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-RNA004). Starting from either poly(A) tailed RNA or total RNA, a second complementary cDNA strand is synthesised for stability by reverse transcription. Sequencing adapters are then attached to the RNA-cDNA hybrid for sequencing on either MinION or PromethION RNA Flow Cells (FLO-MIN004RA / FLO-PRO004RA respectively). Please note, the the complementary cDNA strand is not sequenced, but improves the RNA sequencing output.

It is recommend that a control experiment using the RNA Control Strand (RCS) is completed first to become familiar with the technology.

Steps in the sequencing workflow:

Prepare for your experiment

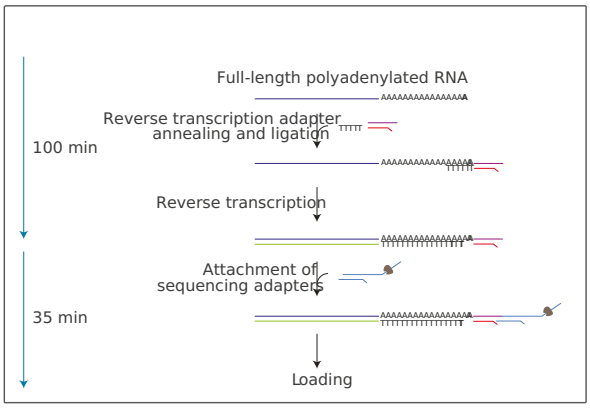
You will need to:

- 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 to the ends of the RNA-cDNA hybrid
- Prime the flow cell, and load your RNA library onto 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 basecall the reads

IMPORTANT

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-RNA004)
- PromethION RNA Flow Cell (FLO-PRO004RA)
- Flow Cell Wash Kit (EXP-WSH004) - Flow Cell Wash Kit (EXP-WSH004) is compatible for removing library between washes but will not remove RNA-related blocking of nanopores.

Equipment and consumables

Materials	• 300 ng of poly(A) tailed RNA or 1 µg of total RNA in 8 µl • Direct RNA Sequencing Kit (SQK-RNA004)
Consumables	• SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, cat # 18080044) • 10 mM dNTP solution (e.g. NEB, cat # N0447) • NEBNext® Quick Ligation Reaction Buffer (NEB, B6058) • T4 DNA Ligase 2M U/ml (NEB, cat # M0202T/M) • RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, cat # 10777019) • Agencourt RNAClean XP beads (Beckman Coulter™, cat # A63987) • Nuclease-free water (e.g. ThermoFisher, AM9937)

- Freshly prepared 70% ethanol in nuclease-free water
- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes
- Qubit RNA HS Assay Kit (ThermoFisher, cat # Q32852)
- Qubit dsDNA HS Assay Kit (ThermoFisher, cat # Q32851)
- Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

- Hula mixer (gentle rotator mixer)
- Magnetic rack, 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
- Eppendorf 5424 centrifuge (or equivalent)

For this protocol, you will need 300 ng of poly(A) tailed RNA or 1 µg of total RNA in 8 µl.

It is possible to start the protocol from a lower sample input, however this will likely yield a lower output.

Please refer to the following input titration graphs for guidance:

Poly(A) tailed RNA

Total RNA

Input RNA

It is important that the input RNA meets the quantity and quality requirements. Using too little or too much RNA, or RNA of poor quality (e.g. fragmented or containing chemical contaminants) can affect your library preparation.

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

For further information on using RNA as input, please read the links below.

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

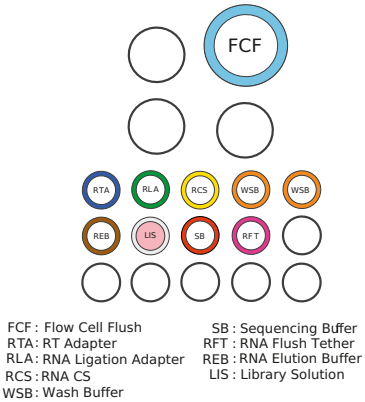
These documents can also be found in the DNA/RNA Handling page.

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.

Direct RNA Sequencing Kit (SQK-RNA004) contents:



Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
RT Adapter	RTA	Blue	1	10
RNA Ligation Adapter	RLA	Green	1	45
RNA CS	RCS	Yellow	1	25
Wash Buffer	WSB	Orange	2	1,200
RNA Elution Buffer	REB	Black	1	300
Library Solution	LIS	White cap, pink label	1	600
Sequencing Buffer	SB	Red	1	700
RNA Flush Tether	RFT	Pink	1	200
Flow Cell Flush	FCF	White	1	8,000

Computer requirements and software

PromethION 24/48 IT requirements

The PromethION device contains all the hardware required to control up to 24 (for the P24 model) or 48 (for the P48 model) sequencing experiments and acquire the data. The device is further enhanced with high performance GPU technology for real-time basecalling. Read more in the PromethION IT Requirements document.

PromethION 2 Solo IT requirements

The PromethION 2 (P2) Solo is a device which directly connects into a GridION Mk1 or a stand-alone computer that meets the miminum specifications for real-time data streaming and analysis. Up to two PromethION flow cells can be can be run and each is independently addressable, meaning experiments can be run concurrently or individually. For information on the computer IT requirements, please see the PromethION 2 Solo IT requirements document.

Software for nanopore sequencing

MinKNOW

The MinKNOW software controls the nanopore sequencing device, collects sequencing data and basecalls in real time. You will be using MinKNOW for every sequencing experiment to sequence, basecall and demultiplex if your samples were barcoded.

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

EPI2ME (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.

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

Check your flow cell

We highly recommend that you check the number of pores in your 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

Library preparation

~130 minutes

Materials

- 300 ng of poly(A) tailed RNA or 1 µg of total RNA in 8 µl
- RT Adapter (RTA)
- RNA CS (RCS)
- Wash Buffer (WSB)
- RNA Ligation Adapter (RLA)
- RNA Elution Buffer (REB)

Consumables

- Agencourt RNAClean XP beads (Beckman Coulter™, cat # A63987)
- SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, cat # 18080044)
- 10 mM dNTP solution (e.g. NEB, cat # N0447)
- NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)
- T4 DNA Ligase 2M U/ml (NEB, cat # M0202T/M)
- RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, cat # 10777019)
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- Freshly prepared 70% ethanol in nuclease-free water
- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes
- Qubit 1x dsDNA HS Assay Kit (ThermoFisher, cat # Q33230)
- Qubit RNA HS Assay Kit (ThermoFisher, cat # Q32852)
- Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

- Hula mixer (gentle rotator mixer)
- Thermal cycler

- Magnetic rack
- Qubit fluorometer (or equivalent for QC check)
- 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

1 Prepare the NEBNext Quick Ligation Reaction Buffer and T4 DNA Ligase according to the manufacturer's instructions, and place on ice:

1. Thaw the reagents at room temperature.
2. Spin down the reagent tubes for 5 seconds.
3. Ensure the reagents are fully mixed by performing 10 full volume pipette mixes.
   Note: Do NOT vortex the T4 DNA Ligase.

The NEBNext Quick Ligation Reaction Buffer may have a little precipitate. Allow the mixture to come to room temperature and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for several seconds to ensure the reagent is thoroughly mixed.

IMPORTANT

We do not recommend using the Quick T4 Ligase for this protocol. We have found that the T4 DNA Ligase (2M U/ml - NEB M0202T/M) works better. It needs to be used in combination with the Quick Ligation Reaction Buffer (NEB B6058).

2 Spin down the RT Adapter (RTA), RNA CS (RCS) (if using), and RNA Ligation Adapter (RLA), pipette mix and place on ice.

3 Thaw the Wash Buffer (WSB) and RNA Elution Buffer (REB) at room temperature and mix by vortexing. Then spin down and place on ice.

4 Prepare the RNA in nuclease-free water:

- Transfer 300 ng of poly(A) tailed RNA or 1 µg of total RNA into a 0.2 ml thin-walled PCR tube.
- Adjust the volume to 8 µl with nuclease-free water.
- Mix thoroughly by flicking the tube to avoid unwanted shearing.
- Spin down briefly in a microfuge.

Optional Action
The use of the RNA CS (RCS) in this preparation is an optional control measure for library preparation QC and troubleshooting.

We recommend the inclusion of the RNA CS (RCS) in the library preparation for troubleshooting purposes.

However, if users chose to not use the RNA CS (RCS) input, replace the volume in the reaction with nuclease-free water.

5 **In the same 0.2 ml thin-walled PCR tube, combine the reagents in the following order:**

Reagent	Volume
RNA	8 µl
NEBNext Quick Ligation Reaction Buffer	3 µl
RNA CS (RCS)	0.5 µl
RNaseOUT™	1 µl
RT Adapter (RTA)	1 µl
T4 DNA Ligase	1.5 µl
Total	**15 µl**

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

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

8 **In a clean 1.5 ml DNA LoBind Eppendorf tube, combine the following reagents together to make the reverse transcription master mix:**

Reagent	Volume
Nuclease-free water	9 µl
10 mM dNTPs	2 µl
5X First-strand buffer	8 µl
DTT	4 µl
Total	**23 µl**

9 **Transfer the reverse transcriptase master mix to the 0.2 ml PCR tube containing your adapter-ligated RNA and mix by pipetting.**

10 **Add 2 µl of SuperScript III Reverse Transcriptase to the reaction and mix by pipetting.**

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

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

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

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

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

16 **Prepare 200 µl of fresh 70% ethanol in nuclease-free water.**

17 **Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless.**

18 **Keep the tube on magnet until the supernatant is clear and colourless before washing the beads with 150 µl of freshly prepared 70% ethanol, as described below:**

1. 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.
2. Rotate the tube 180° again (back to the starting position), and wait for the beads to pellet again.

19 **Carefully remove the 70% ethanol using a pipette and discard.**

20 **Spin down and place the tube back on the magnet until the eluate is clear and colourless. Keep the tubes on the magnet and pipette off any residual ethanol.**

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

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

23 **Remove and retain 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Optional Action
At this stage the RT-RNA sample can be stored at -80°C for later use.

Please note, this is the only pause point in this protocol.

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

Reagent	Volume
RT-RNA sample	20 µl
NEBNext Quick Ligation Reaction Buffer	8 µl
RNA Ligation Adapter (RLA)	6 µl
Nuclease-free water	3 µl
T4 DNA Ligase	3 µl
Total	**40 µl**

25 **Mix by pipetting.**

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

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

28 **Add 16 µl of resuspended Agencourt RNAClean XP beads to the reaction and mix by pipetting.**

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

30 **Spin down the sample and pellet on a magnet. Keep the tube on the magnet for 5 minutes, and pipette off the supernatant when clear and colourless.**

31 **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 the beads to pellet for 5 minutes and pipette off the supernatant when clear and colourless.**

32 **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.**

33 **Spin down the tube and replace onto the magnetic rack until the beads have pelleted to pipette off any remaining Wash Buffer (WSB).**

34 **Remove the tube from the magnetic rack and resuspend pellet in 33 µl RNA Elution Buffer (REB) by the gently flicking the tube. Incubate for 10 minutes at room temperature.**

35 **Pellet the beads on a magnet for 5 minutes until the eluate is clear and colourless.**

36 **Remove and retain 33 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

37 **Quantify 1 µl of reverse-transcribed and adapted RNA using the Qubit fluorometer DNA HS assay.**

The recovery aim in the final eluate is > 30 ng.

Recovery quantities can vary between different inputs and library preparations. However, we always recommend taking forward the full volume of RNA library for the best sequencing results.

END OF STEP

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

IMPORTANT

The RNA library must be sequenced immediately and cannot be stored for later use.

Priming and loading the PromethION flow cell

~5 minutes

Materials	• Sequencing Buffer (SB) • Library Solution (LIS) • Flow Cell Flush (FCF) • RNA Flush Tether (RFT)
Consumables	• PromethION Flow Cell • 1.5 ml Eppendorf DNA LoBind tubes
Equipment	• PromethION 2 Solo device • PromethION 24/48 device • PromethION Flow Cell Light Shield • P1000 pipette and tips • P200 pipette and tips • P20 pipette and tips

IMPORTANT

This kit is only compatible with RNA flow cells (FLO-PRO004RA).

1 **Thaw the Sequencing Buffer (SB), Library Solution (LIS), RNA Flush Tether (RFT) and Flow Cell Flush (FCF) at room temperature. Mix by vortexing and spin down.**

2 **To prepare the flow cell priming mix in a clean 1.5 ml Eppendorf DNA LoBind tube, combine the following reagents. Mix by vortexing and spin down at room temperature.**

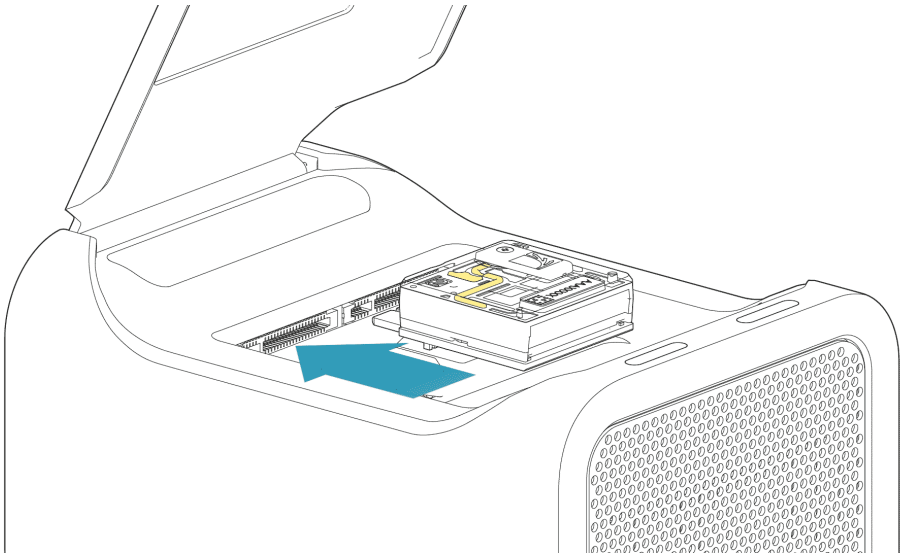
Reagent	Volume per flow cell
RNA Flush Tether (RFT)	30 µl
Flow Cell Flush (FCF)	1,170 µl
Total	**1,200 µl**

IMPORTANT

After taking flow cells out of the fridge, wait 20 minutes before inserting the flow cell into the PromethION for the flow cell to come to room temperature. Condensation can form on the flow cell in humid environments. Inspect the gold connector pins on the top and underside of the flow cell for condensation and wipe off with a lint-free wipe if any is observed. Ensure the heat pad (black pad) is present on the underside of the flow cell.

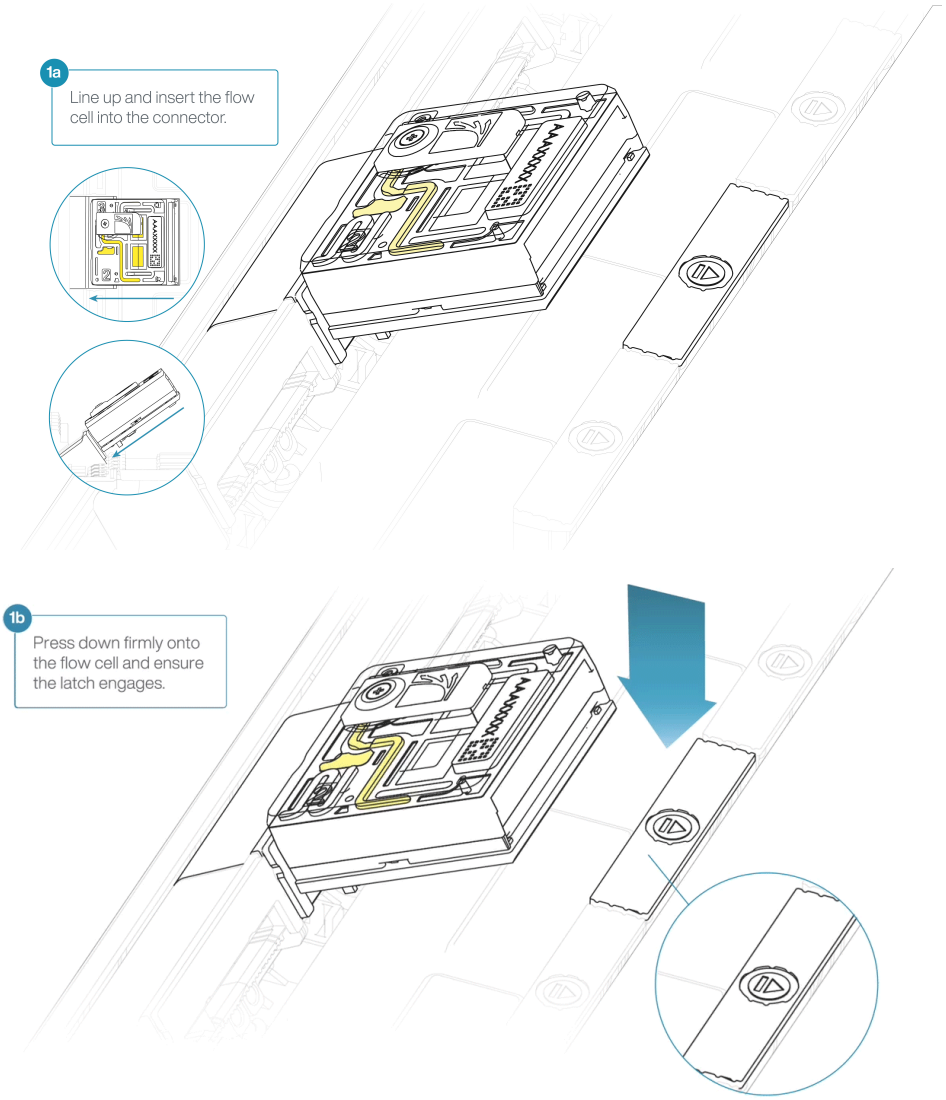
3 **For PromethION 2 Solo, load the flow cell(s) as follows:**

1. Place the flow cell flat on the metal plate.
2. Slide the flow cell into the docking port until the gold pins or green board cannot be seen.



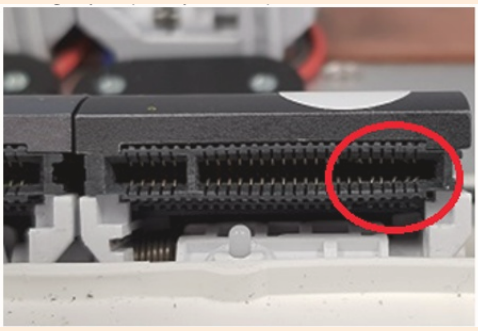
4 For the PromethION 24/48, load the flow cell(s) into the docking ports:

1. Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position.
2. Press down firmly onto the flow cell and ensure the latch engages and clicks into place.

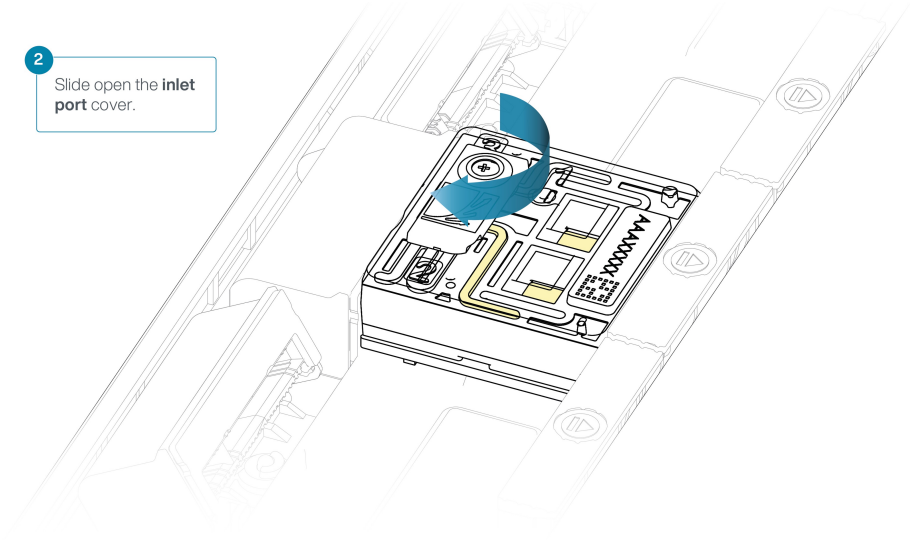


IMPORTANT

Insertion of the flow cells at the wrong angle can cause damage to the pins on the PromethION and affect your sequencing results. If you find the pins on a PromethION position are damaged, please contact support@nanoporetech.com for assistance.



5 **Turn the valve clockwise to expose the inlet port.**

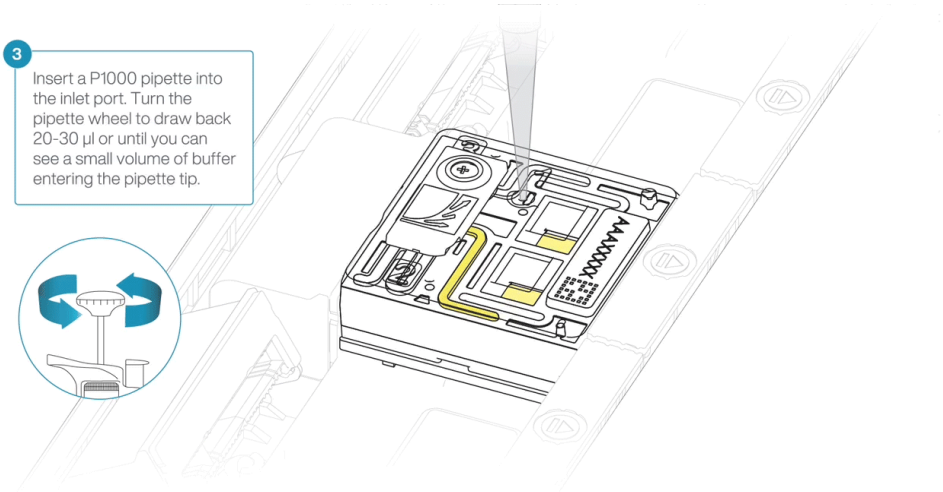


IMPORTANT

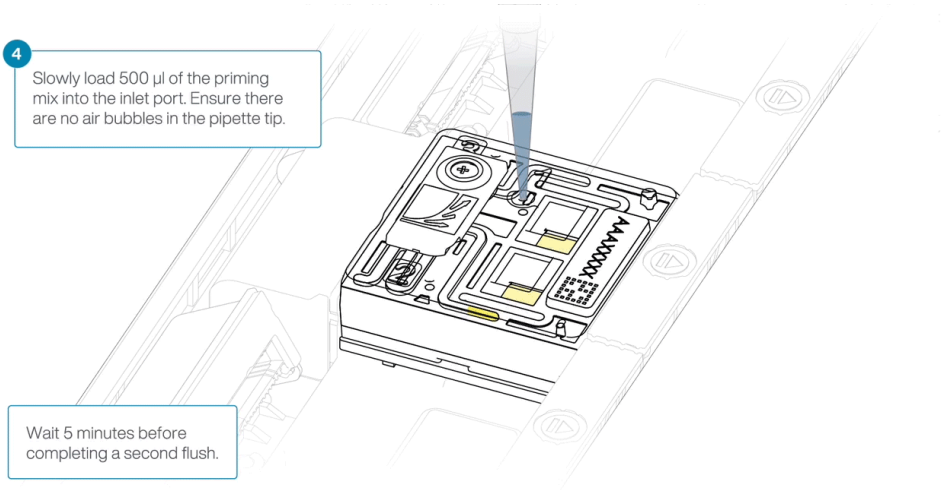
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

6 After opening the inlet port, draw back a small volume to remove any air bubbles:

1. Set a P1000 pipette tip to 200 µl.
2. Insert the tip into the inlet port.
3. Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.



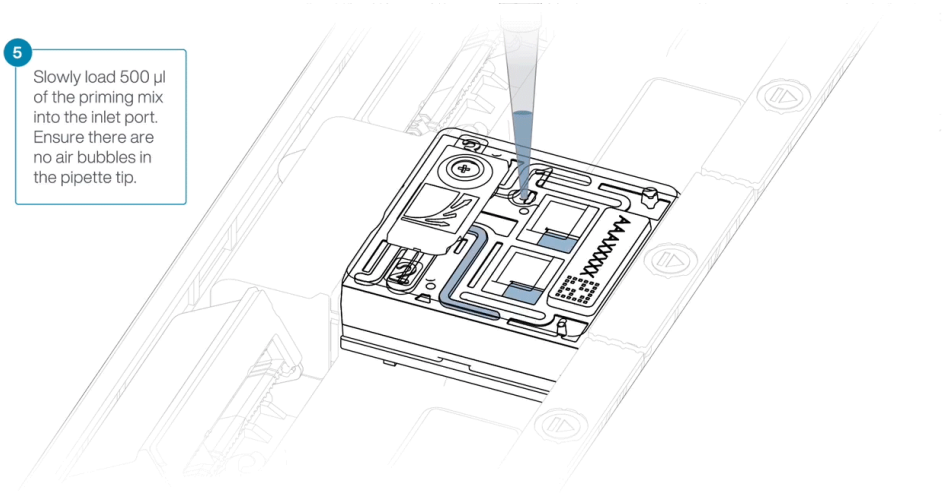
7 Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes. During this time, prepare the library for loading using the next steps in the protocol.



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

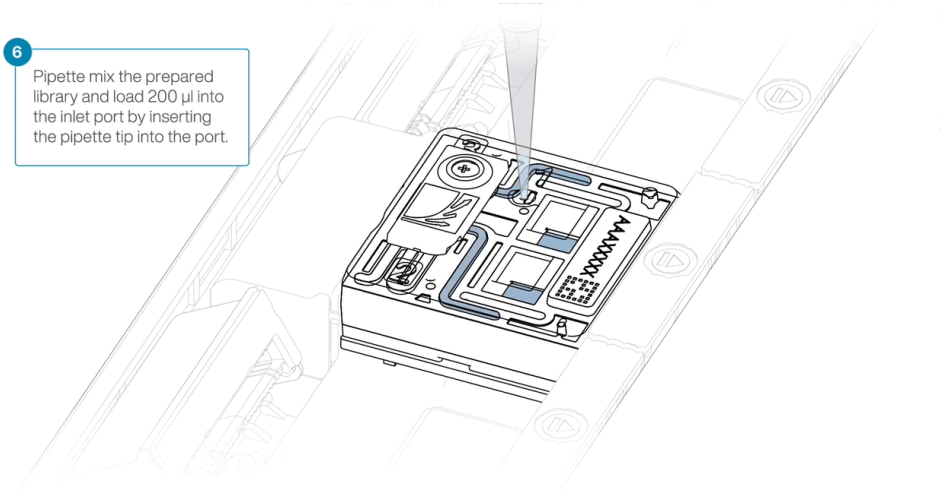
Reagent	Volume per flow cell
Sequencing Buffer (SB)	100 µl
Library Solution (LIS)	68 µl
RNA library	32 µl
Total	**200 µl**

9 Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port.

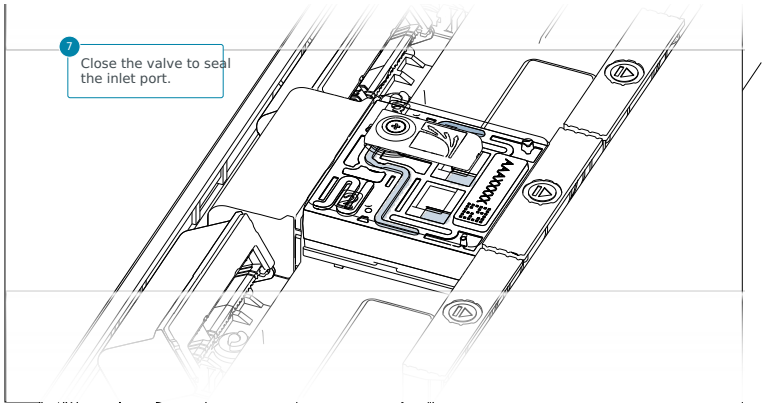


10 Mix the prepared library gently by pipetting up and down just prior to loading.

11 Using a P1000, insert the pipette tip into the inlet port and load 200 µl of library.



12 Close the valve to seal the inlet port.



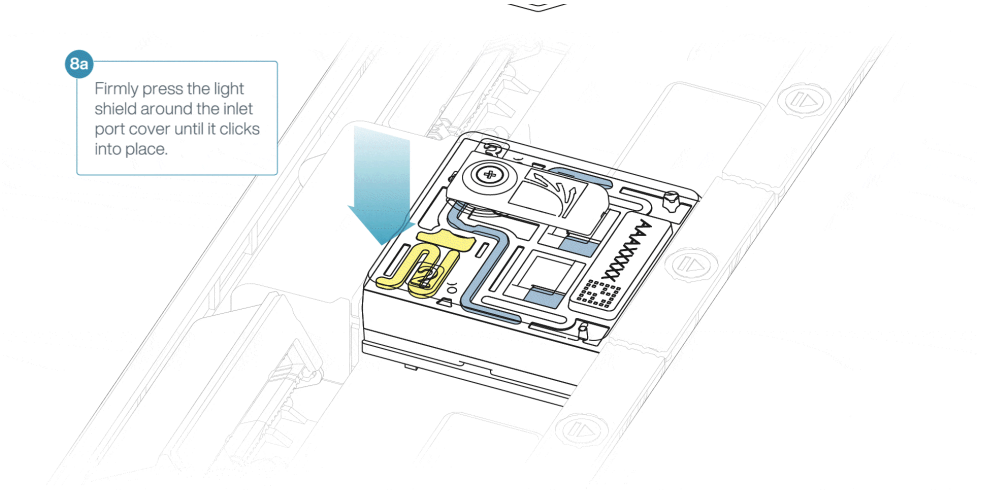
IMPORTANT

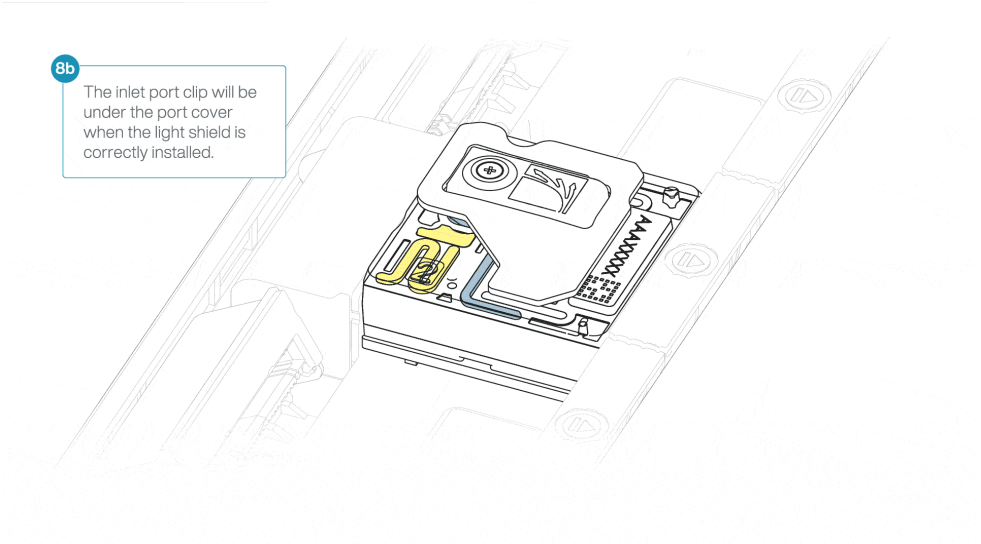
Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.

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 If the light shield has been removed from the flow cell, install the light shield as follows:

1. Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID.
2. Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.





END OF STEP

Close the PromethION lid when ready to start a sequencing run on MinKNOW.

Wait a minimum of 10 minutes after loading the flow cells onto the PromethION before initiating any experiments. This will help to increase the sequencing output.

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 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 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 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 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.

Issues during an RNA 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 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 NEBio Calculator, choosing "RNA ss: mass to moles"

Observation	Possible cause	Comments and actions
Pore occupancy close to 0	No tether on the flow cell	Tethers are adding during flow cell priming (FCT tube). Make sure Flow Cell Tether (FCT) was added to Flow Cell Flush (FCF) before priming.

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	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.