Overview of the protocol

IMPORTANT

This is an Early Access product

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

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

Introduction to the Rapid Barcoding Kit V14 kits

This protocol describes how to carry out rapid barcoding of genomic DNA using the Rapid Barcoding Kit 24 and 96 V14 (SQK-RBK114.24 or SQK-RBK114.96). These kits use our most recent Kit 14 chemistry and are optimised for fast library preparation requiring minimal laboratory equipment.

Steps in the sequencing workflow:

Prepare for your experiment

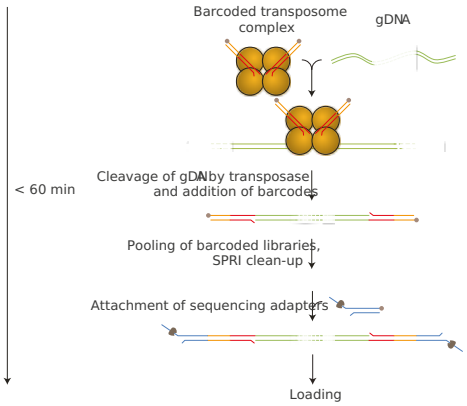
You will need to:

- Extract your DNA, and check its length, quantity and purity using theInput DNA/RNA QC protocol. **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

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

Library preparation step	Process	Time	Stop option
DNA barcoding	Tagmentation of the DNA using the Rapid Barcoding Kit V14	15 minutes	4°C overnight
Sample pooling and clean-up	Pooling of barcoded libraries and AMPure XP Bead clean-up	25 minutes	4°C overnight
Adapter ligation	Attach the sequencing adapters to the DNA ends	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 and convert it into basecalled reads.
- Demultiplex the reads by barcode using MinKNOW, the Guppy software, or the barcoding workflow in EPI2ME.

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	• 200 ng gDNA per sample • Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) OR Rapid Barcoding Kit 96 V14 (SQK-RBK114.96)
Consumables	• MinION and GridION Flow Cell • Qubit dsDNA HS Assay Kit (Invitrogen, Q32851) • (Optional) 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)
- Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, cat # 0030129504) with heat seals
- 2 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- MinION or GridION device
- MinION Flow Cell Light Shield
- Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, 11766427)
- Timer
- Thermal cycler or heat blocks
- Magnetic rack
- Hula mixer (gentle rotator mixer)
- Ice bucket with ice
- Qubit fluorometer (or equivalent for QC check)
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P2 pipette and tips
- Multichannel pipette and tips

Rapid Barcode use requirements

Note: Ensure you are using the correct kit for your desired number of samples:

- Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) contains barcodes RB01-24, allowing you to multiplex up to 24 samples.
- Rapid Barcoding Kit 96 V14 (SQK-RBK114.96) contains barcodes RB01-96, allowing you to multiplex up to 96 samples.

For optimal output, we currently do not recommend using fewer than 4 barcodes. If you wish to multiplex less than 4 samples, please ensure you split your sample(s) across multiple barcodes so at least 4 barcodes are run (e.g. for 2 samples, use RB01-RB02 for sample A and RB03-RB04 for sample B).
Please note that the required sample input for **each barcode** is 200 ng gDNA.

Alternatively, you might want to explore our Rapid Sequencing Kit V14 (SQK-RAD114) for sequencing individual or small sets of samples.

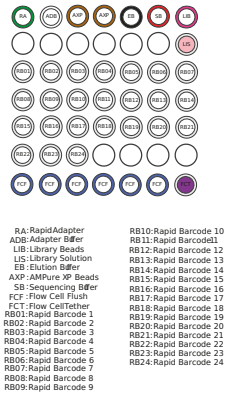
Check your flow cell

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

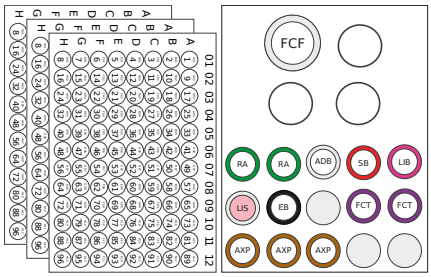
Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) contents



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

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

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



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

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

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

Library preparation

~50 minutes

Materials

- 200 ng gDNA 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) from the Oxford Nanopore sequencing kit

Consumables

- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- Nuclease-free water (e.g. ThermoFisher, AM9937)

- Freshly prepared 80% ethanol in nuclease-free water
- Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, cat # 0030129504) with heat seals
- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes
- 2 ml Eppendorf DNA LoBind tubes
- Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

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

Minimum Rapid Barcode use requirements

For optimal output, we currently do not recommend using fewer than 4 barcodes. If you wish to multiplex less than 4 samples, please ensure you split your sample(s) across barcodes so a minimum of 4 barcodes are run:

- For 1 sample, run your sample across 4 barcodes (e.g. RB01-RB04 using 200ng of sample A per barcode)
- For 2 samples, run each sample across two barcodes. (e.g. RB01-RB02 for sample A and RB03-RB04 for sample B)
- For 3 samples, run two samples individually and one across 2 barcodes. (e.g. RB01 and RB02 for sample A and B respectively, and RB03-RB04 for sample C)

Please note that the required sample input for**each barcode** is 200 ng gDNA.

Alternatively, you might want to explore our Rapid Sequencing Kit V14 (SQK-RAD114) for sequencing individual or small sets of samples.

Check your flow cell.

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

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

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

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

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
Rapid Barcodes (RB01-24) or 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.**

1. Transfer 200 ng of genomic DNA per sample into 0.2 ml thin-walled PCR tubes or an Eppendorf twin.tec® PCR plate 96 LoBind.
2. Adjust the volume of each sample to 10 µl with nuclease-free water.
3. Pipette mix the tubes for 10-15 times to avoid unwanted shearing
4. Spin down briefly in a microfuge

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

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

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

6 **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.**

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

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

	Volume per sample	For 4 samples	For 12 samples	For 24 samples	For 48 samples	For 96 samples
Total volume	11.5 µl	46 µl	138 µl	276 µl	552 µl	1,104 µl

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

IMPORTANT

Ensure you have sufficient capacity in your reaction tube for all the reagents.

Limit the volume taken forward of pooled barcoded sample to 1,000 µl (i.e. half the capacity of the 2 ml Eppendorf DNA LoBind tube) to ensure feasibility of the next step.

10 **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 4 samples	For 12 samples	For 24 samples	For 48 samples	For 96 samples
Volume of AMPure XP Beads (AXP) added	11.5 µl	46 µl	138 µl	276 µl	552 µl	1,000 µl

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

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

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

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

15 **Repeat the previous step.**

16 **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.**

17 **Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB) per 24 barcodes used.**

	For 24 barcodes	For 48 barcodes	For 96 barcodes
Volume of Elution Buffer (EB)	15 µl	30 µl	60 µl

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

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

20 Remove and retain the full volume of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

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

Quantify 1 µl of eluted sample using a Qubit fluorometer.

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

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

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

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

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

25 Incubate the reaction for 5 minutes at room temperature.

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

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

~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
- (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)

- 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- MinION or GridION device
- MinION 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 Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.

IMPORTANT

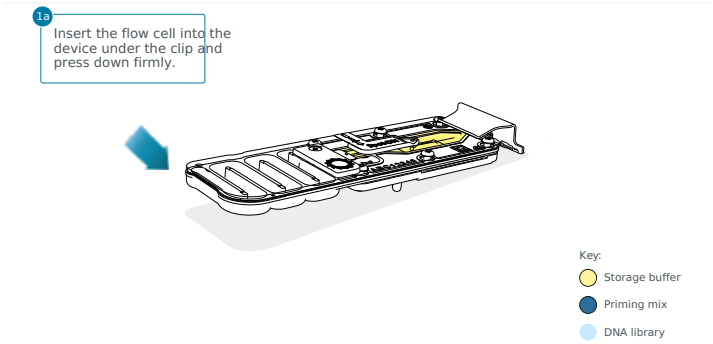
For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), we recommend adding Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.

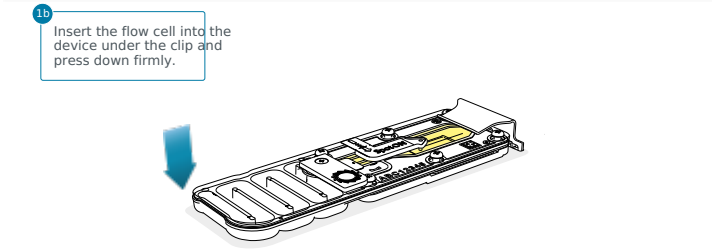
Note: We do not recommend using any other albumin type (e.g. recombinant human serum albumin).

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

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

3 Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the 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.

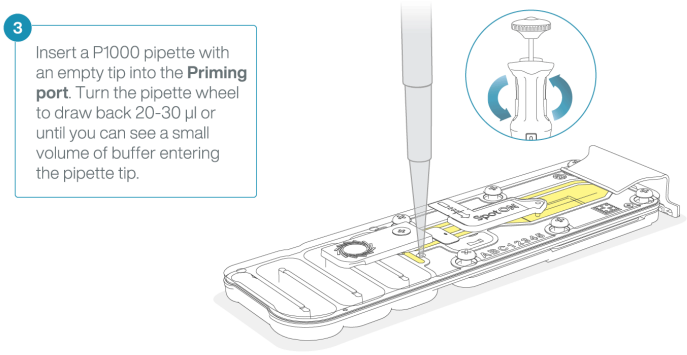
4 Slide the flow cell priming port cover clockwise to open the priming port.

IMPORTANT

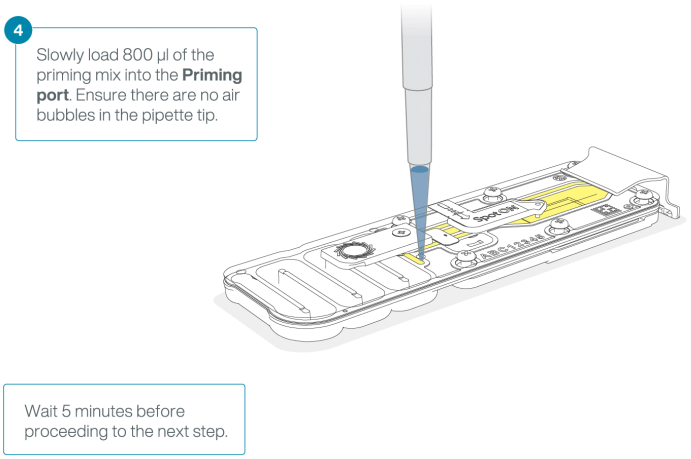
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

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 µl
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 ul, to draw back 20-30 ul, 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.

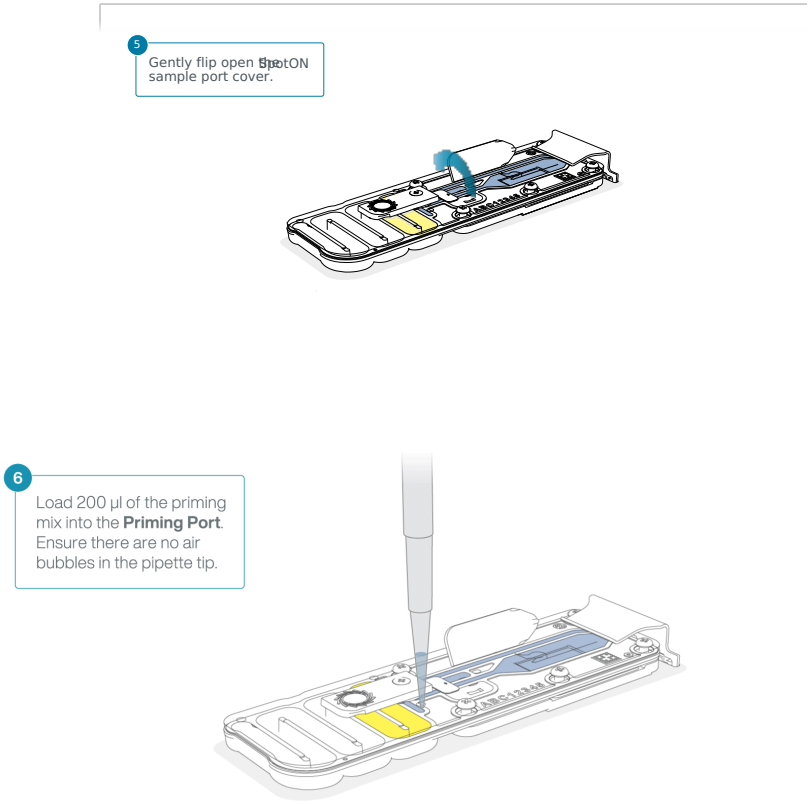
We recommend using the Library Beads (LIB) for most sequencing experiments. However, the Library Solution (LIS) is available for more viscous libraries.

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**

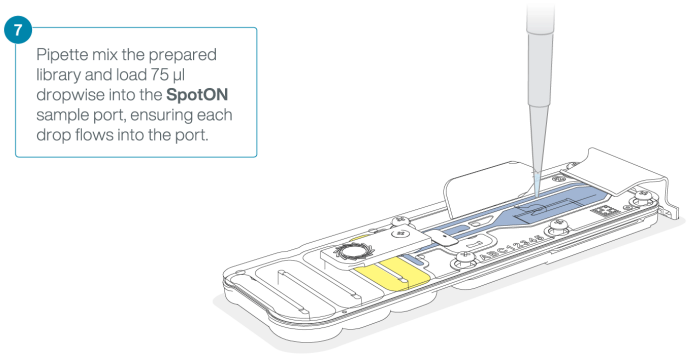
9 Complete the flow cell priming:

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.

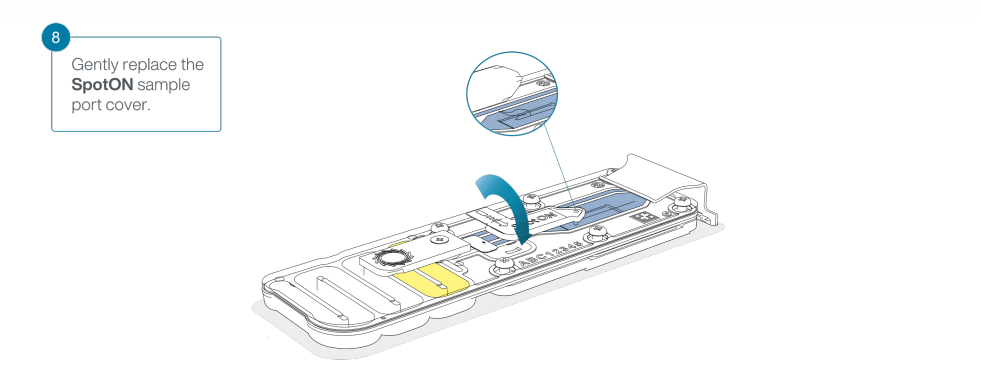


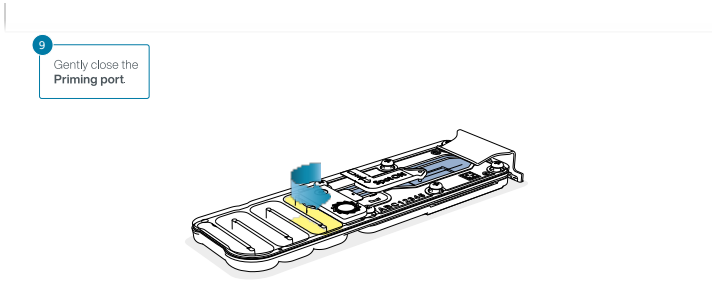
10 Mix the prepared library gently by pipetting up and down just prior to loading.

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.**





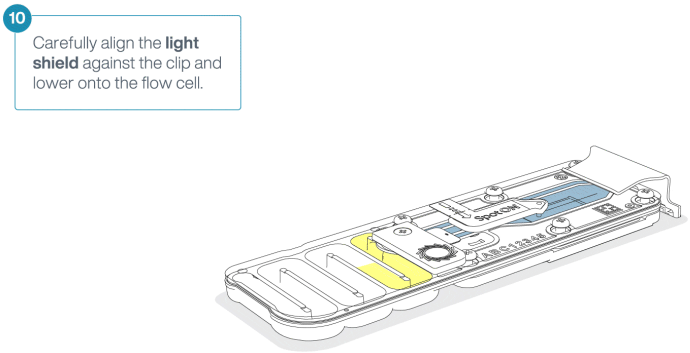
IMPORTANT

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

Overview of nanopore data analysis

For a full overview of nanopore data analysis, which includes options for basecalling and post-basecalling analysis, please refer to the Data Analysis document.

How to start sequencing

The sequencing device control, data acquisition and real-time basecalling are carried out by the MinKNOW software. It is assumed you have already installed MinKNOW on your computer. There are multiple options for how to carry out sequencing:

1. Data acquisition and basecalling in real-time using MinKNOW on a computer

Follow the instructions in the MinKNOW protocol beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section.

2. Data acquisition and basecalling in real-time using the GridION device

Follow the instructions in the GridION user manual.

3. Data acquisition and basecalling in real-time using the MinION Mk1C device

Follow the instructions in the MinION Mk1C user manual.

4. Data acquisition and basecalling in real-time using the PromethION device

Follow the instructions in the PromethION user manual or the PromethION 2 Solo user manual.

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

Follow the instructions in the MinKNOW protocol beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section. **When setting your experiment parameters, set the *Basecalling* tab to OFF.** After the sequencing experiment has completed, follow the instructions in the Post-run analysis section of the MinKNOW protocol.

Downstream analysis

Post-basecalling analysis

There are several options for further analysing your basecalled data:

1. EPI2ME workflows

For in-depth data analysis, Oxford Nanopore Technologies offers a range of bioinformatics tutorials and workflows available in EPI2ME Labs, which are available in the EPI2ME Labs section of the Community. The platform provides a vehicle where workflows deposited in GitHub by our Research and Applications teams can be showcased with descriptive texts, functional bioinformatics code and example data.

2. Research analysis tools

Oxford Nanopore Technologies' Research division has created a number of analysis tools, that are available in the Oxford Nanopore GitHub repository. The tools are aimed at advanced users, and contain instructions for how to install and run the software. They are provided as-is, with minimal support.

3. Community-developed analysis tools

If a data analysis method for your research question is not provided in any of the resources above, please refer to the Bioinformatics section of the Resource centre. Numerous members of the Nanopore Community have developed their own tools and pipelines for analysing nanopore sequencing data, most of which are available on GitHub. Please be aware that these tools are not supported by Oxford Nanopore Technologies, and are not guaranteed to be compatible with the latest chemistry/software configuration.

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.

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

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

Instructions for returning flow cells can be foundhere.

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.

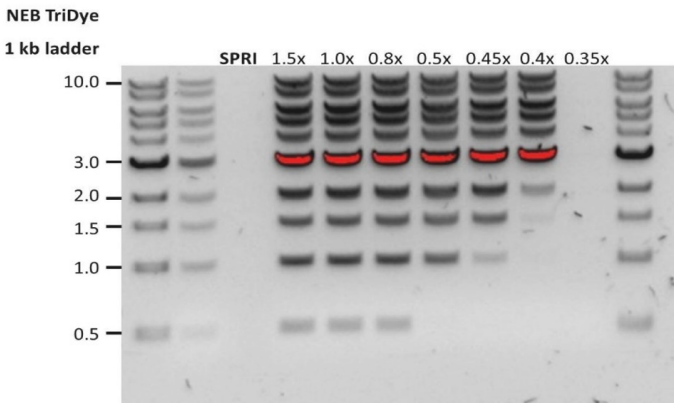
We also have an FAQ section available on theNanopore Community Support section.

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

Issues during the sequencing run using a Rapid-

based sequencing kit

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

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

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

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

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

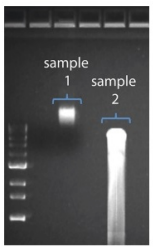
MinKNOW script failed

Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support. If you do not have another sequencing device available, we recommend storing the flow cell and the loaded library at 4°C and contact Technical Support for further storage guidance.

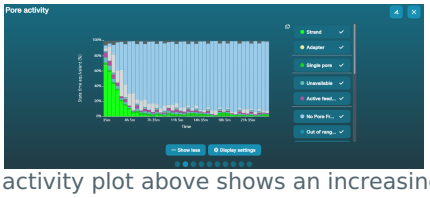
Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	Ensure the correct concentration of good quality library is loaded on to a MinION Mk1B/GridION flow cell. To check the concentration, please refer to the library preparation protocol. Please quantify the library before loading and calculate mols using tools like the Promega Biomath Calculator, choosing "dsDNA: µg to pmol"
Pore occupancy close to 0	The Rapid Sequencing Kit V14/Rapid Barcoding Kit V14 was used, and sequencing adapters did not attach to the DNA	Make sure to closely follow the protocol and use the correct volumes and incubation temperatures. A Lambda control library can be prepared to test the integrity of reagents.
Pore occupancy close to 0	No tether on the flow cell	Tethers are adding during flow cell priming (FCT tube). Make sure FCT was added to FCF before priming.

Shorter than expected read length

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

Large proportion of unavailable pores

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

Large proportion of inactive pores

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

Temperature fluctuation

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

Failed to reach target temperature

Observation	Possible cause	Comments and actions
MinKNOW shows "Failed to reach target temperature"	The instrument was placed in a location that is colder than normal room temperature, or a location with poor ventilation (which leads to the flow cells overheating)	MinKNOW has a default timeframe for the flow cell to reach the target temperature. Once the timeframe is exceeded, an error message will appear and the sequencing experiment will continue. However, sequencing at an incorrect temperature may lead to a decrease in throughput and lower q-scores. Please adjust the location of the sequencing device to ensure that it is placed at room temperature with good ventilation, then re-start the process in MinKNOW. Please refer to this FAQ for more information on MinION Mk 1B temperature control.