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 cDNA-PCR Barcoding Kit 24 V14 protocol

This protocol describes how to carry out sequencing of multiple cDNA samples using a strand-switching method and the cDNA-PCR Barcoding Kit 24 V14 (SQK-PCB114.24). There are 24 unique barcodes available, allowing the user to pool up to 24 different samples in one sequencing experiment. During the strand-switching step, a UMI is incorporated, before the double-stranded cDNA is amplified by PCR using primers containing 5' tags. The amplified and barcoded samples are then pooled together and the Rapid Sequencing Adapters are added to the pooled mix.

A control experiment can be completed first using RNA Control Sample (RCS) from the RNA Control Expansion (EXP-RCS001) as your input to troubleshoot your library preparation or to become familiar with the protocol.

Steps in the sequencing workflow:

Prepare for your experiment

You will need to:

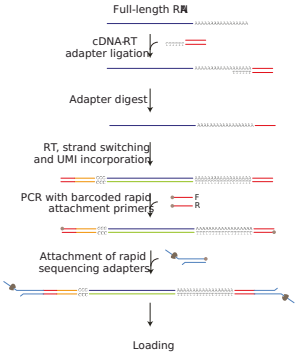
- Extract your RNA, and check its length, quantity and purity using theInput DNA/RNA QC protocol. **The quality checks performed during the protocol are essential in ensuring experimental success**
- Ensure you have your sequencing kit, the correct equipment and third-party reagents
- Download the software 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
Reverse transcription and strand-switching	Prepare full-length cDNA from Poly(A)+ RNA (or total RNA) with the incorporation of the UMI	170 minutes	-20°C overnight
Selecting for full-length transcripts by PCR	Amplify the cDNA by PCR using rapid attachment barcode primers during the PCR step	40 minutes	4°C short-term storage or for repeated use, such as re-loading your flow cell. -80°C for single-use long-term storage.
Adapter ligation	Attach the sequencing adapters to the to the PCR products.	5 minutes	We strongly recommend sequencing your library as soon as it is adapted.

Library preparation step	Process	Time	Stop option
Priming and loading the flow cell	Prime the flow cell and load the prepared cDNA 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
- ***Optional:*** Start the EPI2ME software and select a workflow for further analysis

IMPORTANT

Compatibilities of the protocol

This protocol should only be used in combination with:

- cDNA-PCR Barcoding Kit 24 V14 (SQK-PCB114.24)
- R10.4.1 flow cells (FLO-MIN114)
- Flow Cell Wash Kit (EXP-WSH004)
- RNA Control Expansion (EXP-RCS001)
- Rapid Adapter Auxiliary V14 (EXP-RAA114)
- Sequencing Auxiliary Vials V14 (EXP-AUX003)
- Flow Cell Priming Kit V14 (EXP-FLP004)
- MinION Mk1C - MinION Mk1C IT requirements document
- MinION Mk1B - MinION IT Requirements document

Equipment and consumables

Materials

- 10 ng enriched RNA (Poly(A)+ RNA or ribodepleted) or 500 ng total RNA per sample
- cDNA-PCR Barcoding Kit 24 V14 (SQK-PCB114.24)

Consumables

- MinION and GridION Flow Cell
- NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)
- T4 DNA Ligase 2M U/ml (NEB, cat # M0202T/M)
- RNaseOUT™, 40 U/µl (Life Technologies, cat # 10777019)
- Lambda Exonuclease (NEB, Cat # M0262L)
- Thermolabile Exonuclease I (NEB, cat # M0568)
- USER (Uracil-Specific Excision Reagent) Enzyme (NEB, cat # M5505L)
- 10 mM dNTP solution (e.g. NEB N0447)
- Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher, cat # EP0751)
- LongAmp Hot Start Taq 2X Master Mix (NEB, M0533S)
- Agencourt RNAClean XP beads (Beckman Coulter™, cat # A63987)
- Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
- Qubit dsDNA HS Assay Kit (ThermoFisher, cat # Q32851)
- Qubit RNA HS Assay Kit (ThermoFisher, cat # Q32852)
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- Freshly prepared 70% ethanol in nuclease-free water
- 1.5 ml Eppendorf DNA LoBind tubes
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- 0.2 ml thin-walled PCR tubes

Equipment

- MinION or GridION device
- MinION Flow Cell Light Shield
- Hula mixer (gentle rotator mixer)
- Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- Microfuge
- Vortex mixer
- Thermal cycler
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips
- Ice bucket with ice
- Timer
- Qubit fluorometer (or equivalent for QC check)
- Agilent Bioanalyzer (or equivalent)

For this protocol, you will need 10 ng enriched RNA (Poly(A)+ RNA or ribodepleted) or 500 ng total RNA per sample.

Third-party reagents

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

Oxford Nanopore Technologies.

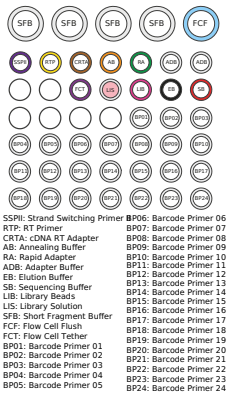
For all third-party reagents, we recommend following the manufacturer's instructions to prepare the reagents for use.

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

cDNA-PCR Barcoding Kit 24 V14 (SQK-PCB114.24) contents



Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Strand Switching Primer II	SSPII	Violet	1	350
RT Primer	RTP	Yellow	1	200
cDNA RT Adapter	CRTA	Amber	1	200
Annealing Buffer	AB	Orange	1	200
Rapid Adapter	RA	Green	1	15
Adapter Buffer	ADB	Clear	1	100
Elution Buffer	EB	Black	2	500
Short Fragment Buffer	SFB	Clear	4	7,500
Sequencing Buffer	SB	Red	1	700

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Library Beads	LIB	Pink	1	600
Library Solution	LIS	White cap, pink label	1	600
Barcode Primers 1-24	BP01-24	White	24	10
Flow Cell Tether	FCT	Purple	1	200
Flow Cell Flush	FCF	Clear cap, light blue label	1	8,000

Reverse transcription and strand-switching

~170 minutes

Materials

- 10 ng enriched RNA (Poly(A)+ RNA or ribodepleted) or 500 ng total RNA per sample
- cDNA RT Adapter (CRTA)
- Annealing Buffer (AB)
- Short Fragment Buffer (SFB)
- RT Primer (RTP)
- Strand Switching Primer II (SSPII)

Consumables

- NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)
- T4 DNA Ligase 2M U/ml (NEB, cat # M0202T/M)
- Lambda Exonuclease (NEB, Cat # M0262L)
- USER (Uracil-Specific Excision Reagent) Enzyme (NEB, cat # M5505L)
- Agencourt RNAClean XP beads (Beckman Coulter™, cat # A63987)
- 10 mM dNTP solution (e.g. NEB cat # N0447)
- Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher, cat # EP0751)
- RNaseOUT™, 40 U/µl (Life Technologies, cat # 10777019)
- Qubit RNA HS Assay Kit (ThermoFisher, cat # Q32852)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- 1.5 ml Eppendorf DNA LoBind tubes
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- 0.2 ml thin-walled PCR tubes

Equipment

- Microfuge
- Thermal cycler
- 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

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.

> **TIP**
>
> **Preparing the laboratory for handling RNA samples:**
>
> For optimal results, we recommend preparing your laboratory space and equipment prior to handling RNA to ensure the presence of RNAse and contaminants is minimal:
>
> - Clean the lab bench space where you will carry out the work with RNaZap and tech wipes.
> - Clean all equipment such as pippettes, tube racks, centrifuge and vortex with RNaZap and tech wipes.
> - Use fresh tip boxes and reagents to minimise risk of contamination.

1 Thaw the following reagents, then spin down briefly using a microfuge and mix as indicated in the table below. Then place the reagents on ice.

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
cDNA RT Adapter (CRTA)	✓	✓	✓
Annealing Buffer (AB)	✓	✓	✓
Short Fragment Buffer (SFB)	✓	✓	✓
RT Primer (RTP)	✓	✓	✓
Strand Switching Primer II (SSPII)	✓	✓	✓
NEBNext® Quick Ligation Reaction Buffer	✓	✓	Mix by vortexing
T4 DNA Ligase 2M U/ml	Not frozen	✓	✓
RNaseOUT	Not frozen	✓	✓
Lambda Exonuclease	Not frozen	✓	✓
Uracil-Specific Excision Reagent (USER)	Not frozen	✓	✓
10 mM dNTP solution	✓	✓	✓
Maxima H Minus Reverse Transcriptase	Not frozen	✓	✓
Maxima H Minus 5x RT Buffer	✓	✓	Mix by vortexing

IMPORTANT

It is important that the NEBNext Quick Ligation Reaction Buffer is mixed well by vortexing.

Check for any visible precipitate; vortexing for at least 30 seconds may be required to solubilise all precipitate.

Optional action
To run a control experiment, replace your sample input with 10 µl diluted RNA Control Sample (RCS) from the RNA Control Expansion (EXP-RCS001) as follows:

- Thaw the RNA Control Sample (RCS) at room temperature, briefly spin down and mix well by pipetting.
- Dilute the RNA Control Sample (RCS) in a 1.5 ml Eppendorf DNA LoBind tube as follows:

Reagent	Volume
RNA Control Sample (RCS)	1 µl
Nuclease-free water	14 µl
Total	**15 µl**

Note: This will provide enough volume for 1 sample, adjust your volumes accordingly for the number of samples you wish to run in your control experiment.

- Mix thoroughly by pipetting 10-20 times and briefly spin down.
- Use the **10 µl** of diluted RNA Control Sample (RCS) as your RNA input.

2 For each sample, prepare the RNA in nuclease-free water:

- Transfer 10 ng Poly(A)+ RNA, or 500 ng total RNA into a 0.2 ml thin-walled PCR tube
- Adjust the volume to up to 10 µl with nuclease-free water
- Mix by flicking the tube to avoid unwanted shearing
- Spin down briefly in a microfuge

3 Prepare the following in a 0.2 ml PCR tube per sample:

Reagent	Volume
RNA	10 µl
cDNA RT Adapter (CRTA)	1 µl
Annealing Buffer (AB)	1 µl
Total volume	**12 µl**

TIP

The cDNA RT Adapter (CRTA) is a double stranded adapter with a poly(T) overhang which anneals to the very end of the poly(A) tail of the RNA strand. This ensures that the full length of the RNA is reverse transcribed and that the poly(A) length can be estimated accurately. Annealing Buffer (AB) has been included to improve CRTA ligation.

4 Mix gently by flicking the tubes, and spin down.

5 Incubate the reactions in the thermal cycler at 60°C for 5 mins, then cool for 5 minutes at room temperature.

6 To each of the 0.2 ml PCR tubes containing you RNA sample(s), add the following:

Reaction	Volume
RNA sample (from previous step)	12 µl
NEBNext® Quick Ligation Reaction Buffer	3.6 µl
T4 DNA Ligase 2M U/ml	1.4 µl
RNaseOUT	1 µl
Total volume (including all reagents)	**18 µl**

7 Ensure the components are thoroughly mixed by pipetting the contents of the tubes 10 times and spin down.

Note: Mix gently to minimise introducing air bubbles to the reactions.

8 Incubate for 10 minutes at room temperature.

9 To each of the 0.2 ml PCR tubes, add the following:

Reagent	Volume
RNA sample (from previous step)	18 µl
Lambda Exonuclease	1 µl
USER (Uracil-Specific Excision Reagent)	1 µl
Total volume (including all reagents)	**20 µl**

TIP

The Lambda Exonuclease and Uracil-Specific Excision Reagent (USER) are third-party reagents used in the preparation of the reverse transcription step. Lambda Exonuclease and USER digest the bottom strand of the ligated CRTA so that the RT Primer (RTP) can bind the CRTA sequence as a primer for the reverse transcription of the RNA.

10 Ensure the components are thoroughly mixed by flicking the tubes and spin down.

11 **Incubate for 5 minutes at 37°C in the thermal cycler.**

12 **Transfer each sample to clean 1.5 ml Eppendorf DNA LoBind tubes.**

13 **Resuspend the RNase-free XP beads by vortexing.**

14 **Add 36 µl of resuspended RNase-free XP beads to each reaction and mix gently by flicking the tubes.**

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

16 **Spin down the samples and pellet on a magnet. Keep the tubes on the magnet, and pipette off the supernatant.**

17 **Keep the tubes on the magnet and wash the beads with 100 µl of Short Fragment Buffer (SFB) as follows:**

1. Wash the beads with 100 µl of Short Fragment Buffer (SFB).
2. Keeping the magnetic rack on the benchtop, rotate the tube by 180°. Wait for the beads to migrate towards the magnet and to form a pellet.
3. Rotate the tube 180° again (back to the starting position), and wait for the beads to pellet again.
4. Without disturbing the pellet, remove the Short Fragment Buffer (SFB) using a pipette and discard.

18 **Repeat the previous step.**

19 **Spin down and place the tubes back on the magnet. Pipette off any residual buffer. Briefly allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.**

20 **Remove the tubes from the magnetic rack and resuspend each pellet in 12 µl of nuclease-free water.**

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

22 **Pellet the beads on a magnet until the eluate is clear and colourless.**

23 **Remove and retain 12 µl of eluate into a clean 0.2 ml thin-walled PCR tube per sample.**

24 To each of the 0.2 ml PCR tubes, add the following:

Reagents	Volume
Eluted sample (from previous step)	12 µl
RT Primer (RTP)	1 µl
dNTPs (10 mM)	1 µl
Total volume (including all reagents)	**14 µl**

TIP

RT Primer (RTP) is a single stranded primer and binds upstream of the poly(A) tail of the RNA transcript to prime for reverse transcription.

25 Ensure the components are thoroughly mixed by flicking the tubes and spin down.

26 Incubate the reaction for 5 minutes at room temperature.

27 To each of the 0.2 ml PCR tubes, add the following:

Reagents	Volume
RT primed sample (from previous step)	14 µl
Maxima H Minus 5x RT Buffer	4.5 µl
RNaseOUT	1 µl
Strand Switching Primer II (SSPII)	2 µl
Total (including all reagents)	**21.5 µl**

TIP

Strand Switching Primer II (SSPII) base pairs to the deoxycytidine present at the 5' end of the first cDNA strand synthesised. This allows the reverse transcriptase to "strand-switch" for synthesis of the second cDNA strand.

28 Mix gently by flicking the tubes, and spin down.

29 Incubate at 42°C for 2 minutes in the thermal cycler.

30 Add 1 µl of Maxima H Minus Reverse Transcriptase to each tube. The total volume will be 22.5 µl per tube.

31 Mix gently by flicking the tubes, and spin down.

32 Incubate using the following protocol using a thermal cycler:

Cycle step	Temperature	Time	No. of cycles
Reverse transcription and strand-switching	42°C	30 mins	1
Heat inactivation	85°C	5 mins	1
Hold	4°C	∞	

END OF STEP

Take your samples forward into the next step. However, at this point it is also possible to store the sample at -20°C overnight.

Selecting for full-length transcripts by PCR

~40 minutes

Materials

- Barcode Primers (BP01-24)
- Elution Buffer (EB)

Consumables

- LongAmp Hot Start Taq 2X Master Mix (NEB, M0533S)
- Thermolabile Exonuclease I (NEB, cat # M0568)
- Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- Freshly prepared 70% ethanol in nuclease-free water
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- 1.5 ml Eppendorf DNA LoBind tubes
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- 0.2 ml PCR tubes

Equipment

- Thermal cycler
- Vortex mixer
- Hula mixer (gentle rotator mixer)
- Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- Ice bucket with ice
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips
- Qubit fluorometer (or equivalent for QC check)
- Agilent Bioanalyzer (or equivalent)

IMPORTANT

This kit enables multiplexing of up to 24 samples. The default method allows you to perform one 25 µl PCR reaction per sample. If multiplexing two or three samples, however, two separate PCR reactions per sample should be performed; if running just one sample, four separate PCR reactions should be performed as per the PCR-cDNA Sequencing Kit V14 (SQK-PCS114) protocol. These recommendations aim to ensure that enough PCR product is generated for optimal flow cell performance.

Reverse transcriptase is a PCR inhibitor and the reverse-transcribed sample must be diluted enough for PCR to take place.

Note: Use one set of Barcode Primers per sample.

1 **Thaw the following reagents, then spin down briefly using a microfuge and mix as indicated in the table below. Then place the reagents on ice.**

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
Barcode Primers (BP01 - BP24)	✓	✓	✓
Elution Buffer (EB)	✓	✓	✓
LongAmp Hot Start Taq 2X Master Mix	✓	✓	✓
Thermolabile Exonuclease I	Not frozen	✓	✓

2 **Spin down the reverse-transcribed RNA samples.**

3 **Prepare a separate 0.2 ml PCR tube for each sample and add 5 µl of reverse-transcribed RNA per tube.**

IMPORTANT

Only 5 µl of the reverse-transcribed sample is to be taken forward. Do NOT use all the 22.5 µl of the reverse transcription reaction in a single PCR reaction.

4 **In each of the 0.2 ml PCR tubes containing reverse-transcribed RNA sample, prepare the following reaction at room temperature:**

Reagent	Volume
Reverse-transcribed sample (from previous step)	5 µl
Unique Barcode Primer (BP01-24)	0.75 µl
Nuclease-free water	6.75 µl
2x LongAmp Hot Start Taq Master Mix	12.5 µl
Total (including all reagents)	**25 µl**

5 **Mix gently by pipetting.**

6 **Amplify using the following cycling conditions.**

Cycle step	Temperature	Time	No. of cycles
Initial denaturation	95°C	30 secs	1
Denaturation	95°C	15 secs	10-18*
Annealing	62°C	15 secs	10-18*
Extension	65°C	60 secs per kb	10-18*
Final extension	65°C	6 mins	1
Hold	4°C	∞	

*We recommend 14 cycles as a starting point. However, the number of cycles can be adjusted between the values shown according to experimental needs.

For further information, please read The effect of varying the number of PCR cycles in the PCR-cDNA Sequencing Kit document.

7 **Add 1 µl Thermolabile Exonuclease I directly to each PCR tube. Mix by flicking the tube and briefly spin down.**

TIP

The Thermolabile Exonuclease I is added to remove any excess primers which have not successfully annealed.

8 **Incubate the reaction at 37°C for 5 minutes, followed by 80°C for 2 minutes in the thermal cycler.**

9 **Transfer each sample to a clean 1.5 ml Eppendorf DNA LoBind tube.**

10 **Resuspend the AMPure XP beads by vortexing.**

11 **Add 18 µl of resuspended AMPure XP beads to each 1.5 ml Eppendorf DNA LoBind tube.**

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

13 **Prepare 5 ml of fresh 70% ethanol in nuclease-free water.**

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

15 **Keep the tubes on the magnet and wash the beads with 100 µl of freshly-prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

16 **Repeat the previous step.**

17 **Spin down and place the tubes back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellets to the point of cracking.**

18 **Remove the tubes from the magnetic rack and resuspend each pellet in 12 µl of Elution Buffer (EB).**

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

20 **Pellet the beads on the magnet until the eluate is clear and colourless.**

21 **Remove and retain 12 µl of each eluate into a separate clean 1.5 ml Eppendorf DNA LoBind tube.**

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

22 **For each sample, analyse 1 µl of the amplified cDNA for size, quantity and quality using a Qubit fluorometer and Agilent Bioanalyzer (or equivalent) for a QC check.**

IMPORTANT

Sometimes a high-molecular weight product is visible in the wells of the gel when the PCR products are run, instead of the expected smear. These libraries are typically associated with poor sequencing performance. We have found that repeating the PCR with fewer cycles can remedy this.

23 Pool together equimolar samples of the amplified cDNA barcoded samples to a total of 50 fmols and make the volume up to 11 µl in Elution Buffer (EB).

Mass	Molarity if fragment length = 0.5 kb	Molarity if fragment length = 1.5 kb	Molarity if fragment length = 3 kb
5 ng	16 fmol	5 fmol	3 fmol
10 ng	32 fmol	11 fmol	5 fmol
15 ng	49 fmol	16 fmol	8 fmol
20 ng	65 fmol	22 fmol	11 fmol
25 ng	81 fmol	27 fmol	13 fmol
50 ng	154 fmol	51 fmol	26 fmol
100 ng	324 fmol	108 fmol	54 fmol

If the quantity of amplified cDNA is above 50 fmol, the remaining cDNA can be frozen and stored for another sequencing experiment (in this case, library preparation would start from the Adapter Addition step). We recommend avoiding multiple freeze-thaw cycles to prevent DNA degradation.

TIP

Library storage recommendations

We recommend storing libraries in Eppendorf DNA LoBind tubes at**-20°C for short term storage** or repeated use, for example, re-loading flow cells between washes.
For single use and **long term storage** of more than 3 months, we recommend storing libraries at**-80°C** in Eppendorf DNA LoBind tubes.

Adapter addition

~5 minutes

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

Consumables
- 1.5 ml Eppendorf DNA LoBind tubes

Equipment
- Microfuge
- Ice bucket with ice
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

- P2 pipette and tips

IMPORTANT

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

Thaw the 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 Adapter (RA)	Not frozen	✓	✓
Adapter Buffer (ADB)	Not frozen	✓	✓

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

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

2 Add 1 µl of the diluted Rapid Adapter (RA) to the amplified cDNA library, making the total volume 12 µl.

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

4 Incubate the reaction for 5 minutes at room temperature.

5 Spin down briefly.

END OF STEP

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

TIP

Library storage recommendations

We recommend storing libraries in Eppendorf DNA LoBind tubes at**4°C for short-term** storage or repeated use, for example, re-loading flow cells between washes.
For single use and **long-term storage** of more than 3 months, we recommend storing libraries at**-80°C** in Eppendorf DNA LoBind tubes.

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

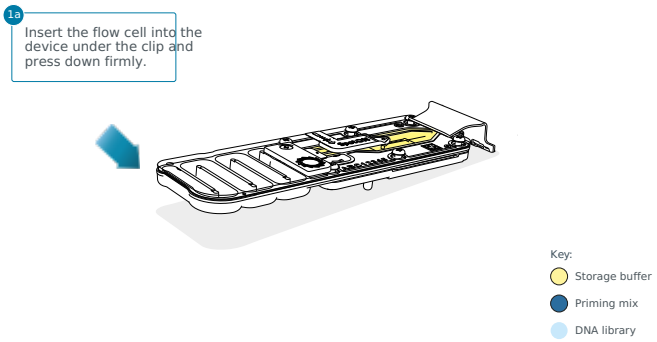
For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), we recommend adding Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.

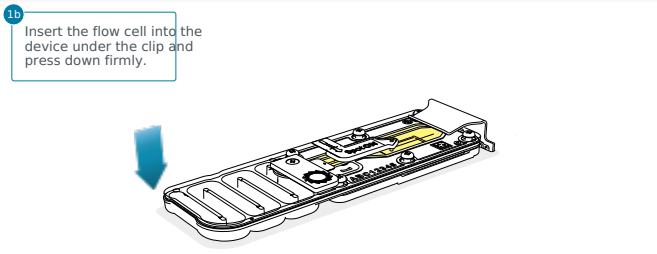
Note: We do not recommend using any other albumin type (e.g. recombinant human serum albumin).

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.

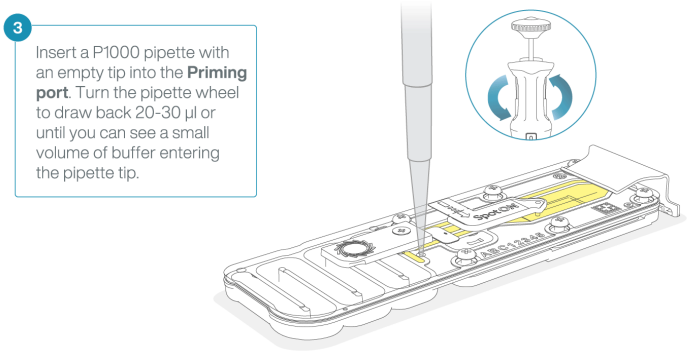
4 Slide the flow cell priming port cover clockwise to open the priming port.

IMPORTANT

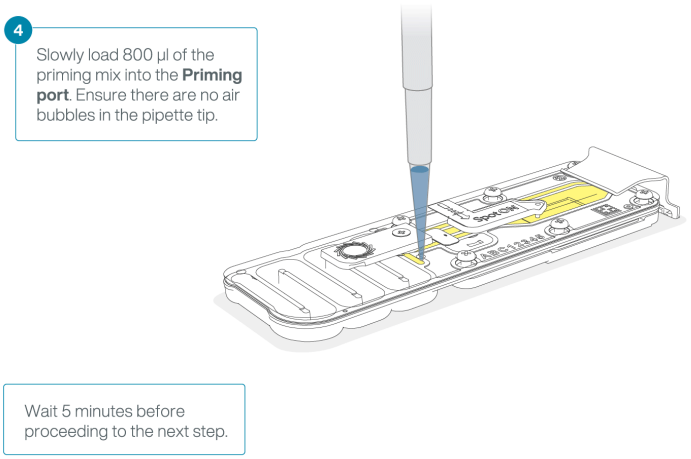
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

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.

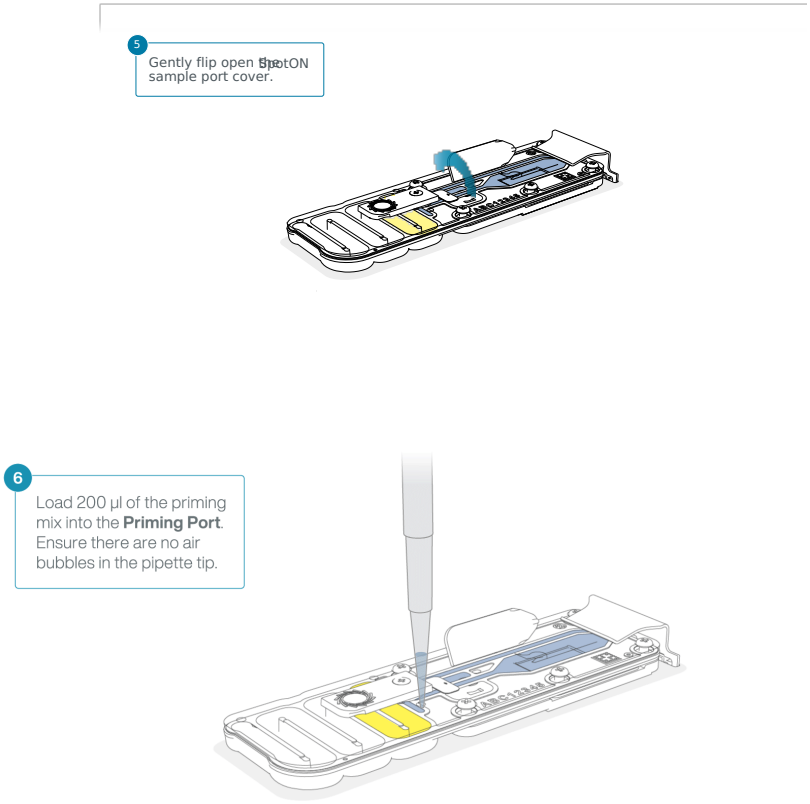
We recommend using the Library Beads (LIB) for most sequencing experiments. However, the Library Solution (LIS) is available for more viscous libraries.

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**

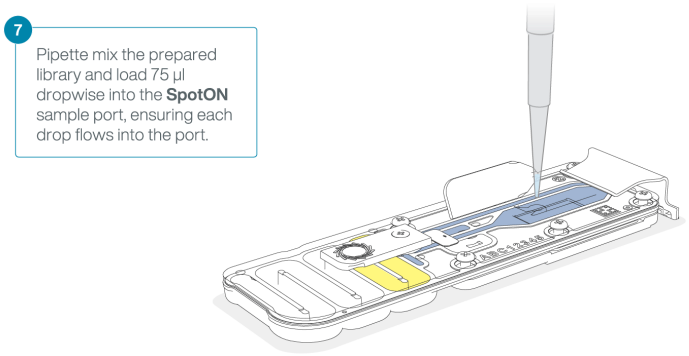
9 Complete the flow cell priming:

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.

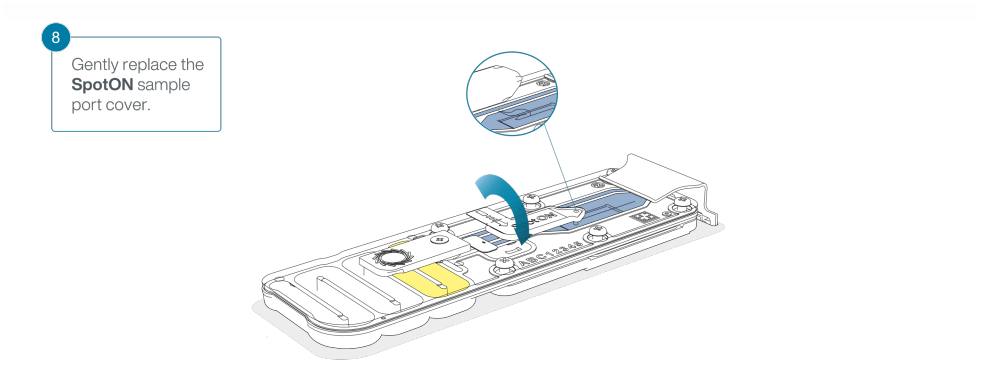


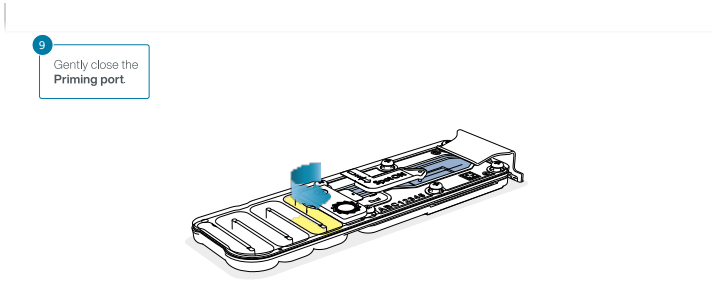
10 Mix the prepared library gently by pipetting up and down just prior to loading.

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.**





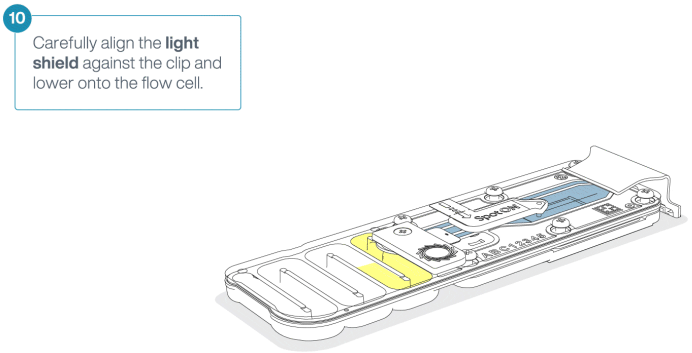
IMPORTANT

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

How to start sequencing

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

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

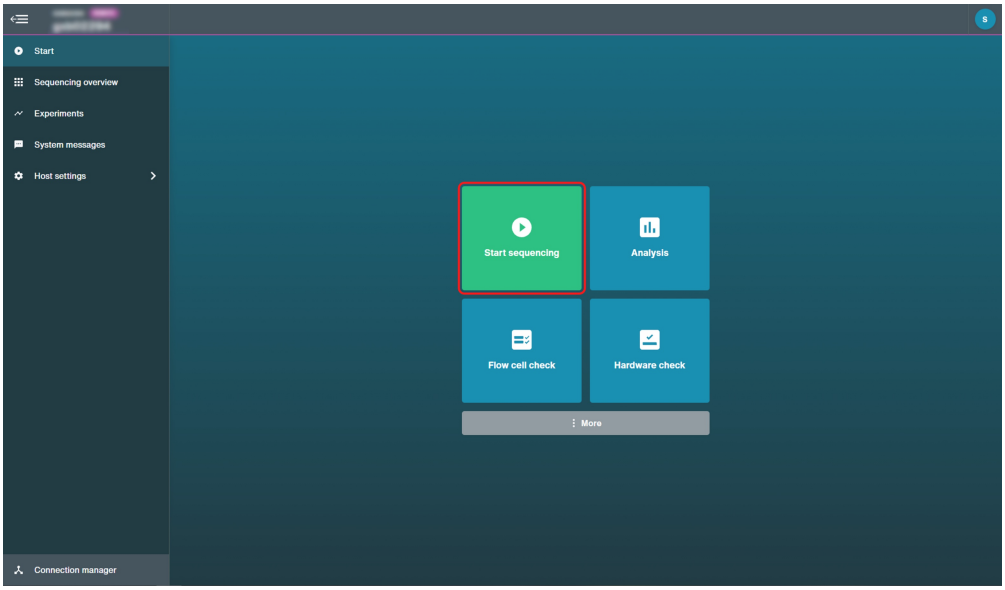
- On a computer either directly or remotely connected to a sequencing device.
- Directly on a GridION, MinION Mk1C or PromethION 24/48 sequencing device.

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

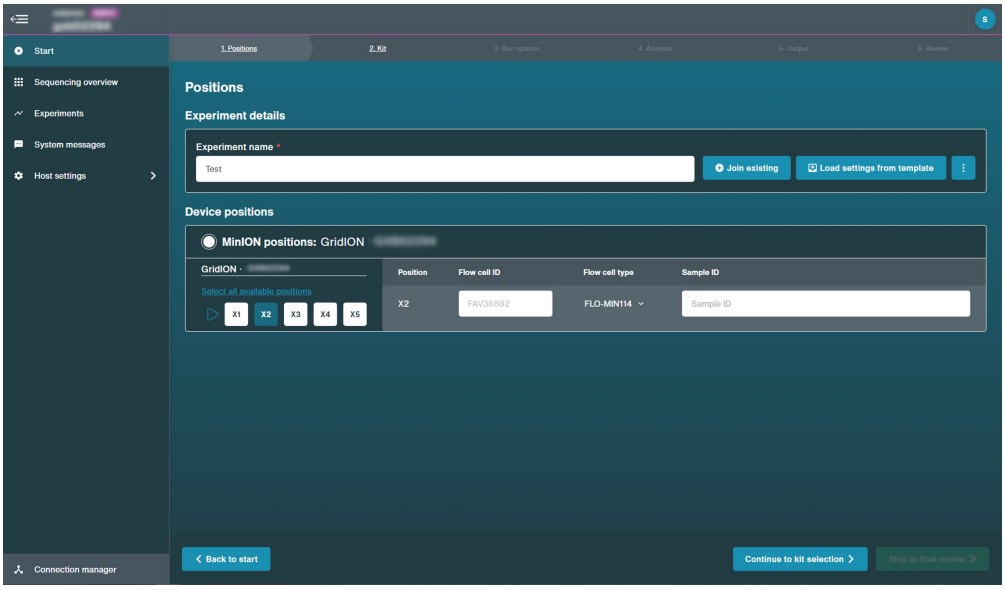
- MinION Mk1B user manual
- MinION Mk1C user manual
- GridION user manual

To start a sequencing run on MinKNOW:

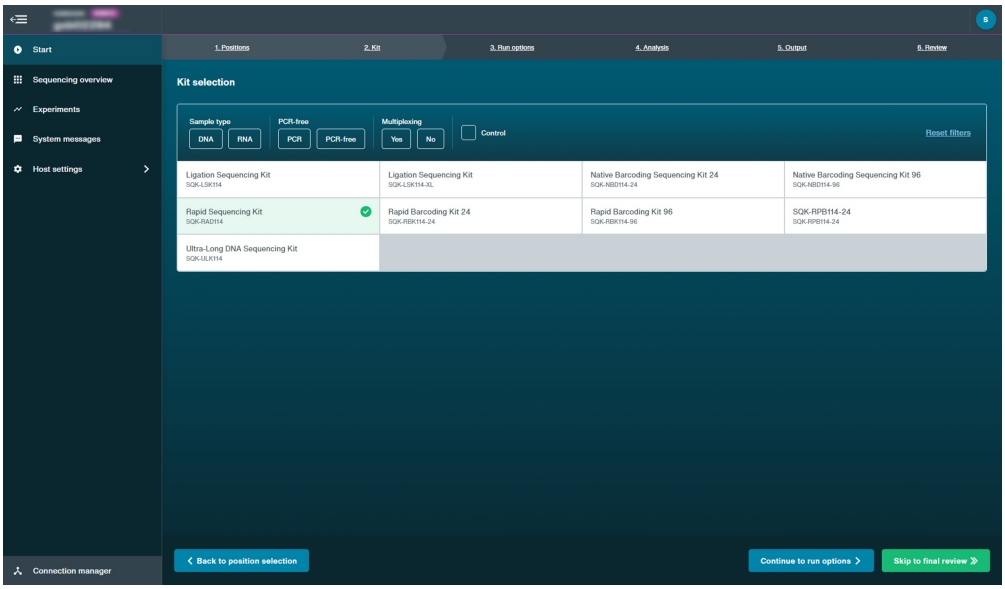
1. Navigate to the start page and click **Start sequencing.**



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



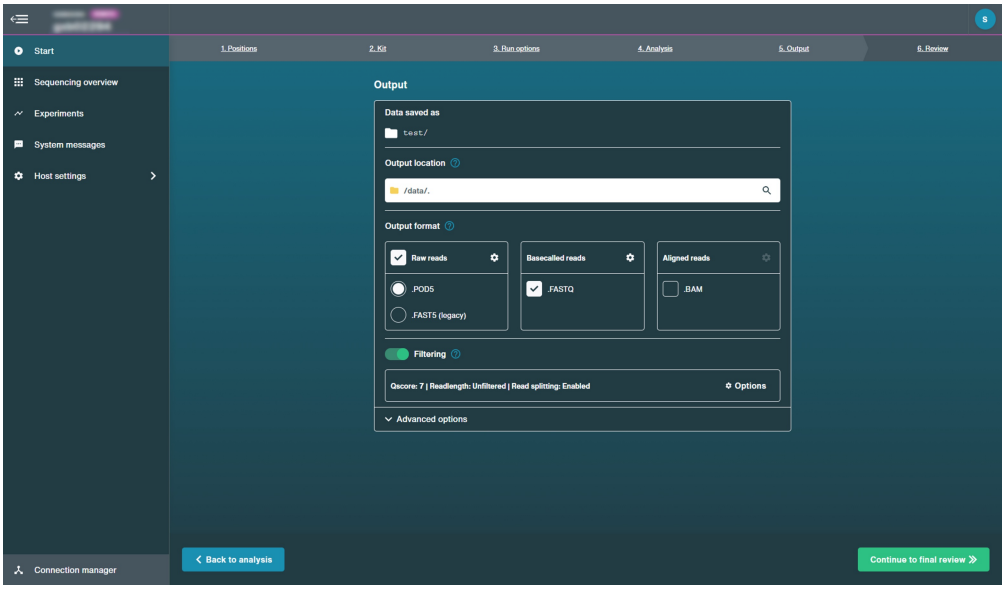
3. Select the sequencing kit used in the library preparation on the Kit page.



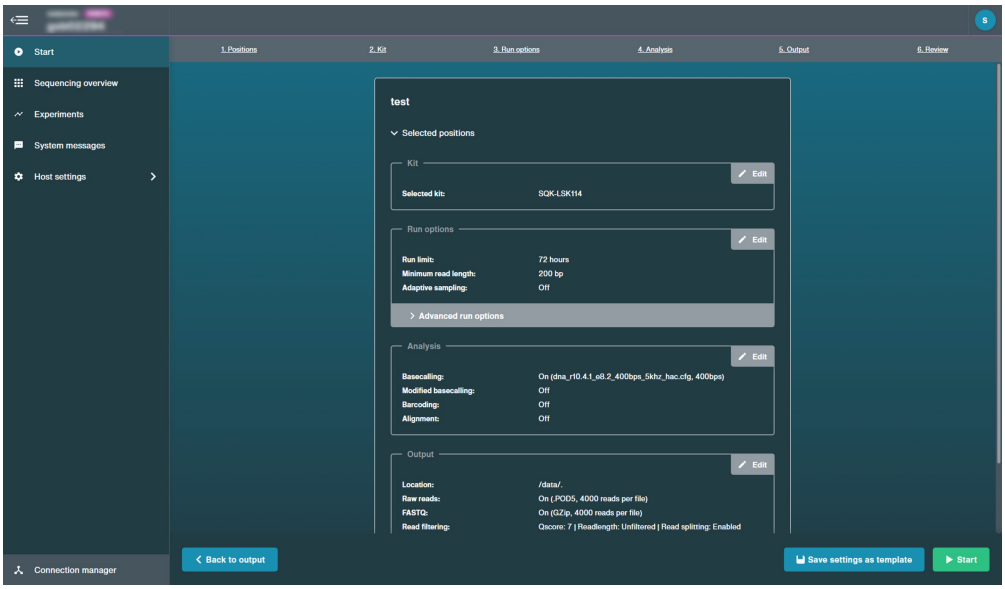
4. Configure the sequencing parameters for your sequencing run or keep to the default settings on the Run options and Analysis tabs.

Note: If basecalling was turned off when a sequencing run was set up, basecalling can be performed post-run on MinKNOW. For more information, please see the MinKNOW protocol.

5. On the Output page, set up the output parameters or keep to the default settings.



6. 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

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 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.

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.

Issues during DNA/RNA extraction and library preparation

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

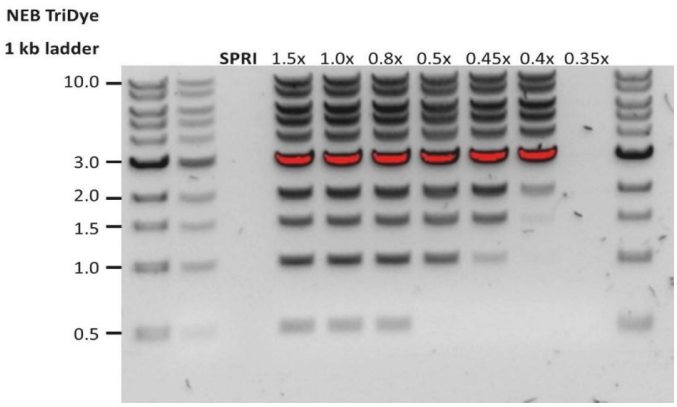
We also have an FAQ section available on the Nanopore Community Support section.

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.
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

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

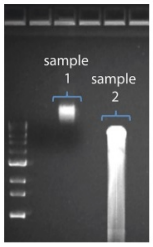
Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support. If you do not have another sequencing device available, we recommend storing the flow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

Pore occupancy below 40%

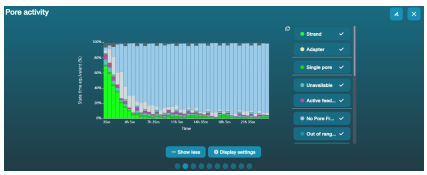
Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	Ensure you load the recommended amount of good quality library in the relevant library prep protocol onto your flow cell. Please quantify the library before loading and calculate mols using 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 adding 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

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

Observation	Possible cause	Comments and actions
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.

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.

Guppy – no input .fast5 was found or basecalled

Observation	Possible cause	Comments and actions
No input .fast5 was found or basecalled	*input_path* did not point to the .fast5 file location	The *--input_path* has to be followed by the full file path to the .fast5 files to be basecalled, and the location has to be accessible either locally or remotely through SSH.
No input .fast5 was found or basecalled	The .fast5 files were in a subfolder at the *input_path* location	To allow Guppy to look into subfolders, add the*--recursive* flag to the command

Guppy – no Pass or Fail folders were generated after basecalling

Observation	Possible cause	Comments and actions
No Pass or Fail folders were generated after basecalling	The *--qscore_filtering* flag was not included in the command	The *--qscore_filtering* flag enables filtering of reads into Pass and Fail folders inside the output folder, based on their strand q-score. When performing live basecalling in MinKNOW, a q-score of 7 (corresponding to a basecall accuracy of ~80%) is used to separate reads into Pass and Fail folders.

Guppy – unusually slow processing on a GPU computer

Observation	Possible cause	Comments and actions
Unusually slow processing on a GPU computer	The *--device* flag wasn't included in the command	The *--device* flag specifies a GPU device to use for accelerate basecalling. If not included in the command, GPU will not be used. GPUs are counted from zero. An example is *--device cuda:0 cuda:1*, when 2 GPUs are specified to use by the Guppy command.