cell priming:

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 SpotON sample port cover.

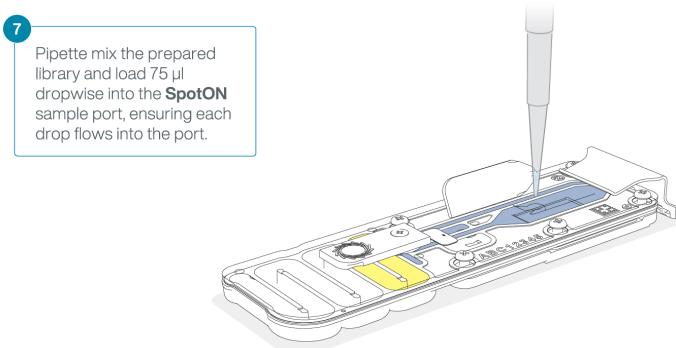


6
Load 200 µl of the priming mix into the **Priming Port**. Ensure there are no air bubbles in the pipette tip.

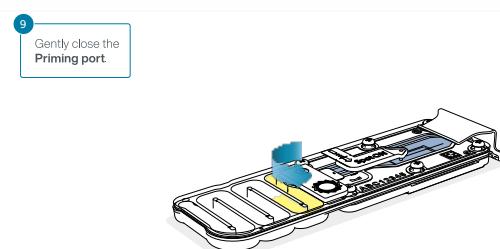
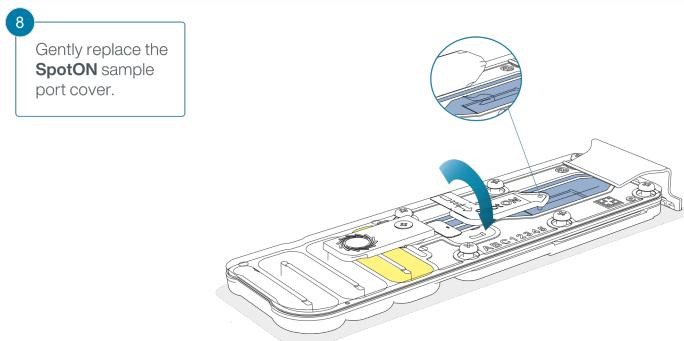


10 Mix the prepared library gently by pipetting up and down just prior to loading.

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.



IMPORTANT

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

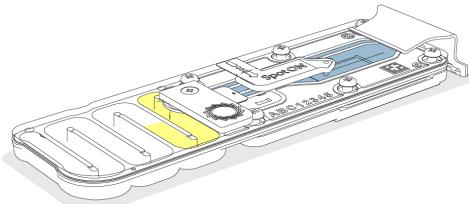
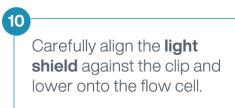
We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

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.



CAUTION

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

END OF STEP

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

Data acquisition and basecalling

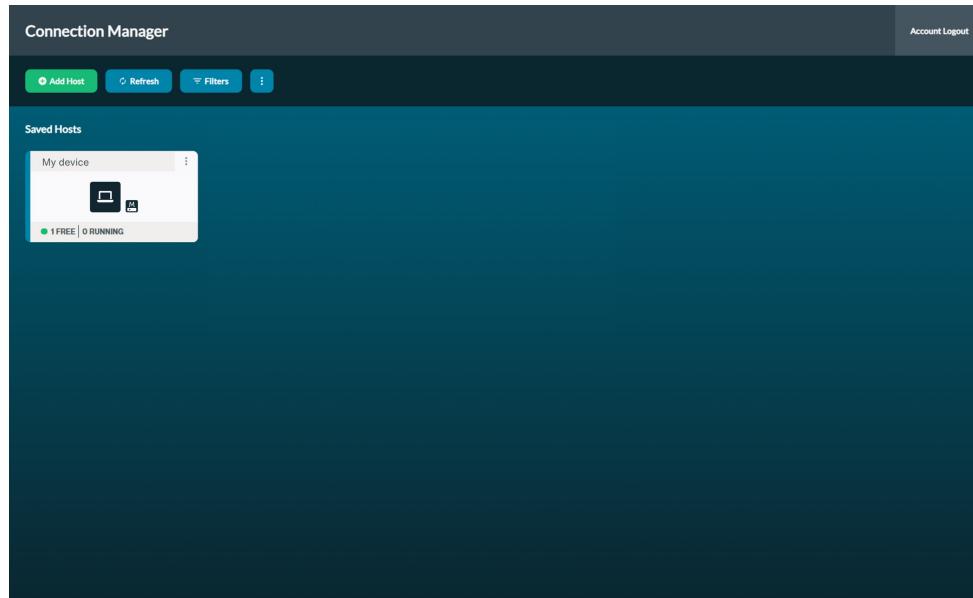
How to start sequencing

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. Further instructions for setting up a sequencing run can be found in the [MinKNOW protocol](#).

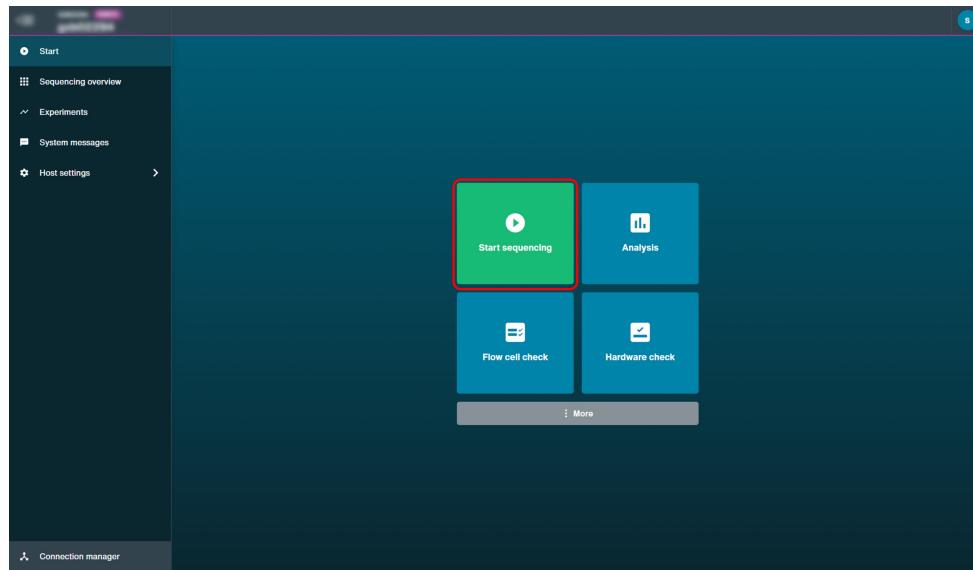
We recommend setting up a sequencing run on a MinION or GridION device using the basecalling and barcoding recommendations outlined below. All other parameters can be left to their default settings.

1 Open the MinNOW software using the desktop shortcut and log into the MinNOW 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 position and enter sample ID. Choose FLO-MIN114 flow cell type from the drop-down menu.**

Click **Continue to kit selection**.

The screenshot shows the 'Positions' screen of the MinION Flow Controller. The experiment name is 'Test'. The flow cell position is set to X1 with flow cell ID 'FAV36835' and flow cell type 'FLO-MIN114'. The sample ID is 'Sample_1'.

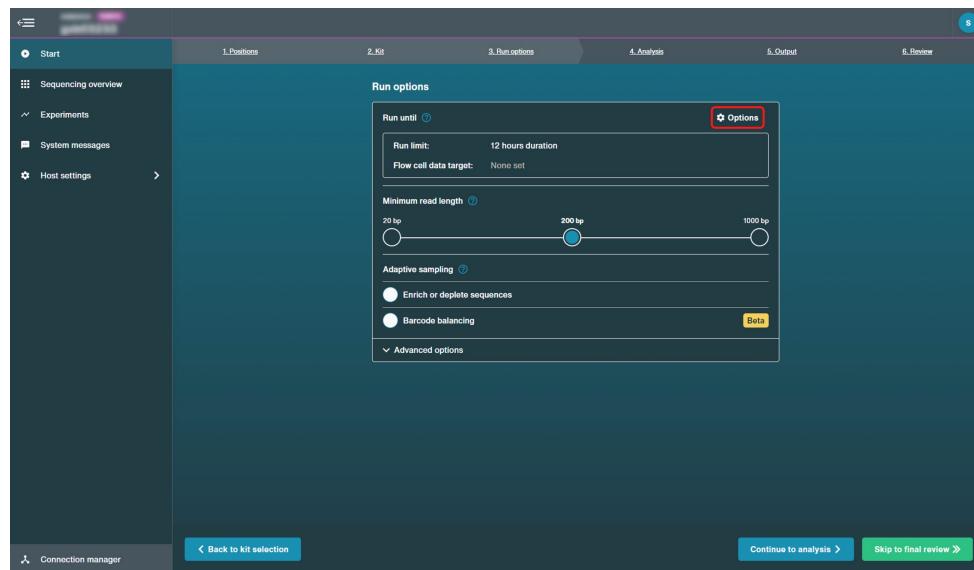
- 5 Select the Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) or Rapid Barcoding Kit 96 V14 (SQK-RBK114.96).**

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

The screenshot shows the 'Kit selection' screen of the MinION Flow Controller. The 'Rapid Sequencing Kit SQK-RAD014' is selected.

6 Change the run limit to 12 hours by clicking "Options" and changing the run limit value to 12. The other run settings can be left at the defaults.

Click **Continue to basecalling** to continue.

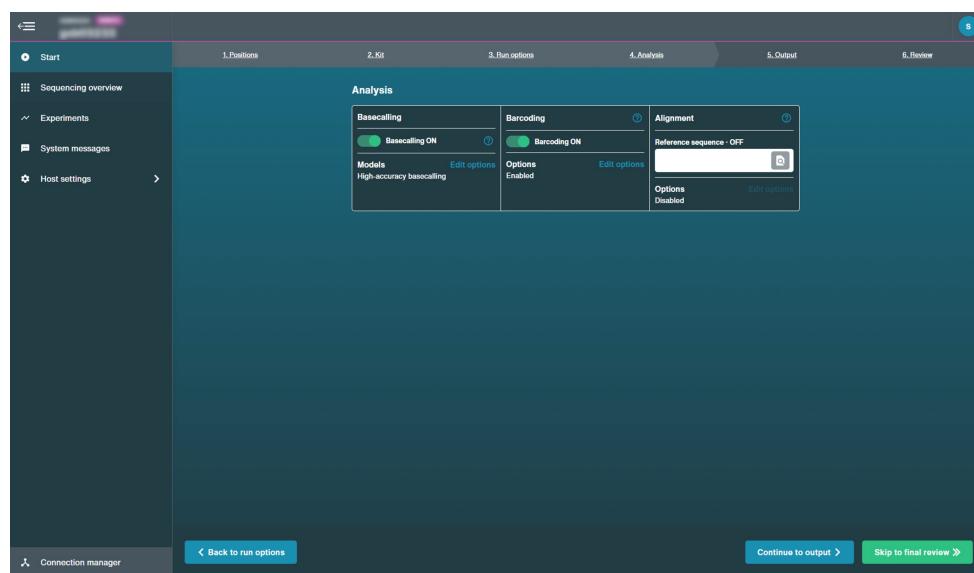


This is a detailed view of the 'Run until' configuration dialog. It shows the 'Run limit' section where 'Stop run when' is set to 'Time equals' with a value of '12 Hrs'. The 'Flow cell data target (optional)' section is also shown, with 'Stop run when' set to 'Select' and 'Input' set to 'Gb'. At the bottom, there are 'Cancel' and 'Apply rules' buttons.

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 is ON.
4. All other options can be kept to their default settings.

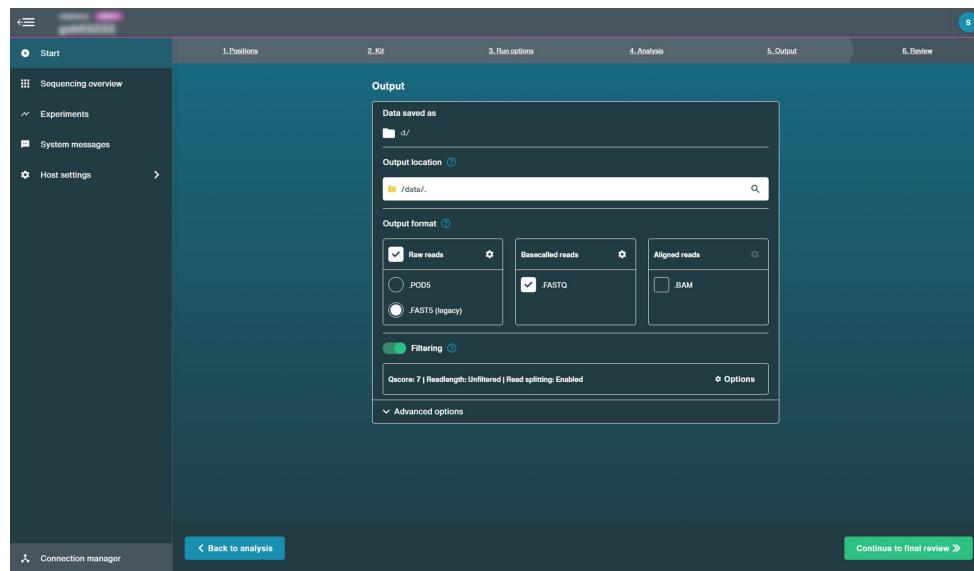
Click **Continue to output** and continue.



8 Set up the output format and filtering as follows:

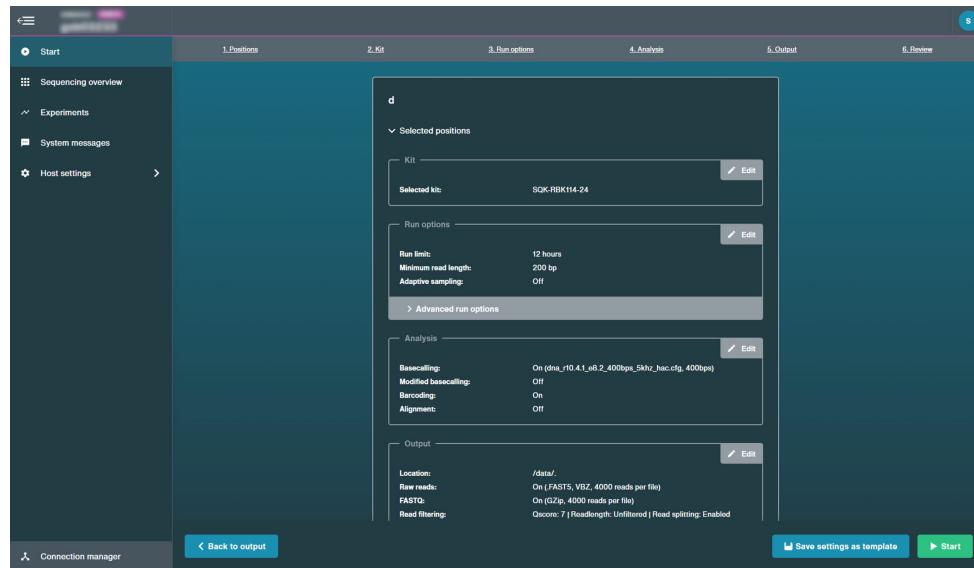
1. Select either **.POD5** or **.FAST5 (legacy)** as the output format.
2. Ensure **.FASTQ** is selected for basecalled reads.
3. Ensure filtering is ON and read splitting is enabled. Other parameters can be kept to their default settings.

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.



Downstream analysis using EPI2ME Labs

Post-basecalling analysis

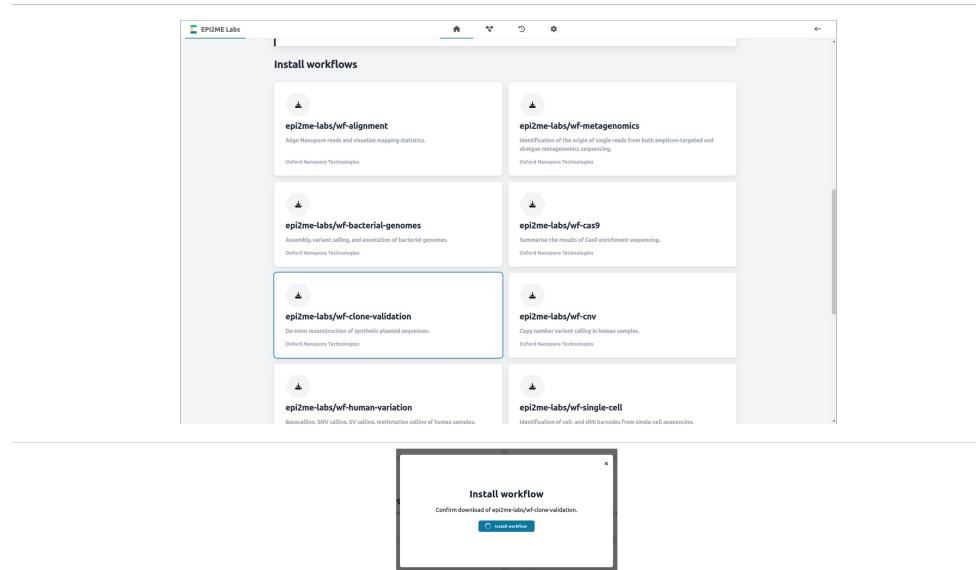
We recommend performing downstream analysis using EPI2ME Labs which facilitates bioinformatic analyses by allowing users to run Nextflow workflows in a desktop application. EPI2ME Labs maintains a collection of bioinformatic workflows which are curated and actively maintained by experts in long-read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

Further information about the available EPI2ME Labs workflows are available [here](#), along with the [Quick Start Guide](#) to start your first bioinformatic workflow.

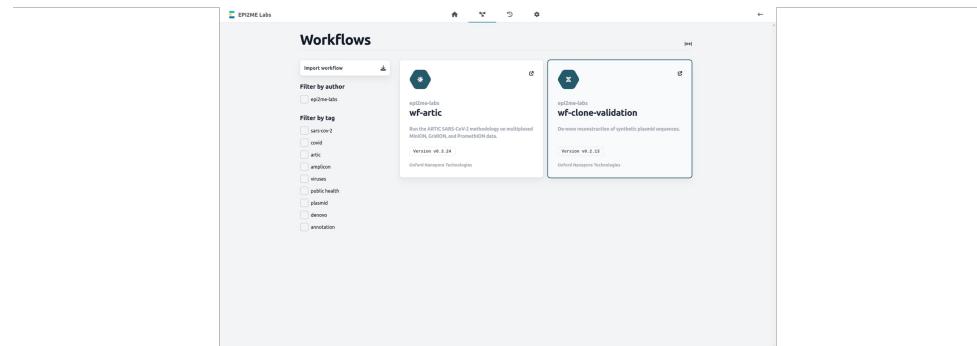
For the assembly of small plasmid sequences, we recommend using the [wf-clone-validation workflow](#) which requires [Nextflow](#) and [Docker](#) to be installed before running the workflow.

1 Open the EPI2ME Labs app using the desktop shortcut.

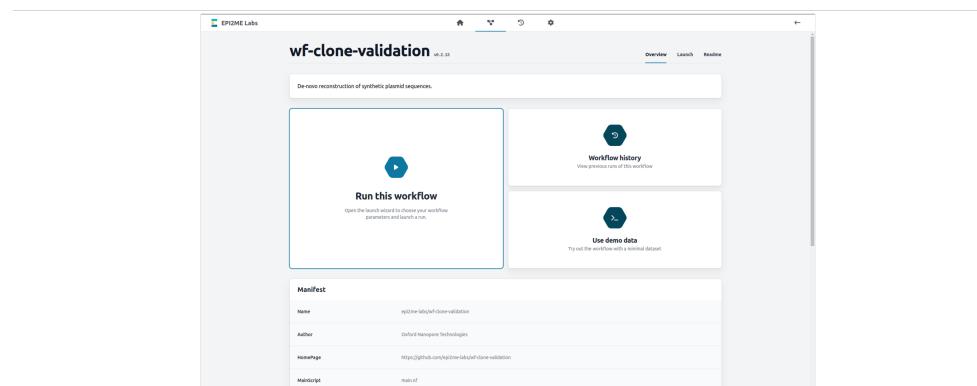
2 Scroll down on the landing page and click on the wf-clone-validation workflow to download and confirm to install.



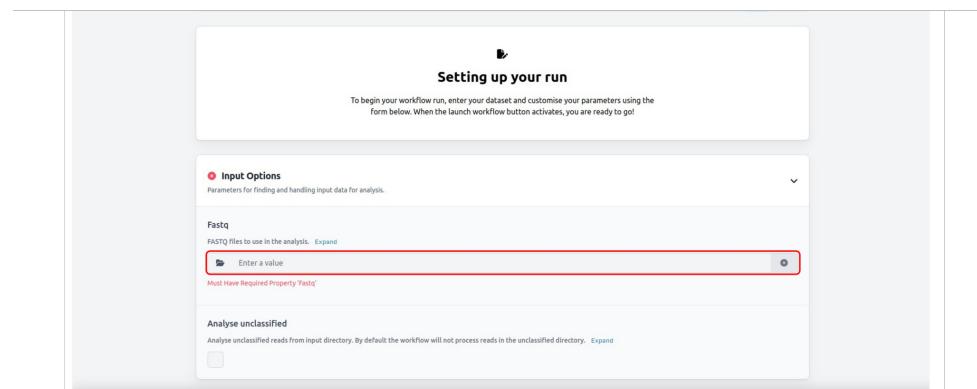
3 Navigate to the Workflows tab and click on wf-clone-validation.



4 Click on "Run this workflow" to open the launch wizard.

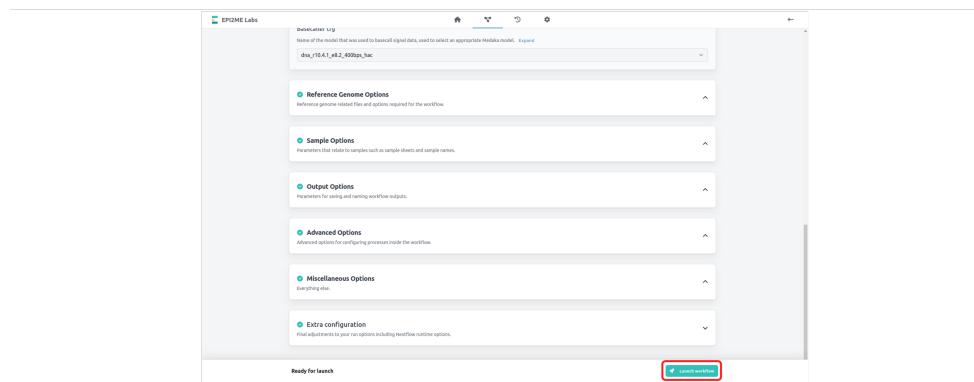


5 Set up your run by uploading your FASTQ file in the "Input Options". We recommend keeping the default settings for the other parameter options.



6 Click "Launch workflow".

Ensure all parameter options have green ticks.



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

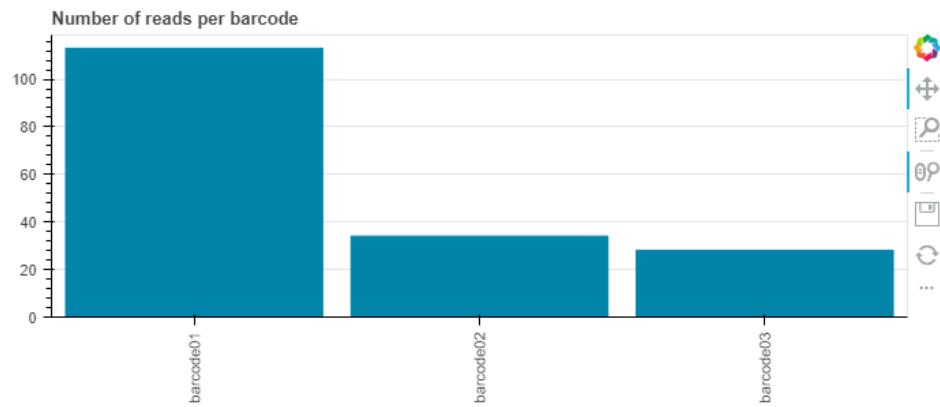
Clone validation workflow report

A report is produced containing the results of the assembled plasmid sequences. The primary outputs of the workflow include:

- a consensus .fasta file for each sample
- a .csv showing the pass or fail status of each sample
- a feature table containing annotations for each of the samples
- an HTML report document detailing the primary findings of the workflow

A sample report can be viewed [here](#).

Summary



Sample status

10 ▾ entries per page

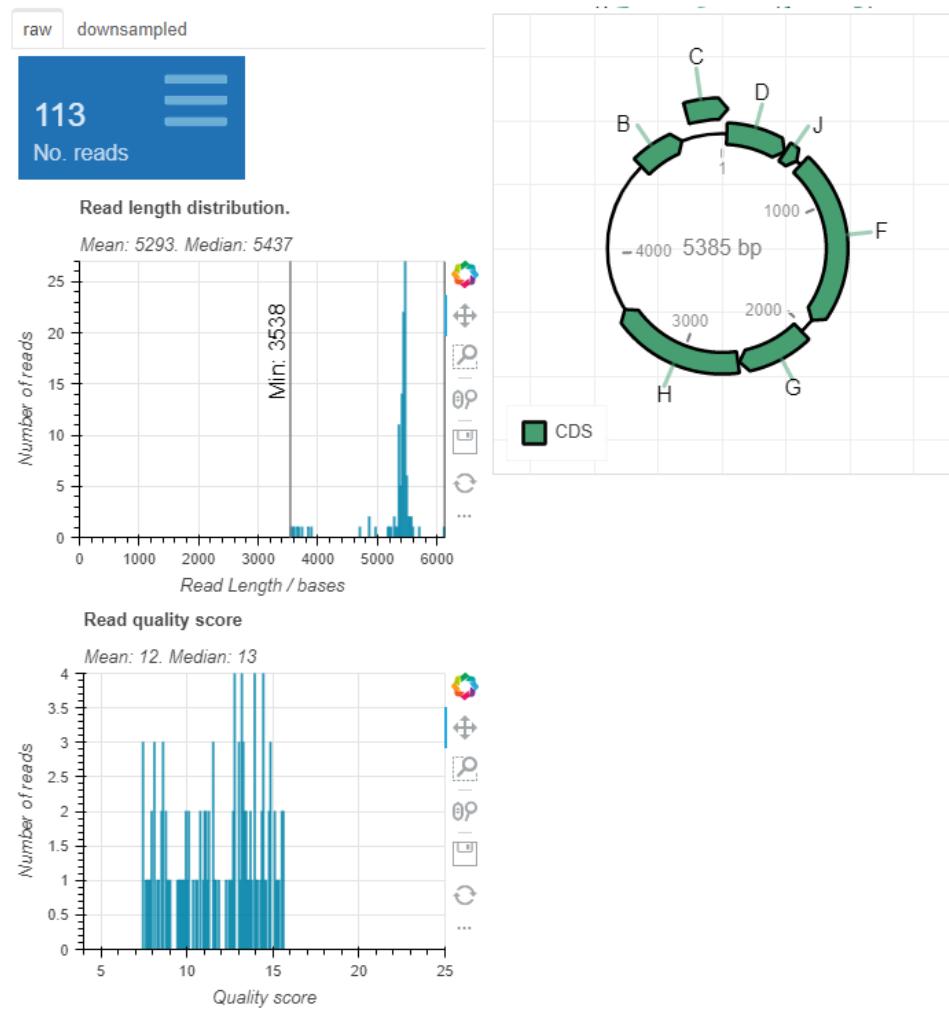
Search...

Sample	pass/failed reason	Length
barcode01	Completed successfully	5385
barcode02	Completed successfully	5385
barcode03	Completed successfully	5382

The summary graph shows the number of reads per barcode and the table shows the length of the consensus sequence for each barcode. These data may be used to identify the samples that may have dropped out of the sequence analysis due to insufficient sequence reads.

Sample: barcode01

Completed successfully



For each barcode, read length statistics and a pLAnnotate plot is presented to illustrate the polished plasmid consensus sequence. In the sample report, barcode01 has been assembled into a 5385 bp consensus sequence. The unfilled features on the plot are incomplete features.

Feature	Database	Identity	Match Length	Description	Start Location	End Location	Length	Strand
F	swissprot	100.0%	100.0%	CAPS_D_BPPHS - Experimental evidence at protein level: Swiss-Prot protein existence level 1. Assembles to form an icosahedral capsid with a T=1 symmetry, about 30 nm in diameter, and consisting of 60 capsid proteins F (PubMed:11991963, PubMed:1370343, PubMed:8158636). Upon virus binding to host cell, one of the spikes dissociates from the capsid and the virus interacts with LPS through the exposed EF loops on the F proteins (PubMed:29229840). After the genome had been ejected, the channel formed by the F proteins at the unique fivefold axis remains open (PubMed:29229840). From Enterobacteria phage phiX174 (Isolate Sanger) (Bacteriophage phi-X174).	645	1926	1281	1
D	swissprot	100.0%	100.0%	SCAFD_BPPHS - Experimental evidence at protein level: Swiss-Prot protein existence level 1. Assembles the procapsid by joining twelve 12S pre-assembly complex into a T=1 icosahedral particle, called 108S procapsid. Ten proteins D bind each 12S complex, which are formed by three pentamers of F, G, B protein and a H protein. The scaffolding protein is released from the provirion after genome packaging to form the mature virion. From Enterobacteria phage phiX174 (Isolate Sanger) (Bacteriophage phi-X174).	34	490	456	1
B	swissprot	100.0%	100.0%	SCAFB_BPPHS - Experimental evidence at protein level: Swiss-Prot protein existence level 1. Participates in the assembly of the viral procapsid in the cytoplasm. Forms first a 12S pre-assembly complex with protein H, and F and G pentamers, then twelve 12S complexes are joined by the D protein to form the procapsid. Internal scaffold protein B is released from the procapsid upon genome packaging (PubMed:159449). Autoproteolytic activity cleaves protein B and probably facilitates its removal through the pores of the procapsid (PubMed:12473449). From Enterobacteria phage phiX174 (Isolate Sanger) (Bacteriophage phi-X174).	4718	5078	360	1

A feature table is also provided for each barcode to give descriptions of the annotated sequence which can be used to identify the precise location of the annotated features.

Ending the experiment

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 2-8°C.

The [Flow Cell Wash Kit](#) protocol is available on the Nanopore Community.

2 Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore.

Instructions for returning flow cells can be found [here](#).

Note: All flow cells must be flushed with deionised water before returning the product.

IMPORTANT

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.

Issues during DNA/RNA extraction and library preparation

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

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

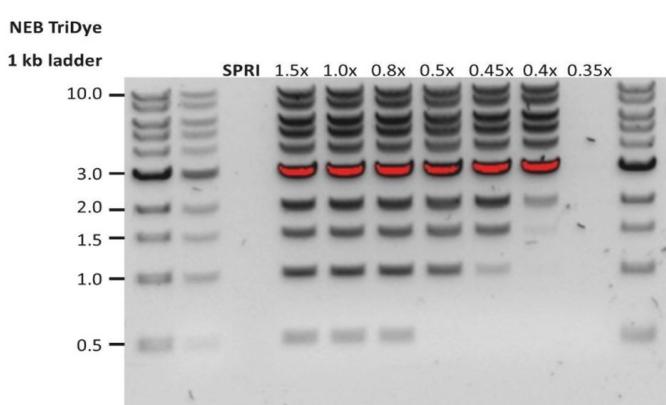
If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via [LiveChat](#) in the Nanopore Community.

Low sample quality

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

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

The VolTRAX run terminated in the middle of the library prep

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

The VolTRAX software shows an inaccurate amount of reagents loaded

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

Issues during the sequencing run

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

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

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

Fewer pores at the start of sequencing than after Flow Cell Check

Observation	Possible cause	Comments and actions
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	An air bubble was introduced into the nanopore array	After the Flow Cell Check it is essential to remove any air bubbles near the priming port before priming the flow cell. If not removed, the air bubble can travel to the nanopore array and irreversibly damage the nanopores that have been exposed to air. The best practice to prevent this from happening is demonstrated in this video .
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	The flow cell is not correctly inserted into the device	Stop the sequencing run, remove the flow cell from the sequencing device and insert it again, checking that the flow cell is firmly seated in the device and that it has reached the target temperature. If applicable, try a different position on the device (GridION/PromethION).
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	Contaminations in the library damaged or blocked the pores	The pore count during the Flow Cell Check is performed using the QC DNA molecules present in the flow cell storage buffer. At the start of sequencing, the library itself is used to estimate the number of active pores. Because of this, variability of about 10% in the number of pores is expected. A significantly lower pore count reported at the start of sequencing can be due to contaminants in the library that have damaged the membranes or blocked the pores. Alternative DNA/RNA extraction or purification methods may be needed to improve the purity of the input material. The effects of contaminants are shown in the Contaminants Know-how piece . Please try an alternative extraction method that does not result in contaminant carryover.

MinKNOW script failed

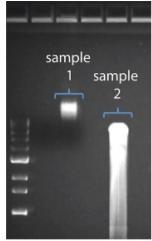
Observation	Possible cause	Comments and actions
MinNOW shows "Script failed"		Restart the computer and then restart MinNOW. If the issue persists, please collect the MinNOW log files and contact Technical Support. If you do not have another sequencing device available, we recommend storing the flow cell 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 you load the recommended amount of good quality library in the relevant library prep protocol onto your flow cell. Please quantify the library before loading and calculate mols using tools like the Promega Biomath Calculator , choosing "dsDNA: µg to pmol"
Pore occupancy close to 0	The Ligation Sequencing Kit was used, and sequencing adapters did not ligate to the DNA	Make sure to use the NEBNext Quick Ligation Module (E6056) and Oxford Nanopore Technologies Ligation Buffer (LNB, provided in the sequencing kit) at the sequencing adapter ligation step, and use the correct amount of each reagent. A Lambda control library can be prepared to test the integrity of the third-party reagents.
Pore occupancy close to 0	The Ligation Sequencing Kit was used, and ethanol was used instead of LFB or SFB at the wash step after sequencing adapter ligation	Ethanol can denature the motor protein on the sequencing adapters. Make sure the LFB or SFB buffer was used after ligation of sequencing adapters.
Pore occupancy close to 0	No tether on the flow cell	Tethers are added during flow cell priming (FLT/FCT tube). Make sure FLT/FCT was added to FB/FCF before priming.

Shorter than expected read length

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

Large proportion of 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	<p>Some contaminants can be cleared from the pores by the unblocking function built into MinNOW. If this is successful, the pore status will change to "sequencing pore". If the portion of unavailable pores stays large or increases:</p> <ol style="list-style-type: none"> 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

Observation	Possible cause	Comments and actions
Large proportion of inactive/unavailable pores	Certain compounds co-purified with DNA	<p>Known compounds, include polysaccharides, typically associate with plant genomic DNA.</p> <ol style="list-style-type: none"> 1. Please refer to the Plant leaf DNA extraction method. 2. Clean-up using the QIAGEN PowerClean Pro kit. 3. Perform a whole genome amplification with the original gDNA sample using the QIAGEN REPLI-g kit.
Large proportion of inactive/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.

Reduction in sequencing speed and q-score later into the run

Observation	Possible cause	Comments and actions
Reduction in sequencing speed and q-score later into the run	For Kit 9 chemistry (e.g. SQK-LSK109), fast fuel consumption is typically seen when the flow cell is overloaded with library (please see the appropriate protocol for your DNA library to see the recommendation).	Add more fuel to the flow cell by following the instructions in the MinKNOW protocol . In future experiments, load lower amounts of library to the flow cell.

Temperature fluctuation

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

Failed to reach target temperature

Observation	Possible cause	Comments and actions
MinKNOW shows "Failed to reach target temperature"	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.

