

Overview of the protocol

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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.

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

This protocol describes how to carry out rapid barcoding of amplicon DNA using the Rapid Barcoding Kit 24 or 96 V14 (SQK-RBK114.24 or SQK-RBK114.96) to sequence up to 96 single amplicon samples. This method allows you to perform your own amplicon sequencing and validation with a quick turn-around time, without the need for primers and a reference, and with competitive price per sample. Using this method, PCR amplicons ranging from 500 bp to 5 kb can be sequenced, which allows users to check that each amplicon is the correct size and no mutations have been introduced during PCR amplification.

The analysis workflow is not intended for marker gene sequencing of mixtures/communities of different organisms (e.g. 16S sequencing). In de-novo consensus mode it expects a single amplicon per barcode. When running in variant calling mode, multiple amplicons per barcode can be processed, but their sequences need to be sufficiently different from each other so that most reads only align to one of the provided references.

We recommend new users to sequence for 12 hours, although a shorter run-time (e.g. 4 hours) may be sufficient to generate enough reads per target. We suggest generating 150X or ~1500 reads per target. In most cases, this should be sufficient data to perform analysis.

After sequencing, we recommend performing downstream analysis using the [EPI2ME Labs amplicon workflow \(wf-amplicon\)](#).

The results of the workflow include an interactive HTML report, FASTQ files with the consensus sequences of the amplicons, and BAM files with alignments of the input reads re-aligned against the consensus. Optionally, a reference with the expected amplicon sequences can be supplied to the workflow, in which case VCF files with variants called against that reference are additionally emitted.

Detailed instructions for setting up MinKNOW and the EPI2ME Labs workflow are included in this protocol.

Steps in the sequencing workflow:

Prepare for your experiment

You will need to:

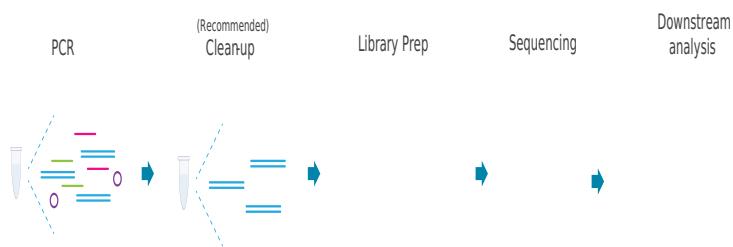
- Extract your DNA, and check its length, quantity and purity using the [Input DNA/RNA QC protocol](#). **The quality checks performed during the protocol are essential in ensuring experimental success.**
- Generate your amplicon sample(s) by PCR amplification.
- Ensure you have your sequencing kit, 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

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
PCR clean-up	AMPure XP Bead purification (or equivalent) of amplicon samples to remove PCR artifacts	25 minutes	4°C overnight
Amplicon DNA barcoding	Tagmentation of the amplicon 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	10 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 from the device into basecalled reads and will perform barcode demultiplexing.
- Start the EPI2ME software and use the [EPI2ME Labs amplicon workflow \(wf-amplicon\)](#) 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)
- MinION Mk1C - [MinION Mk1C IT requirements document](#)
- MinION Mk1B - [MinION IT Requirements document](#)

Equipment and consumables

Materials

- 50 ng amplicon DNA per sample (500 bp – 5 kb amplicon size)
- Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) OR Rapid Barcoding Kit 96 V14 (SQK-RBK114.96)

Consumables

- MinION and GridION Flow Cell
- Agencourt AMPure XP beads (Beckman Coulter™, A63881) (or equivalent for DNA purification)
- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- 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)
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes or 0.2 ml 96-well PCR plate
- 2 ml Eppendorf DNA LoBind tubes

Equipment

- MinION or GridION device
- MinION and GridION Flow Cell Light Shield
- Hula mixer (gentle rotator mixer)
- Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, 11766427)
- Microfuge
- Vortex mixer
- Thermal cycler or heat blocks
- Qubit fluorometer (or equivalent for QC check)

- Magnetic rack
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P2 pipette and tips
- Multichannel pipette and tips
- Ice bucket with ice
- Timer

For this protocol, you will need 50 ng of single species amplicon DNA per sample.

We recommend performing a purification step following the PCR reaction used to generate your amplicons. Please use standard methods appropriate for the amplicon size, such as AMPure XP beads. This step ensures the removal of proteins, salts, dNTPs, and primers, which could potentially impact the library preparation and subsequent analysis workflow.

Please note that while alternative methods exist, they have not been validated by our internal teams.

This method is intended for barcoding single species amplicon samples and is not suitable for mixed species amplicons.

IMPORTANT

The wf-amplicon workflow is optimised for 500 bp - 5 kb amplicons.

Sequencing amplicons <500 bp or >5 kb may result in sub-optimal performance.

Further work is ongoing to broaden the range of amplicons compatible with this end-to-end workflow.

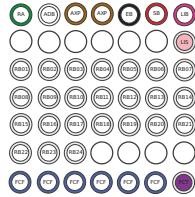
Check your flow cell

We highly recommend that you check the number of pores in your flow cell prior to starting a sequencing experiment. This should be done within three months of purchasing for MinION/GridION/PromethION or within four weeks of purchasing Flongle Flow Cells. Oxford Nanopore Technologies will replace any flow cell with fewer than the number of pores in the table below, when the result is reported within two days of performing the flow cell check, and when the storage recommendations have been followed. To do the flow cell check, please follow the instructions in the [Flow Cell Check document](#).

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

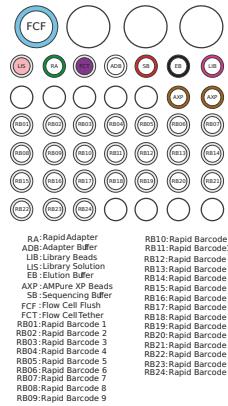
Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) contents

Single-use tubes format:



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

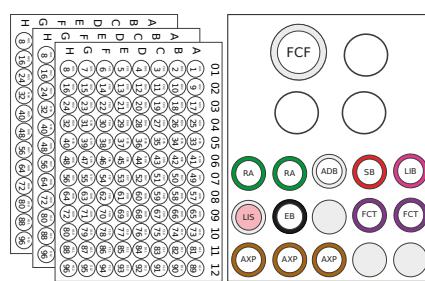
Bottle format:



RA: Rapid Adapter	RB10:Rapid Barcode 10
ADB: Adapter Buffer	RB11:Rapid Barcode11
LIB: Library Beads	RB12:Rapid Barcode 12
LIS: Library Solution	RB13:Rapid Barcode 13
EB :Elution Buffer	RB14:Rapid Barcode 14
AXP :AMPure XP Beads	RB15:Rapid Barcode 15
SB :Sequencing Buffer	RB16:Rapid Barcode 16
FCF :Flow Cell Flush	RB17:Rapid Barcode 17
FCT :Flow CellTether	RB18:Rapid Barcode 18
RB01:Rapid Barcode 1	RB19:Rapid Barcode 19
RB02:Rapid Barcode 2	RB20:Rapid Barcode 20
RB03:Rapid Barcode 3	RB21:Rapid Barcode 21
RB04:Rapid Barcode 4	RB22:Rapid Barcode 22
RB05:Rapid Barcode 5	RB23:Rapid Barcode 23
RB06:Rapid Barcode 6	RB24:Rapid Barcode 24
RB07:Rapid Barcode 7	
RB08:Rapid Barcode 8	
RB09:Rapid Barcode 9	

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

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



AXP: AMPure XP Beads	EB: Elution Buffer
FCF: Flow Cell Flush	LIS: Library Solution
ADB Adapter Buffer	FCT: Flow CellTether
RA: Rapid Adapter	SB: Sequencing Buffer
LIB: Library Beads	

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

Library preparation

~50 minutes

Materials

- 50 ng amplicon DNA per sample (500 bp – 5 kb amplicon size)
- Rapid Barcodes (RB01-24) or Rapid Barcode Plate (RB01-96)
- Rapid Adapter (RA)
- Adapter Buffer (ADB)
- AMPure XP Beads (AXP)
- Elution Buffer (EB)

Consumables

- Agencourt AMPure XP beads (Beckman Coulter™, A63881) (or equivalent for DNA purification)
- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- Freshly prepared 80% ethanol in nuclease-free water
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes or 0.2 ml 96-well PCR plate
- 2 ml Eppendorf DNA LoBind tubes

Equipment

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

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.

IMPORTANT**PCR clean-up of amplicon DNA samples**

We recommend performing a purification step following the PCR reaction used to generate your amplicons. Please use standard methods appropriate for the amplicon size, such as AMPure XP beads. This step ensures the removal of proteins, salts, dNTPs, and primers, which could potentially impact the library preparation and subsequent analysis workflow.

Please note that while alternative methods exist, they have not been validated by our internal teams.

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 amplicon DNA is required in 9 µl of volume for each sample for barcoding.

- Dilute your amplicon 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 10 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 MinION and GridION Flow Cell

Priming and loading the MinION and GridION Flow Cell

~10 minutes

Materials

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

Consumables

- MinION and GridION Flow Cell
- Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
- Nuclease-free water

- 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- MinION or GridION device
- MinION and GridION 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.

- 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.

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 Flow Cell Flush (FCF) and Flow Cell Tether (FCT), as directed below. Mix by pipetting at room temperature.

Note: We are in the process of reformatting our kits with single-use tubes into a bottle format. Please follow the instructions for your kit format.

Single-use tubes format:

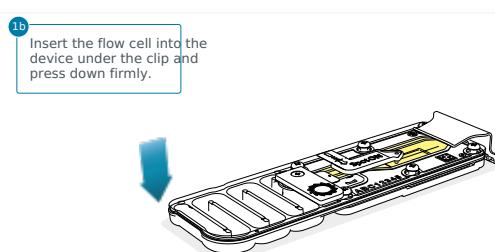
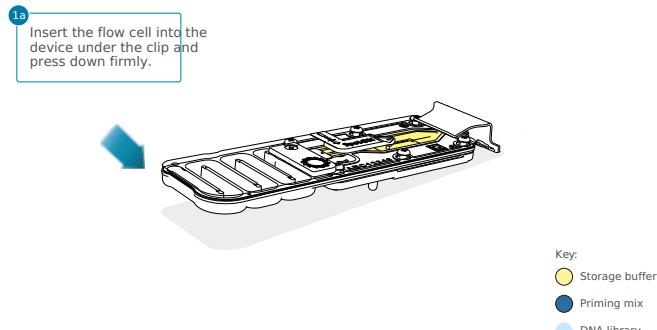
Add 5 µl Bovine Serum Albumin (BSA) at 50 mg/ml and 30 µl Flow Cell Tether (FCT) directly to a tube of Flow Cell Flush (FCF).

Bottle format:

In a suitable tube for the number of flow cells, combine the following reagents:

Reagent	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.

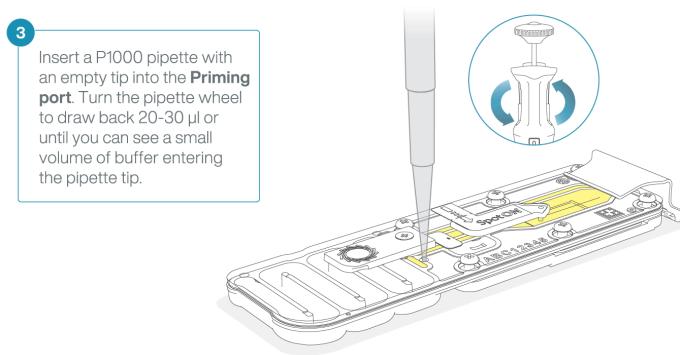
IMPORTANT

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

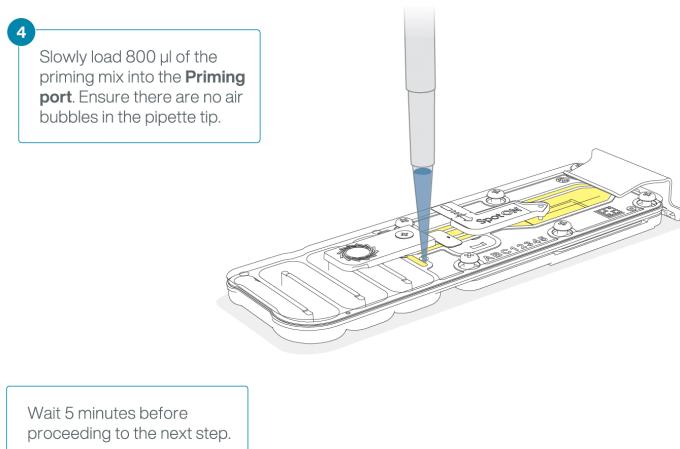
5 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:

1. Set a P1000 pipette to 200 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 μ l 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.

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

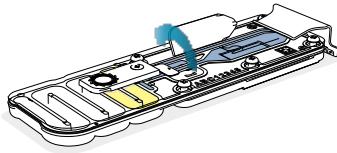
8 In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:

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

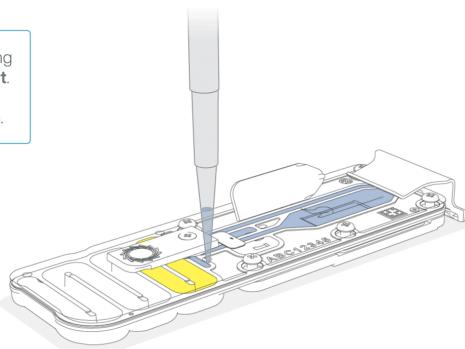
9 Complete the flow cell priming:

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 SpotON sample port cover.

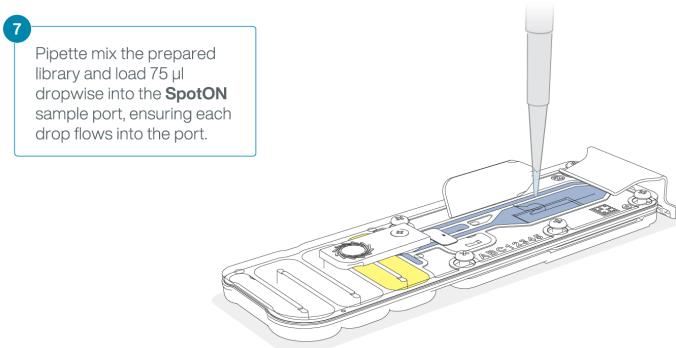


6
Load 200 µl of the priming mix into the **Priming Port**. Ensure there are no air bubbles in the pipette tip.

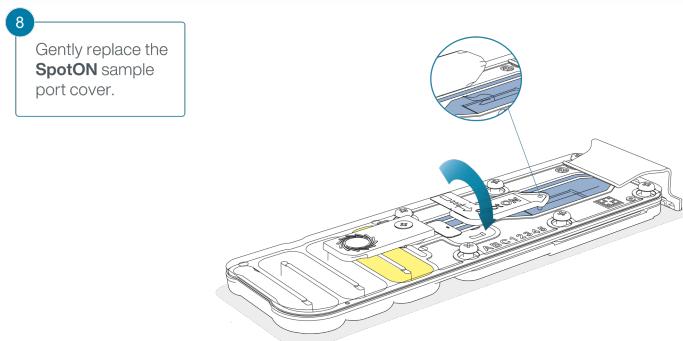


10 Mix the prepared library gently by pipetting up and down just prior to loading.

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.

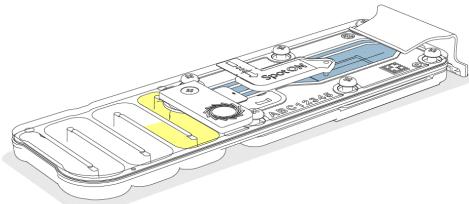
We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

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 MinNOW.

Data acquisition and basecalling

Data acquisition and basecalling

How to start sequencing

Once you have loaded your flow cell, the sequencing run can be started on MinNOW, our sequencing software that controls the device, data acquisition and real-time basecalling. For more detailed information on setting up and using MinNOW, please see the [MinNOW protocol](#).

MinNOW can be used and set up to sequence in multiple ways:

- On a computer either directly or remotely connected to a sequencing device.

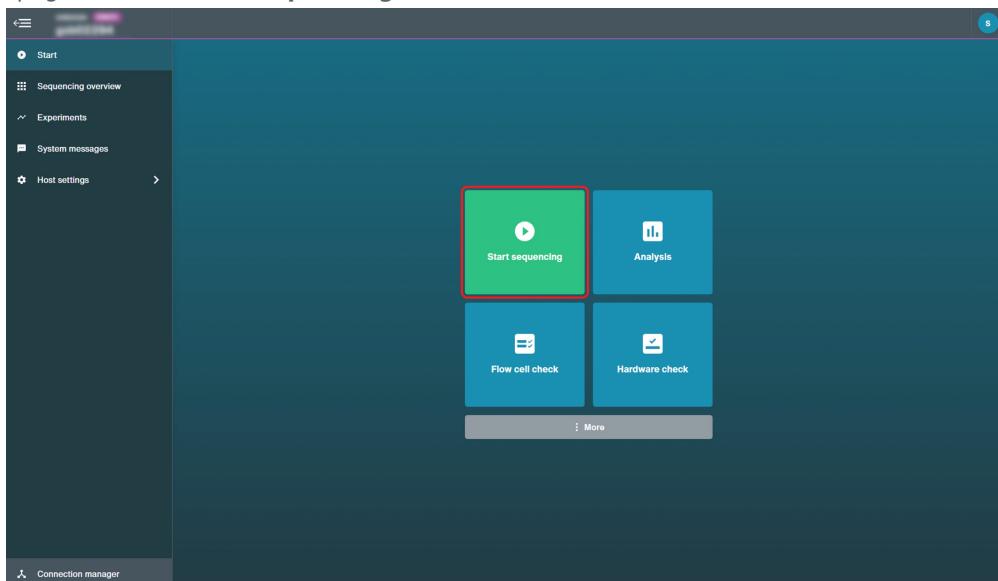
- Directly on a GridION, MinION Mk1C or PromethION 24/48 sequencing device.

For more information on using MinKNOW on a sequencing device, please see the device user manuals:

- [GridION user manual](#)
- [MinION Mk1C user manual](#)
- [PromethION user manual](#)
- [PromethION 2 Solo user manual](#)

To start a sequencing run on MinKNOW:

1. Navigate to the start page and click **Start sequencing**.



2. Fill in your experiment details, such as name and flow cell position and sample ID.

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

4. 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.

Run until

Run limit

Action	Condition	Value
Stop run when	Time equals	12 Hrs

Must be between 0.05–1,000

Flow cell data target (optional)

Action	Condition	Value
Stop run when	Select	Input Gb

Cancel **Apply rules**

Note: We recommend a run-time of 12 hours for new users to ensure sufficient data is generated for downstream analysis. However, shorter run-times may be sufficient depending on your sample input and analysis requirements.

If using custom sequencing time, for optimal results, we recommend allowing the sequencing run to generate 1000-1500 reads per sample before proceeding to the analysis workflow.

5. Set up basecalling and barcoding using the following parameters:

- Toggle basecalling to ON.
- Next to "Models", click Edit options and choose High accuracy basecaller (HAC) from the drop-down menu.
- Toggle barcoding to ON.
- Keep all other options at their default settings.
- Click **Continue to output** and continue.

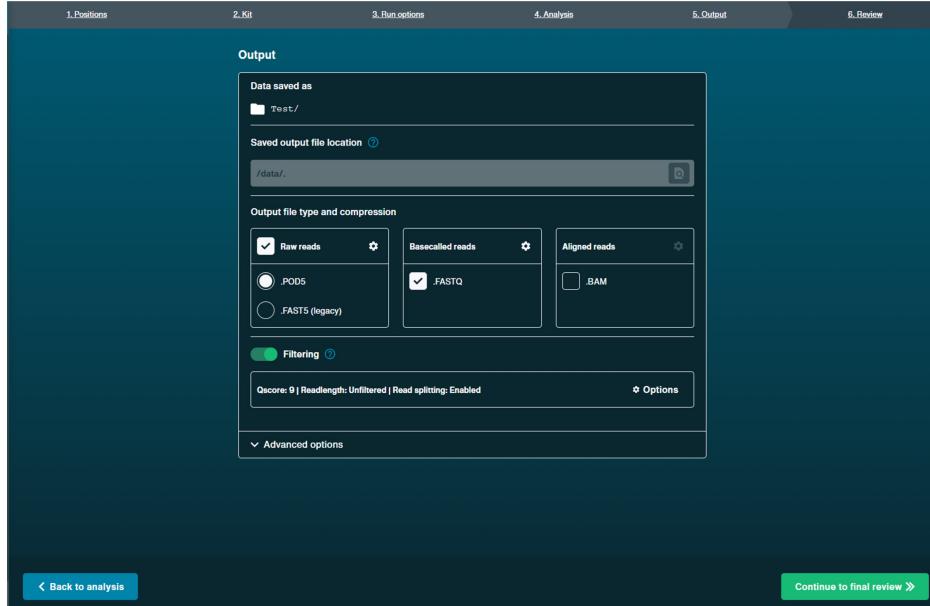
Analysis

Basecalling	Barcode	Alignment
<input checked="" type="checkbox"/> Basecalling ON	<input checked="" type="checkbox"/> Barcoding ON	Reference sequence - DISABLED
<input type="radio"/> Modified bases OFF	<input type="checkbox"/> Options Enabled	Options Edit options
Models High-accuracy basecalling		Disabled on remote host connection

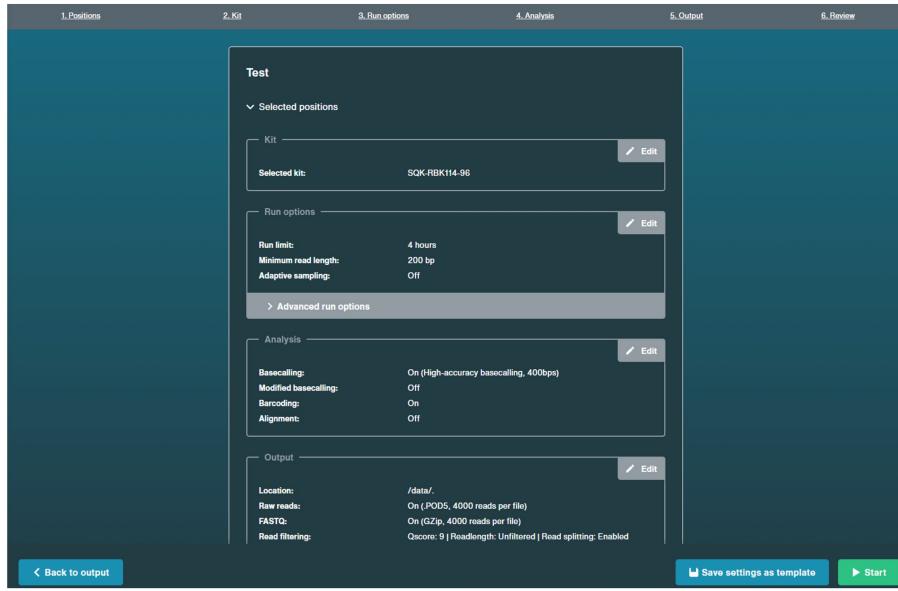
< Back to run options **Continue to output** **Skip to final review**

6. Set up the output format and filtering as follows:

- Select either **.POD5** or **.FAST5 (legacy)** as the output format.
- Ensure **.FASTQ** is selected for basecalled reads.
- 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.



7. Click **Start** on the Review page to start the sequencing run.



Data analysis after sequencing

After sequencing has completed on MinKNOW, the flow cell can be reused or returned, as outlined in the Flow cell reuse and returns section.

After sequencing and basecalling, the data can be analysed. For further information about options for basecalling and post-basecalling analysis, please refer to the [Data Analysis](#) document.

In the Downstream analysis section, we outline further options for analysing your data.

Flow cell reuse and returns

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 2-8°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.

Downstream analysis using EPI2ME Labs

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 analysis of amplicon sequences, we recommend using the [wf_amplicon workflow](#) which requires [Nextflow](#) and [Docker](#) or [Singularity](#) to be installed before running the workflow.

For installation instructions please click [here](#).

IMPORTANT

The wf-amplicon workflow is optimised for 500 bp - 5 kb amplicons.

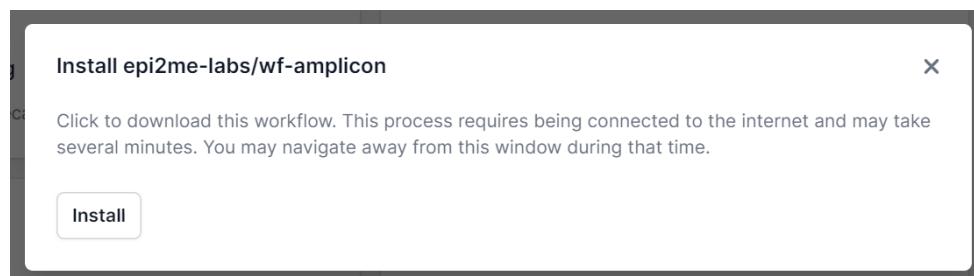
Sequencing amplicons <500 bp or >5 kb may result in sub-optimal performance.

We recommend generating 150X or 1500 reads per target, which should be enough to perform the analysis in most cases.

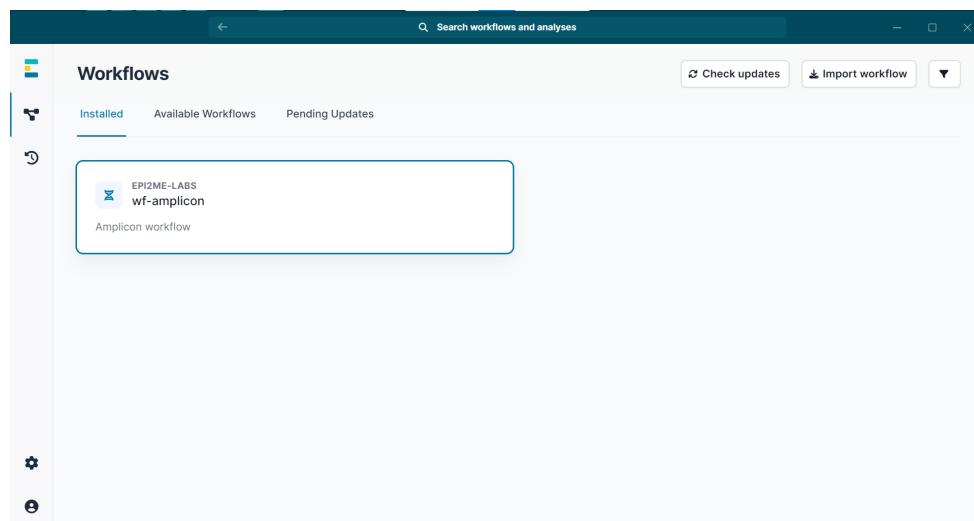
Further work is ongoing to broaden the range of amplicons compatible with this end-to-end workflow.

1 Open the EPI2ME app using the desktop shortcut.

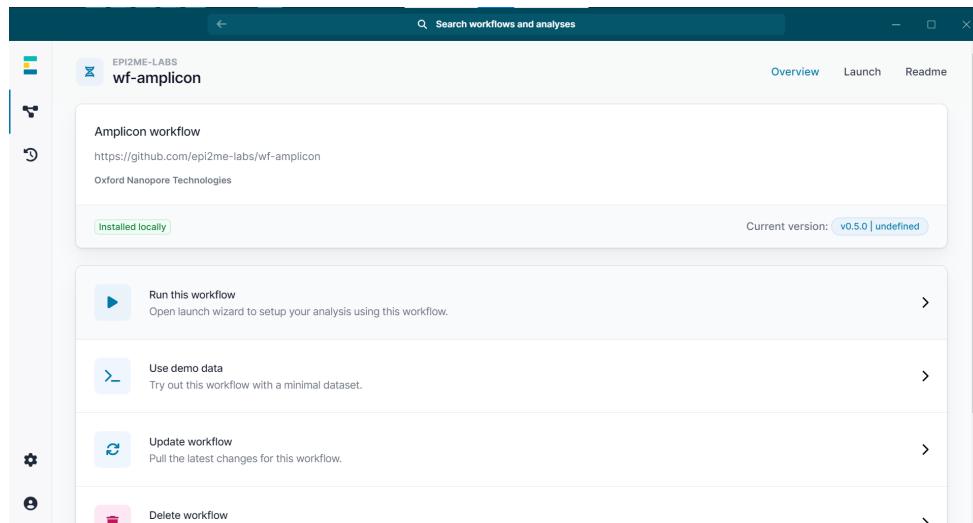
2 Navigate to the workflow downloads page. Click on the wf-amplicon workflow to download and confirm to install.



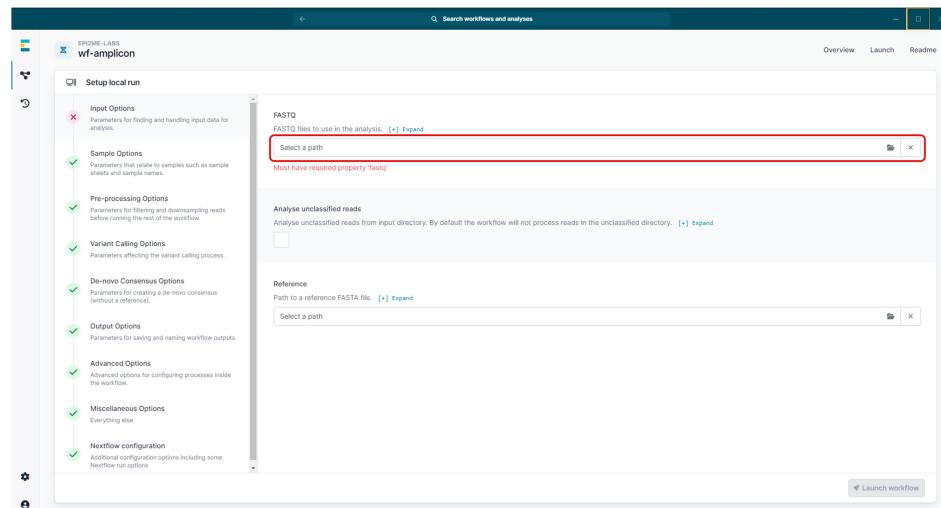
3 Navigate to the Workflows tab and click on wf-amplicon.



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

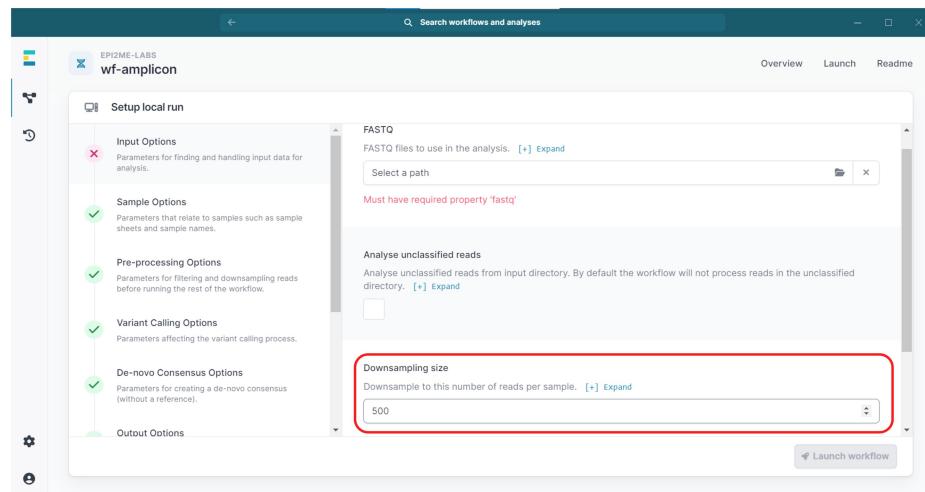


5 Set up your run by selecting your sequencing data in the "Input Options".



6 To speed up the analysis, you can set "Downsampling size" to 500.

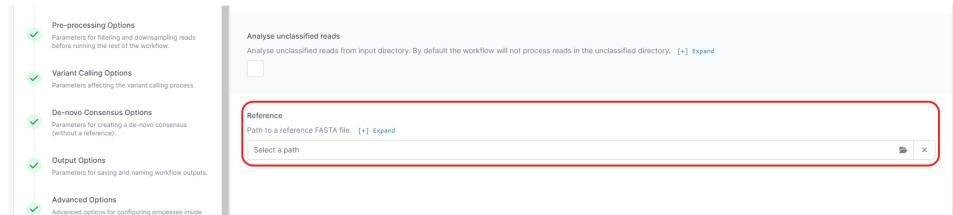
Unless your amplicons are very long (>5 kb), this should provide sufficient coverage.



Optional action

The amplicon workflow can be run with a sequence reference file if required.

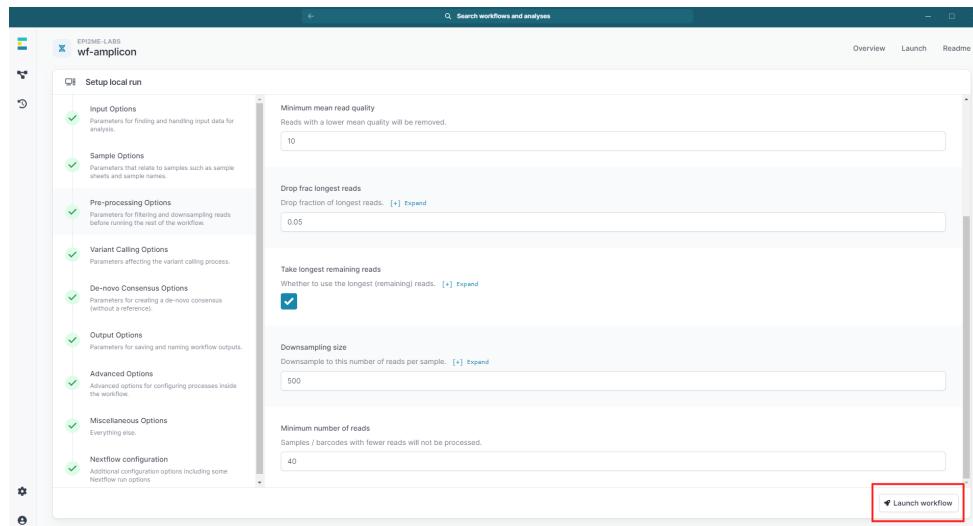
Set up the reference FASTA by uploading the file in the following location:



7 For the remaining parameter options we recommend keeping the default settings.

8 Click "Launch workflow".

Ensure all parameter options have green ticks.



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

Amplicon workflow report

The primary outputs of the workflow include:

- an interactive HTML report with tables and plots detailing the results.
- FASTQ files (one per barcode) with the de-novo consensus sequence and per-base consensus qualities (as calculated by Medaka).
- BAM files (one per barcode) of input reads re-aligned against the consensus.

Example reports:

- When a reference file has been uploaded, reads are aligned to the reference (containing the expected sequence for each amplicon) for variant calling. An example of a **sample variant calling report** can be viewed [here](#).
- When no reference file has been uploaded, the amplicon's consensus sequence is generated de novo. An example of **sample de novo consensus report** can be viewed [here](#).

Report contents

The report consists of several sections. The introduction section gives a brief overview of key results for the individual samples analysed, while the preprocessing section illustrates the number of reads removed during downsampling / filtering. It also contains read length and quality histograms as well as a plot showing base yield vs. read length.

The remaining sections of the report depend on the mode in which the workflow was run. In **variant calling mode**, they summarise the mapping and variant calling stages of the workflow, showing the depth of coverage of aligned reads and providing further details on the variants called. In **de novo consensus mode**, results of the draft consensus QC stage and re-alignment of input reads against the consensus are described. Additionally, a depth of coverage plot of the re-aligned reads along the consensus is shown.

Issues during DNA/RNA extraction and library preparation

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.

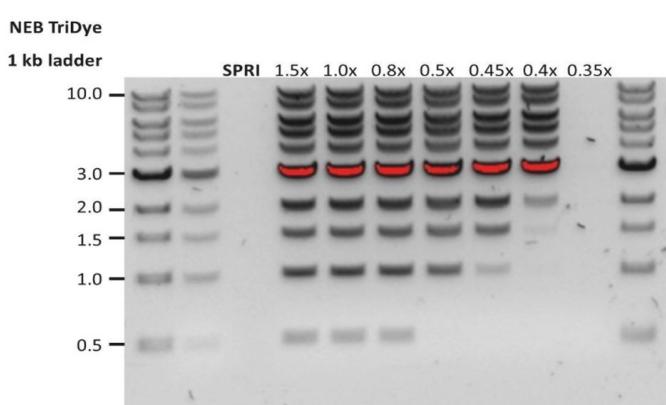
If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via [LiveChat](#) in the Nanopore Community.

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

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

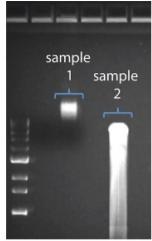
Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support. If you do not have another sequencing device available, we recommend storing the flow 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 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.