Overview of the protocol

Direct RNA Sequencing Kit features

This kit is highly recommended for users who:

- are exploring attributes of native RNA such as modified bases
- would like to remove RT or PCR bias
- have transcripts that are difficult to reverse transcribe

Introduction to the Direct RNA Sequencing protocol

This protocol describes how to carry out sequencing of native RNA using the Direct RNA Sequencing Kit (SQK-RNA002).

Steps in the sequencing workflow:

Prepare for your experiment

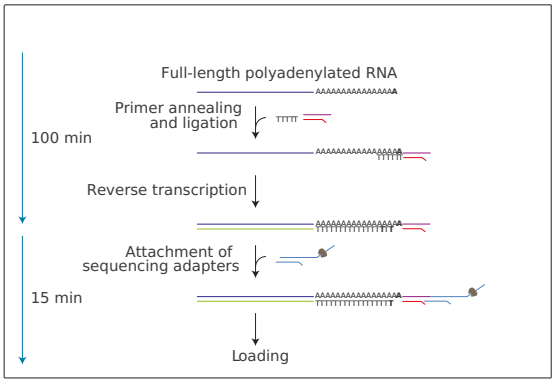
You will need to:

- Extract your RNA, and check its length, quantity and purity. **The quality checks performed during the protocol are essential in ensuring experimental success.**
- Ensure you have your sequencing kit, the correct equipment and third-party reagents
- Download the software for acquiring and analysing your data
- Check your flow cell(s) to ensure it has enough pores for a good sequencing run

Library preparation

You will need to:

- Synthesise the complementary strand of the RNA
- Attach sequencing adapters supplied in the kit to the ends of the RNA-cDNA hybrid
- Prime the flow cell, and load your RNA library into the flow cell



Sequencing and analysis

You will need to:

- Start a sequencing run using the MinKNOW software, which will collect raw data from the device and convert it into basecalled

reads

IMPORTANT

Please note that, unlike DNA, RNA is translocated through the nanopore in the 3'-5' direction. However, the basecalling algorithms automatically flip the data, and the reads are displayed 5'-3'.

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Direct RNA Sequencing Kit (SQK-RNA002)
- FLO-MIN106 flow cells (FLO-MIN107 not advised)
- Guppy software for basecalling
- Flow Cell Wash Kit (EXP-WSH004)

Equipment and consumables

Materials

- 50 ng of poly(A)-tailed RNA or 500 ng of total RNA in 9 µl
- Direct RNA Sequencing Kit (SQK-RNA002)
- Flow Cell Priming Kit (EXP-FLP002)

Consumables

- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 70% ethanol in nuclease-free water
- SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, 18080044)
- 10 mM dNTP solution (e.g. NEB N0447)
- NEBNext® Quick Ligation Reaction Buffer (NEB B6058)
- T4 DNA Ligase 2M U/ml (NEB M0202)
- Agencourt RNAClean XP beads
- Qubit RNA HS Assay Kit (ThermoFisher Q32852)
- Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)

Equipment

- Hula mixer (gentle rotator mixer)
- Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- Microfuge
- Vortex mixer
- Ice bucket with ice
- Timer
- Thermal cycler
- Qubit fluorometer (or equivalent for QC check)

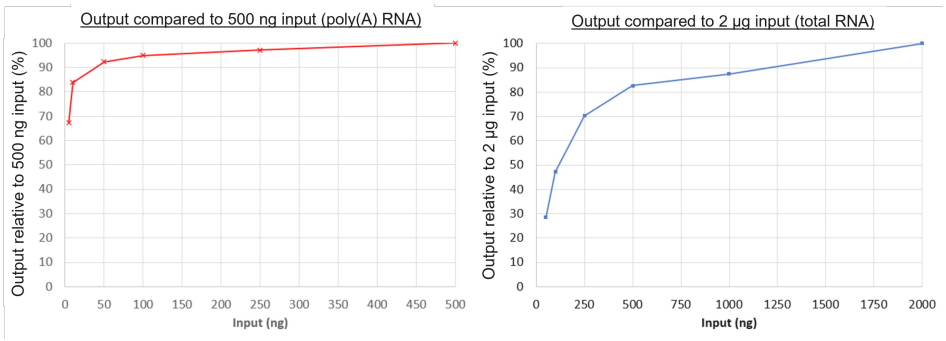
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips

Optional Equipment	• Agilent Bioanalyzer (or equivalent) • Eppendorf 5424 centrifuge (or equivalent)

For this protocol, you will need 50 ng of poly(A)-tailed RNA or 500 ng of total RNA in 9 µl.

Recommended RNA input

Input recommendations are 50 ng of poly(A)-tailed RNA or 500 ng of total RNA in 9 µl. For certain applications, lower input can be used but depending on the input, users may experience a drop in output.



We recommend reading our Know How documents about RNA handling to ensure the highest success with this protocol. Available documents include:

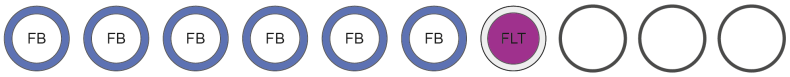
- Enrichment of polyadenylated RNA molecules
- Polyadenylation of non-poly(A) transcripts using *E. coli* poly(A) polymerase
- RNA contaminants
- RNA stability
- RNA Integrity Number (RIN)
- Input DNA/RNA QC

Direct RNA Sequencing Kit contents

RTA : RT adapter
RMX : RNA adapter mix
RCS : RNA CS
WSB : Wash buffer
ELB : Elution buffer
RRB : RNA running buffer

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
RT Adapter	RTA	Blue	1	10
RNA Adapter Mix	RMX	Green	1	45
RNA Calibrant Strand	RCS	Yellow	1	25
Wash Buffer	WSB	Orange	2	1,200
Elution Buffer	ELB	Black	1	300
RNA Running Buffer	RRB	Red	1	600

Flow Cell Priming Kit contents (EXP-FLP002)



FLB : Flush buffer
FLT : Flush tether

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Flush Buffer	FB	Blue	6	1,170
Flush Tether	FLT	Purple	1	200

Computer requirements and software

MinION Mk1B IT requirements

Unless you are using a MinIT device, sequencing on a MinION Mk1B requires a high-spec computer or laptop to keep up with the rate of data acquisition. Read more in the MinION IT Requirements document.

MinION Mk1C IT requirements

The MinION Mk1C contains fully-integrated compute and screen, removing the need for any accessories to generate and analyse nanopore data. Read more in the MinION Mk1C IT requirements document.

Software for nanopore sequencing

MinKNOW

The MinKNOW software controls the nanopore sequencing device, collects sequencing data in real time and processes it into basecalls. You will be using MinKNOW for every sequencing experiment. MinKNOW can also demultiplex reads by barcode, and basecall/demultiplex data after a sequencing run has completed.

MinKNOW use

For instructions on how to run the MinKNOW software, please refer to the relevant section in the MinKNOW protocol.

EPI2ME (optional)

The EPI2ME cloud-based platform performs further analysis of basecalled data, for example alignment to the Lambda genome, barcoding, or taxonomic classification. You will use the EPI2ME platform *only* if you would like further analysis of your data post-basecalling.

EPI2ME installation and use

For instructions on how to create an EPI2ME account and install the EPI2ME Desktop Agent, please refer to the EPI2ME Platform protocol.

Guppy (optional)

The Guppy command-line software can be used for basecalling and demultiplexing reads by barcode instead of MinKNOW. You can use it if you would like to re-analyse old data, or integrate basecalling into your analysis pipeline.

Guppy installation and use

If you would like to use the Guppy software, please refer to the Guppy protocol.

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 flow cells, or within four weeks of purchasing for 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

Library preparation

~115 minutes

Materials

- 50 ng of poly(A)-tailed RNA or 500 ng of total RNA in 9 µl
- RT Adapter (RTA)
- RNA CS (RCS)
- RNA Adapter (RMX)
- Wash Buffer (WSB)
- Elution Buffer (ELB)

Consumables

- NEBNext® Quick Ligation Reaction Buffer (NEB B6058)

	• T4 DNA Ligase 2M U/ml (NEB M0202) • 0.2 ml thin-walled PCR tubes • Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • Agencourt RNAClean XP beads • Freshly prepared 70% ethanol in nuclease-free water • 1.5 ml Eppendorf DNA LoBind tubes • SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, 18080044) • 10 mM dNTP solution (e.g. NEB N0447) • Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)
Equipment	• Magnetic separator, suitable for 1.5 ml Eppendorf tubes • Hula mixer (gentle rotator mixer) • Thermal cycler
Optional Equipment	• Qubit fluorometer (or equivalent for QC check)

1 **Prepare the RNA in nuclease-free water.**

- Transfer 50 ng of poly(A)-tailed RNA or 500 ng of total RNA into a 1.5 ml Eppendorf DNA LoBind tube
- Adjust the volume to 9 µl with nuclease-free water
- Mix thoroughly by flicking the tube to avoid unwanted shearing
- Spin down briefly in a microfuge

2 **In a 0.2 ml thin-walled PCR tube, mix the reagents in the following order:**

Reagent	Volume
NEBNext Quick Ligation Reaction Buffer	3.0 µl
RNA	9.0 µl
RNA CS (RCS), 110 nM	0.5 µl
RT Adapter (RTA)	1.0 µl
T4 DNA Ligase	1.5 µl
Total	**15 µl**

3 **Mix by pipetting and spin down.**

4 **Incubate the reaction for 10 minutes at room temperature.**

5 **Mix the following reagents together to make the reverse transcription master mix:**

Reagent	Volume
Nuclease-free water	9.0 µl
10 mM dNTPs	2.0 µl
5x first-strand buffer	8.0 µl
0.1 M DTT	4.0 µl
Total	**23.0 µl**

6 **Add the master mix to the 0.2 ml PCR tube containing the RT adapter-ligated RNA from the "RT Adapter ligation" step. Mix by pipetting.**

7 **Add 2 µl of SuperScript III reverse transcriptase to the reaction and mix by pipetting.**

8 **Place the tube in a thermal cycler and incubate at 50°C for 50 min, then 70°C for 10 min, and bring the sample to 4°C before proceeding to the next step.**

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

10 **Resuspend the stock of Agencourt RNAClean XP beads by vortexing.**

11 **Add 72 µl of resuspended RNAClean XP beads to the reverse transcription reaction and mix by pipetting.**

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

13 **Prepare 200 µl of fresh 70% 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 magnet, and wash the beads with 150 µl of freshly prepared 70% ethanol without disturbing the pellet as described below.**

1. Keeping the magnetic rack on the benchtop, rotate the bead-containing tube by 180°. Wait for the beads to migrate towards the magnet and form a pellet.
2. Rotate the tube 180° again (back to the starting position), and wait for the beads to pellet.

16 **Remove the 70% ethanol using a pipette, and discard.**

17 **Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol.**

18 **Remove the tube from the magnetic rack and resuspend pellet in 20 µl nuclease-free water. Incubate for 5 minutes at room temperature.**

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

20 **Pipette 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

21 **In the same 1.5 ml Eppendorf DNA LoBind tube, mix the reagents in the following order:**

Reagent	Volume
NEBNext Quick Ligation Reaction Buffer	8.0 µl
RNA Adapter (RMX)	6.0 µl
Nuclease-free water	3.0 µl
T4 DNA Ligase	3.0 µl
Total (including all reagents)	**40 µl**

22 **Mix by pipetting.**

23 **Incubate the reaction for 10 minutes at room temperature.**

24 **Resuspend the stock of Agencourt RNAClean XP beads by vortexing.**

25 **Add 16 µl of resuspended RNAClean XP beads to the adapter ligation reaction and mix by pipetting.**

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

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

28 **Add 150 µl of the Wash Buffer (WSB) to the beads. Close the tube lid, and resuspend the beads by flicking the tube. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant.**

29 **Repeat the previous step.**

IMPORTANT

Agitating the beads results in a more efficient removal of free adapter, compared to adding the wash buffer and immediately aspirating.

30 **Remove the tube from the magnetic rack and resuspend pellet in 21 µl Elution Buffer by the gently flicking the tube. Incubate for 10 minutes at room temperature.**

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

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

Quantify 1 µl of reverse-transcribed and adapted RNA using the Qubit fluorometer DNA HS assay - recovery aim ~20 ng.

If you have omitted the reverse transcription step, please use the Qubit RNA HS Assay Kit instead. However, please note that the kit will measure all RNA present, including any non-adapted RNA that has been carried through in the RNAClean XP bead clean-up. The reported quantity of RNA may therefore not fully represent the amount of sequenceable RNA.

END OF STEP

The reverse-transcribed and adapted RNA is now ready for loading into the flow cell.

Priming and loading the SpotON flow cell

~15 minutes

Materials	• Prepared RNA library • RNA Running Buffer (RRB) • Flow Cell Priming Kit (EXP-FLP002)
Consumables	• MinION Mk1B • SpotON Flow Cell • Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • 1.5 ml Eppendorf DNA LoBind tubes

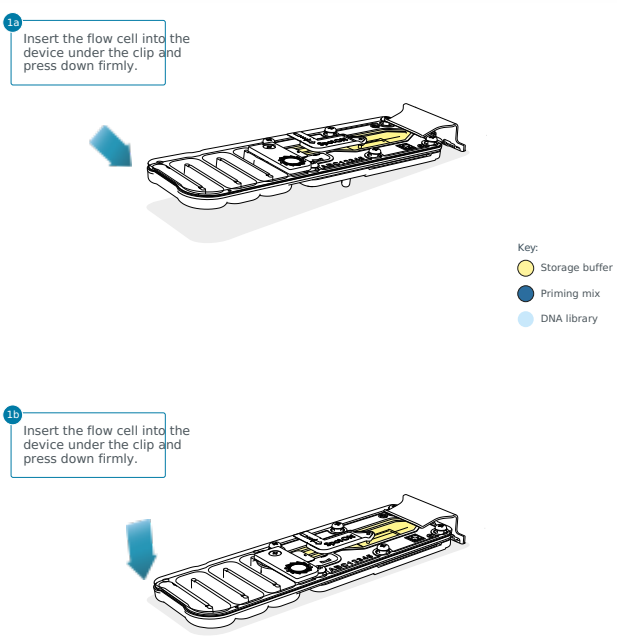
1 **Thaw the RNA Running Buffer (RRB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature.**

2 **Mix the RNA Running Buffer (RRB), Flush Buffer (FB) and Flush Tether (FLT) tubes thoroughly by vortexing and spin down at room temperature.**

3 **To prepare the flow cell priming mix, add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at room temperature.**

4 Open the MinION Mk1B 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.

Please see the MinKNOW Flow cell check protocol for more information

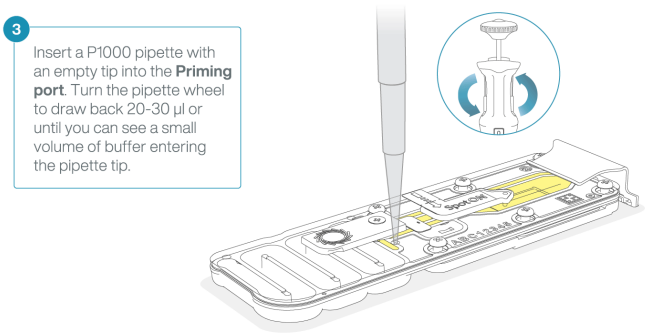
5 Slide the priming port cover clockwise to open the priming port.

IMPORTANT

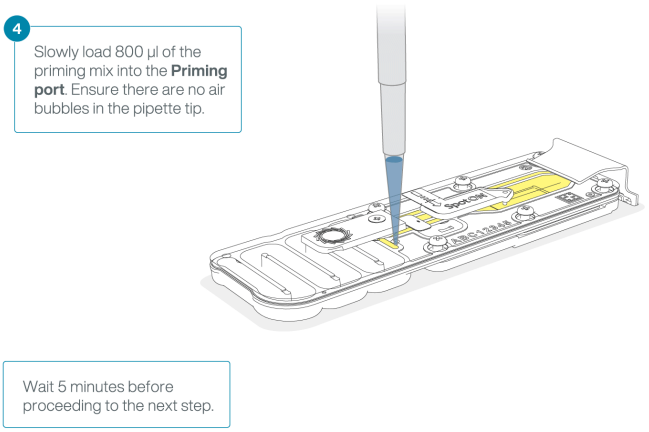
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

6 **After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µl):**

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.



7 **Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes. During this time, prepare the library for loading by following the steps below.**



IMPORTANT

Thoroughly mix the contents of the RRB tube by vortexing or pipetting, and spin down briefly.

8 **Take 20 µl of the prepared RNA library and mix it with 17.5 µl of nuclease-free water.**

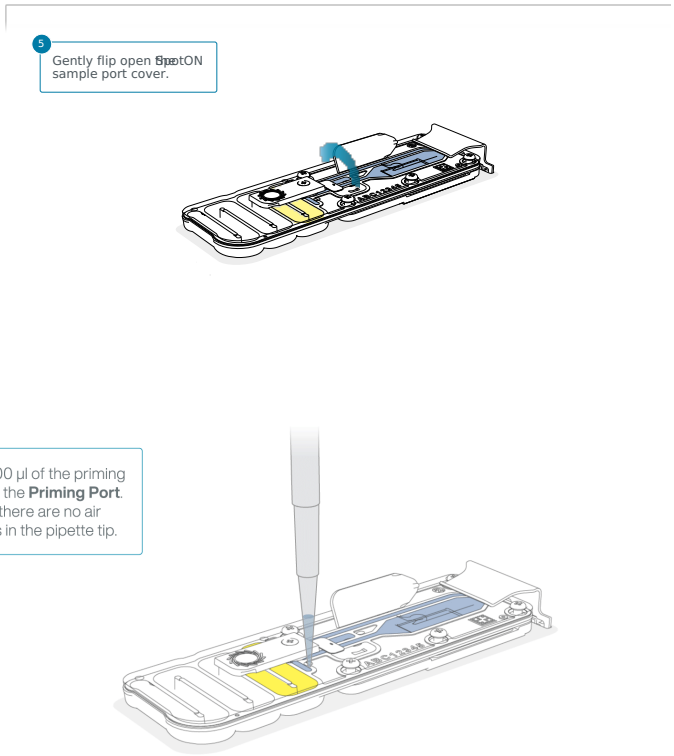
9 **In a new tube, prepare the library for loading as follows:**

Reagent	Volume
RRB	37.5 µl
RNA library in nuclease-free water	37.5 µl
Total	**75 µl**

10 Complete the flow cell priming:

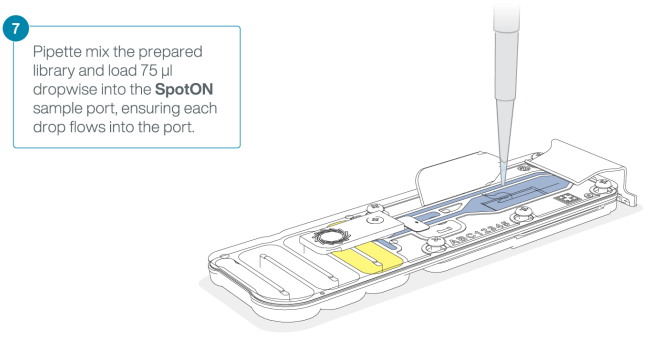
1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load **200 µl** of the priming mix into the flow cell via the priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.

Note: Load the library as soon as possible after this step.

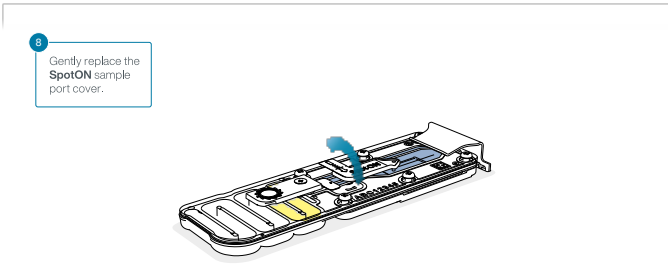


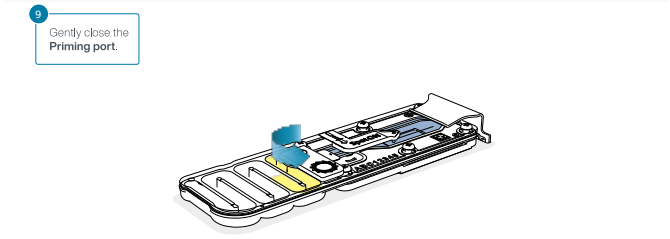
11 Mix the prepared library gently by pipetting up and down just prior to loading.

12 **Add 75 µl of sample to the Flow Cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.**



13 **Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION Mk1B lid.**





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, or that you are using the MinIT device for data acquisition and basecalling. There are three 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 MinIT device

Follow the instructions in the MinIT protocol.

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

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 or the Guppy protocol starting from the "Quick Start Guide for Guppy" section.

Downstream analysis

Post-basecalling analysis

There are several options for further analysing your basecalled data:

1. EPI2ME platform

The EPI2ME platform is a cloud-based data analysis service developed by Metrichor Ltd., a subsidiary of Oxford Nanopore Technologies. The EPI2ME platform offers a range of analysis workflows, e.g. for metagenomic identification, barcoding, alignment,

and structural variant calling. The analysis requires no additional equipment or compute power, and provides an easy-to-interpret report with the results. For instructions on how to run an analysis workflow in EPI2ME, please follow the instructions in the EPI2ME protocol, beginning at the "Starting data analysis" step.

2. EPI2ME Labs tutorials and workflows

For more 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.

3. Research analysis tools

Oxford Nanopore Technologies' Research division has created a number of analysis tools, which 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.

4. 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.

Ending the experiment

Materials

- Flow Cell Wash Kit (EXP-WSH004)

1 **After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Wash Kit instructions and store the washed flow cell at 2-8°C, OR**

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 **Follow the returns procedure by washing out the flow cell ready to send back to Oxford Nanopore.**

Instructions for returning flow cells can be foundhere.

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

Issues during DNA/RNA extraction and library preparation

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

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

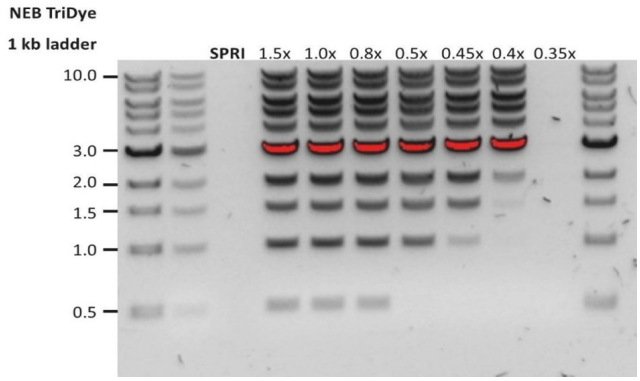
If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via LiveChat in the Nanopore Community.

Low sample quality

Observation	Possible cause	Comments and actions
Low DNA purity (Nanodrop reading for DNA OD 260/280 is <1.8 and OD 260/230 is <2.0–2.2)	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the Contaminants Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover. Consider performing an additional SPRI clean-up step.
Low 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 Know-how piece.
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 Know-how piece. 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	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.

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	TRainin 20 µl or 30 µl and Gilson 10 µl, 20 µl or 30 µl pipette tips are compatible with loading reagents into the VolTRAX cartridge. Rainin 20 µl is the most suitable.
The VolTRAX software shows an inaccurate amount of reagents loaded	The angle at which reagents are pipetted into the cartridge is incorrect	The pipetting angle should be slightly greater than the cartridge inlet angle. Please watch the demo video included in the VolTRAX software before loading.

Issues during the sequencing run

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

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

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

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

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

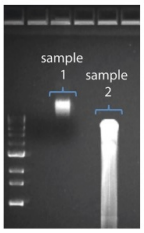
MinKNOW script failed

Observation	Possible cause	Comments and actions
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.

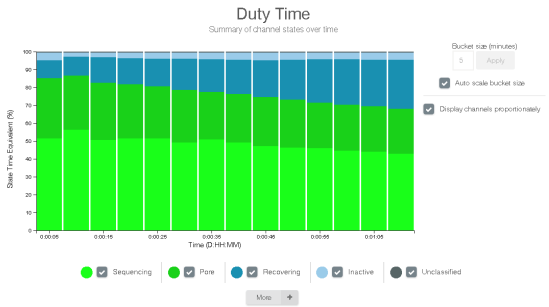
Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	5–50 fmol of good quality library can be loaded on to a MinION Mk1B/GridION 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 SQK-LSK109 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 tube). Make sure FLT was added to FB 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 recovering pores

Observation	Possible cause	Comments and actions
Large proportion of recovering pores (shown as dark blue in the channels panel and duty time plot)	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 "single pores". If the portion of recovering pores (unavailable pores in the extended view) stays large or increases: 1. A nuclease flush can be performed, or 2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.  The duty time plot above shows an increasing proportion of "recovering" pores over the course of a sequencing experiment

Large proportion of inactive pores

Observation	Possible cause	Comments and actions
Large proportion of inactive pores (shown as light blue in the channels panel and duty time 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 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 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	Fast fuel consumption is typically seen when the flow cell is overloaded with library (~5–50 fmol of library is recommended).	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" (37°C for Flow Cell Check, 34°C for sequencing on MinION Mk 1B/PromethION flow cells, and 35°C for sequencing on Flongle)	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

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.

MinIT – the MinKNOW interface is not shown in the web browser

Observation	Possible cause	Comments and actions
The MinKNOW interface is not shown in the web browser	Browser compatibility issue	Always use Google Chrome as the browser to view MinKNOW. Alternatively, instead of typing //mt-xxxxxx (x is a number) in the address bar, type in in the generic IP address, 10.42.0.1, which identifies the MinIT Wi-Fi router.
The MinKNOW interface is not shown in the web browser	The MinIT Wi-Fi was not used for connecting to the computer or mobile device	Make sure the computer or mobile device is using the MinIT Wi-Fi. It should be shown as MT-xxxxxx (x is a number) on the underside label on the MinIT:  Disable the Ethernet connection from the computer or mobile device as needed. If necessary, contact your IT department to determine if the MinIT Wi-Fi is blocked (MinIT generic IP: 10.42.0.1). Please white-list MinIT as needed.
The MinKNOW interface is not shown in the web browser	The MinIT was not on the same network that the computer was connected to.	Make sure that the wall sockets used by the Ethernet cables from the MinIT and computer belong to the same local network.

MinIT – the MinIT software cannot be updated

Observation	Possible cause	Comments and actions
The MinIT software cannot be updated	The firewall is blocking IPs for update	Please consult your IT department, as the MinIT software requires access to the following AWS IP ranges. Access to the following IP addresses is also needed: 178.79.175.200 96.126.99.215

Observation	Possible cause	Comments and actions
The MinIT software cannot be updated	The device already has the latest version of the software	Occassionaly, the MinIT software admin page displays "updates available" even when the software is already up-to-date. Please compare the version listed on the admin page with the one on the Software Downloads page. Alternatively, SSH into the MinIT through a SSH Client (e.g. Bitvise or Putty, as described in the MinIT protocol) on a Windows computer or the terminal window on a Mac, run the command, *dpkg -l | grep minit*, to find out the version of the MinIT software and *sudo apt update* if an update is needed. If the issue still persists, please contact Technical Services with details of the error.