Oxford Nanopore Technologies

PROMETHION: PROTOCOL

Direct RNA sequencing (SQK-RNA004)

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This protocol:

- Is for sequencing native RNA
- Can be used with total RNA or an enriched sample (e.g. poly(A) tailed or ribo-depleted) as a starting input material
- Requires no fragmentation
- Takes approximately 140 minutes for library preparation
- Is only compatible with RNA flow cells

For Research Use Only

FOR RESEARCH USE ONLY

Contents

1. Overview of the protocol

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

Direct RNA Sequencing Kit features

This kit is highly recommended for:

- Exploring attributes of native RNA, such as modified bases
- Removing RT or PCR bias
- 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™/GridION™ or PromethION™ Flow Cells RNA (FLO-MIN004RA or FLO-PRO004RA respectively). Please note, 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

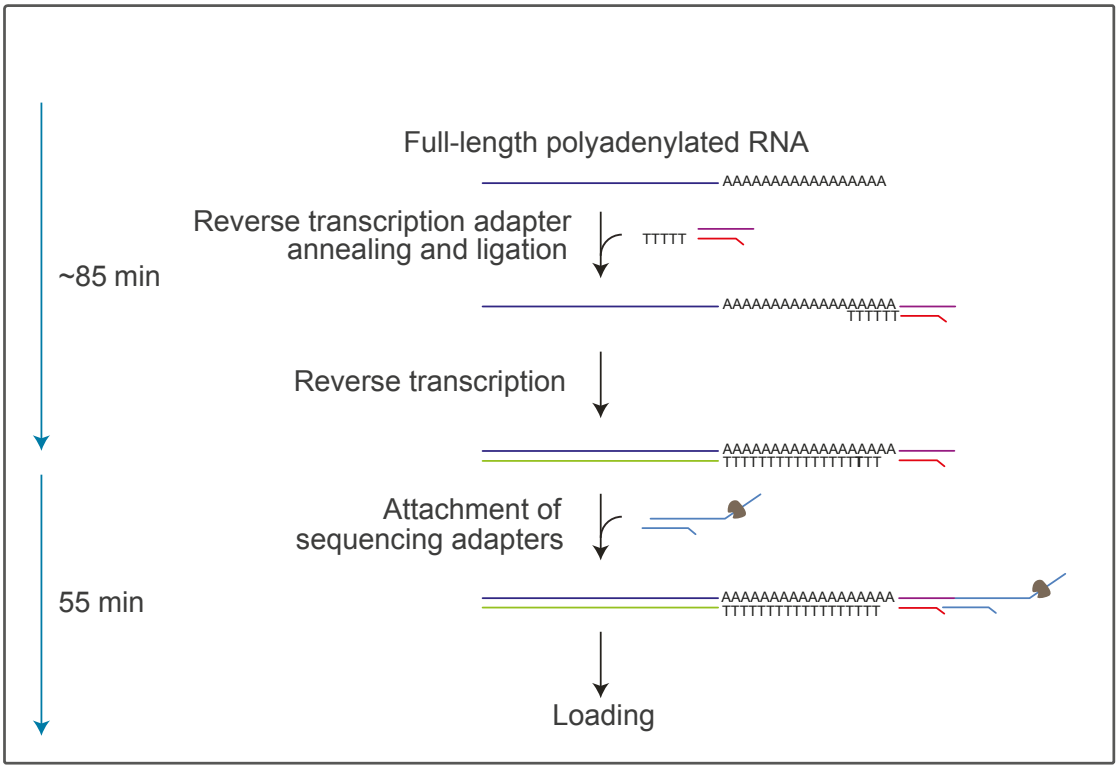
You will need to:

- 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 sufficient 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	Process	Time	Stop option
Reverse transcription	Synthesise the complementary strand of the RNA	~85 minutes	At this stage the RT-RNA can be stored at -80°C for later use. Please note, this is the only pause point in this protocol.
Adapter ligation and clean-up	Attach the sequencing adapters to the RNA-cDNA hybrid ends	45 minutes	We strongly recommend sequencing your library as soon as it is adapted.
Priming and loading the flow cell	Prime the flow cell and load the prepared library for sequencing	10 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 basecall the reads.

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'.

Compatibility of this protocol

This protocol should only be used in combination with:

- Direct RNA Sequencing Kit (SQK-RNA004)
- PromethION Flow Cell RNA (FLO-PRO004RA)
- Flow Cell Wash Kit (EXP-WSH004) - this Wash Kit is compatible for removing library between washes but will not remove RNA-related blocking of nanopores.
- PromethION™ 24/48 - PromethION IT requirements
- PromethION™ 2 Solo - PromethION 2 Solo IT requirements
- PromethION 2 Integrated - PromethION 2 Integrated IT requirements

2. 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

MinION/GridION Flow Cells RNA (FLO-MIN004RA) or PromethION Flow Cells RNA (FLO-PRO004RA)
Flow Cell Wash Kit (EXP-WSH004) or Flow Cell Wash Kit XL (EXP-WSH004-XL)
Induro® Reverse Transcriptase and 5x Induro® RT Reaction Buffer (NEB, M0681)
10 mM dNTP solution (e.g. NEB N0447)
NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)
T4 DNA Ligase 2M U/ml (NEB, M0202M)
Murine RNase Inhibitor (NEB, M0314)
Agencourt RNAClean XP beads (Beckman Coulter®, A63987)
Nuclease-free water (e.g. Thermo Scientific, 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, Q32852)
Qubit™ dsDNA HS Assay Kit (ThermoFisher, Q32851)
Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

MinION, GridION or PromethION device
MinION/GridION Flow Cell Light Shield or PromethION Flow Cell Light Shield
Vortex mixer
Microfuge

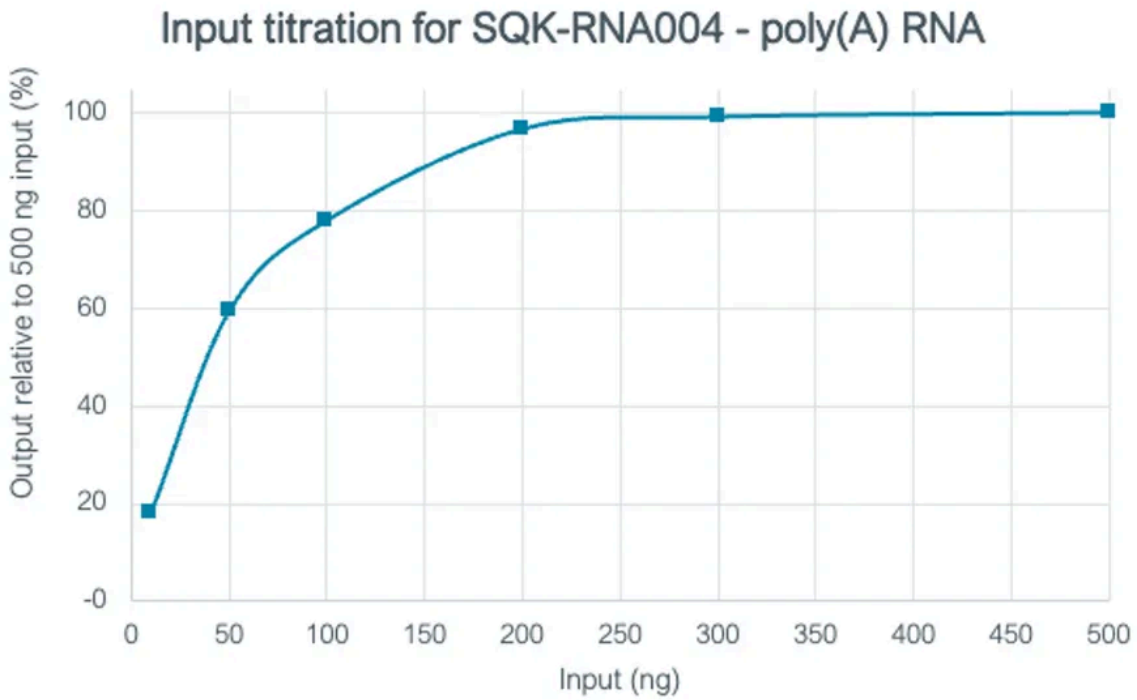
Hula mixer (gentle rotator mixer)
Magnetic separation rack, suitable for 1.5 ml Eppendorf tubes
Eppendorf 5424 centrifuge (or equivalent)
Ice bucket with ice
Timer
Thermal cycler
Qubit™ fluorometer (or equivalent for QC check)
P1000 pipette and tips
P200 pipette and tips
P100 pipette and tips
P20 pipette and tips
P10 pipette and tips
P2 pipette and tips

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

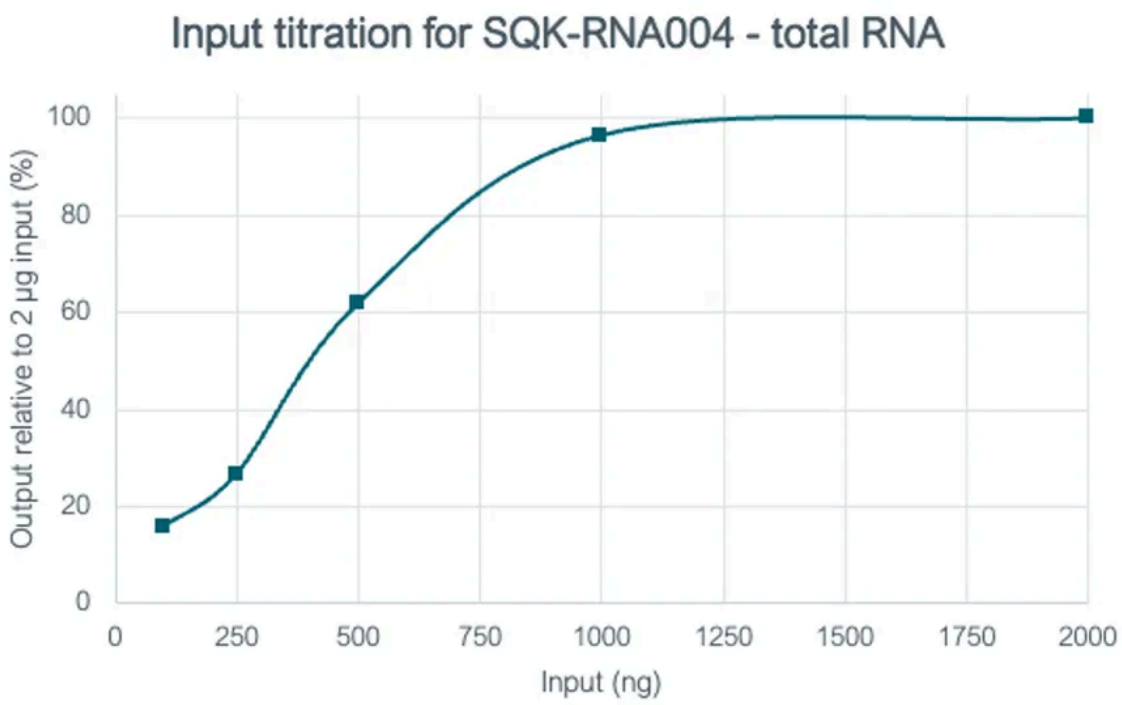
It is possible to start the protocol from a lower sample input, however this will likely yield a lower output.

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 for 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

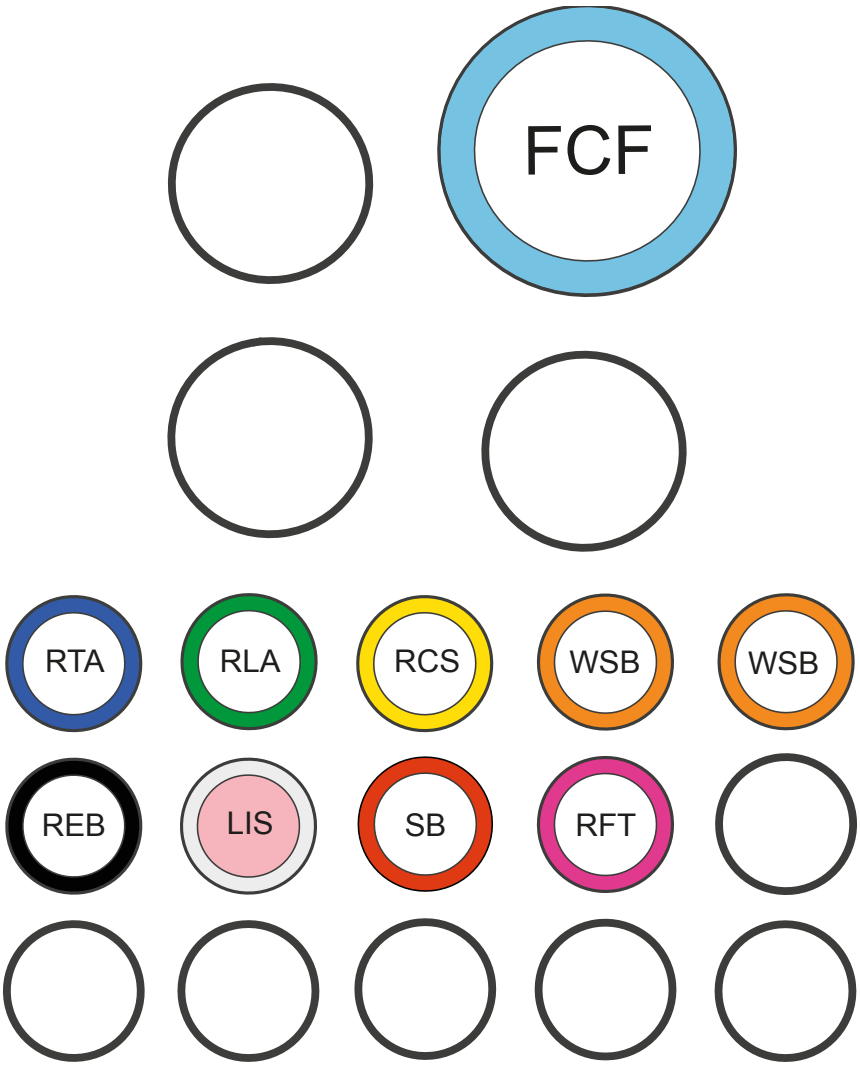
We highly recommend that you check the number of pores in your flow cell prior to starting a sequencing experiment. This should be done within 12 weeks of purchasing your MinION/GridION or PromethION Flow Cells RNA. Oxford Nanopore Technologies will replace any unused flow cell with fewer than the number of pores listed 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 perform the flow cell check, please follow the instructions in the Flow Cell Check document.

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

Direct RNA Sequencing Kit (SQK-RNA004) contents:

Note: We have increased the concentration of the RNA CS (RCS) in vials found in newer batches of SQK-RNA004. Please ensure you are following the correct method and inputs for your RCS concentration:

Batch RNA004.20.xxxx or older	Batch RNA004.30.0001 or newer
Lower concentration in the RNA CS (RCS) vial:	Higher concentration in the RNA CS (RCS) vial:
15 ng/µl	**50 ng/µl**



FCF: Flow Cell Flush
RTA: RT Adapter
RLA: RNA Ligation Adapter
RCS: RNA CS
WSB: Wash Buffer
SB: Sequencing Buffer
RFT: RNA Flush Tether
REB: RNA Elution Buffer
LIS: Library Solution

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

Note: The RNA CS (RCS) is the control strand and contains the Enolase II from YHR174W, extracted from the yeast *Saccharomyces cerevisiae*. The reference FASTA files for the yeast is available here.

3. Library preparation

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®, A63987)
Induro® Reverse Transcriptase and 5x Induro® RT Reaction Buffer (NEB, M0681)
10 mM dNTP solution (e.g. NEB N0447)
NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)
T4 DNA Ligase 2M U/ml (NEB, M0202M)
Murine RNase Inhibitor (NEB, M0314)
Nuclease-free water (e.g. Thermo Scientific, 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, Q32852)
Qubit™ dsDNA HS Assay Kit (ThermoFisher, Q32851)
Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

Vortex mixer
Microfuge
Thermal cycler
Hula mixer (gentle rotator mixer)
Magnetic separation 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
Eppendorf 5424 centrifuge (or equivalent)

Perform a flow cell check

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

To perform a flow cell check, please follow the instructions in the flow cell check document.

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 Quick Ligation Reaction Buffer at room temperature and place the T4 DNA Ligase on ice.
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 buffer to come to room temperature and then 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.

We do not recommend using the Quick T4 Ligase for this protocol. We have found that the T4 DNA Ligase (2M U/ml - NEB M0202M) works better. It needs to be used in combination with the Quick Ligation Reaction Buffer (NEB B6058).

2 Thaw and 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 as follows:

- 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.

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.

Please note: Depending on the concentration of RNA CS (RCS) in your kit, please ensure you are following the correct inputs for your RCS concentration:

We have increased the concentration of the RNA CS (RCS) in vials found in newer batches of SQK-RNA004 and EXP-RCS001.

Batch RNA004.20.xxxx or older	Batch RNA004.30.0001 or newer
Lower concentration of the RNA CS (RCS) in vial:	Higher concentration of the RNA CS (RCS) in vial:
15 ng/µl	**50 ng/µl**

Batch RCS001.10.xxxx or older	Batch RCS001.20.0001 or newer
Lower concentration of the RNA CS (RCS) in vial:	Higher concentration of the RNA CS (RCS) in vial:
15 ng/µl	**50 ng/µl**

5 Prepare the RT Adapter (RTA) reaction as follows:

If you choose not to use the RNA CS (RCS) input, replace the RCS volume in the RTA reaction mix with nuclease-free water.

For the higher concentration of RNA CS (RCS): kit batch RNA004.30.0001 or newer:

1. In a separate, clean 0.2 ml thin-walled PCR tube, dilute the RNA CS (RCS) input as follows:

Reagent	Volume
RNA CS (RCS)	1 µl
Nuclease-free water	2 µl
Total	**3 µl**

2. Ensure the sample is thoroughly mixed by pipette mixing 10 times.

3. In the 0.2 ml thin-walled PCR tube containing your RNA sample, combine the reagents in the following order:

Reagent	Volume
RNA from previous step	8 µl
NEBNext Quick Ligation Reaction Buffer	3 µl
Diluted RNA CS (RCS) or nuclease-free water	0.5 µl
Murine RNase Inhibitor	1 µl
RT Adapter (RTA)	1 µl
T4 DNA Ligase	1.5 µl
Total	**15 µl**

For the lower concentration of RNA CS (RCS): kit batch RNA004.20.xxxx or older:

1. In the 0.2 ml thin-walled PCR tube containing your RNA sample, combine the reagents in the following order:

Reagent	Volume
RNA	8 µl
NEBNext Quick Ligation Reaction Buffer	3 µl
RNA CS (RCS) or nuclease free water	0.5 µl
Murine RNase Inhibitor	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 Eppendorf DNA LoBind tube, combine the following reagents together to prepare the reverse transcription mix:**

Reagent	Volume
Nuclease-free water	13 µl
10 mM dNTPs	2 µl
5x Induro RT reaction buffer	8 µl
Total	**23 µl**

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

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

11 **Place the tubes in a thermal cycler and incubate at 60°C for 30 minutes, then 70°C for 10 minutes. Bring the samples to 4°C before proceeding to the next step.**

12 **Spin down the tubes and transfer each 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 each reverse transcribed reaction and mix by pipetting.**

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

16 **For each sample, freshly prepare 200 µl of 70% ethanol in nuclease-free water.**

17 **Spin down the samples and pellet on a magnetic rack. Keeping the tubes on the magnetic rack, pipette off the supernatant when clear and colourless.**

18 **Keep the tubes on the magnetic rack and wash the beads in each tube with 150 µl of freshly prepared 70% ethanol, as described below:**

1. Add 150 µl of freshly prepared 70% ethanol to each tube and ensure the beads are pelleted on one side of the tube.
2. Keeping the magnetic rack on the benchtop, rotate the tubes by 180°. Wait for the beads to migrate towards the magnet and to form a pellet.
3. Rotate the tubes 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 tubes back on the magnetic rack and wait until the eluate is clear and colourless. Keep the tube(s) on the magnetic rack and pipette off any residual ethanol.**

21 **Remove the tubes from the magnetic rack and resuspend each pellet in 23 µl nuclease-free water. Incubate for 5 minutes at room temperature.**

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

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

> **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	23 µl
NEBNext Quick Ligation Reaction Buffer	8 µl
RNA Ligation Adapter (RLA)	6 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 each reaction and mix by pipetting.**

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

30 **Spin down the samples and pellet on the magnetic rack. Keep the tubes on the magnetic rack for 5 minutes and pipette off the supernatant when clear and**

colourless.

31 **Remove the tubes from the magnetic rack and add 150 µl of the Wash Buffer (WSB) to the beads in each tube. Close the tube lids and resuspend the beads by flicking the tube. Return the tubes to the magnetic rack and allow the beads to pellet for 5 minutes. Pipette off the supernatant when clear and colourless.**

32 **Repeat the previous step.**

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

33 **Spin down the tubes and replace onto the magnetic rack and wait until the beads have pelleted. Pipette off any remaining Wash Buffer (WSB).**

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

35 **Spin down and pellet the beads on the magnetic rack 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 on a Qubit™ fluorometer using the Qubit™ dsDNA 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.

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

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

4. Priming and loading the PromethION Flow Cell

Materials

Sequencing Buffer (SB)
Library Solution (LIS)
Flow Cell Flush (FCF)
RNA Flush Tether (RFT)

Consumables

PromethION Flow Cell RNA (FLO-PRO004RA)
1.5 ml Eppendorf DNA LoBind tubes

Equipment

PromethION 2 Solo or PromethION 24/48 device
PromethION Flow Cell Light Shield
Vortex mixer
Microfuge
P1000 pipette and tips
P200 pipette and tips
P100 pipette and tips
P20 pipette and tips

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

After taking the flow cells out of the fridge, wait 20 minutes for the flow cells to reach room temperature before inserting into the PromethION. 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.

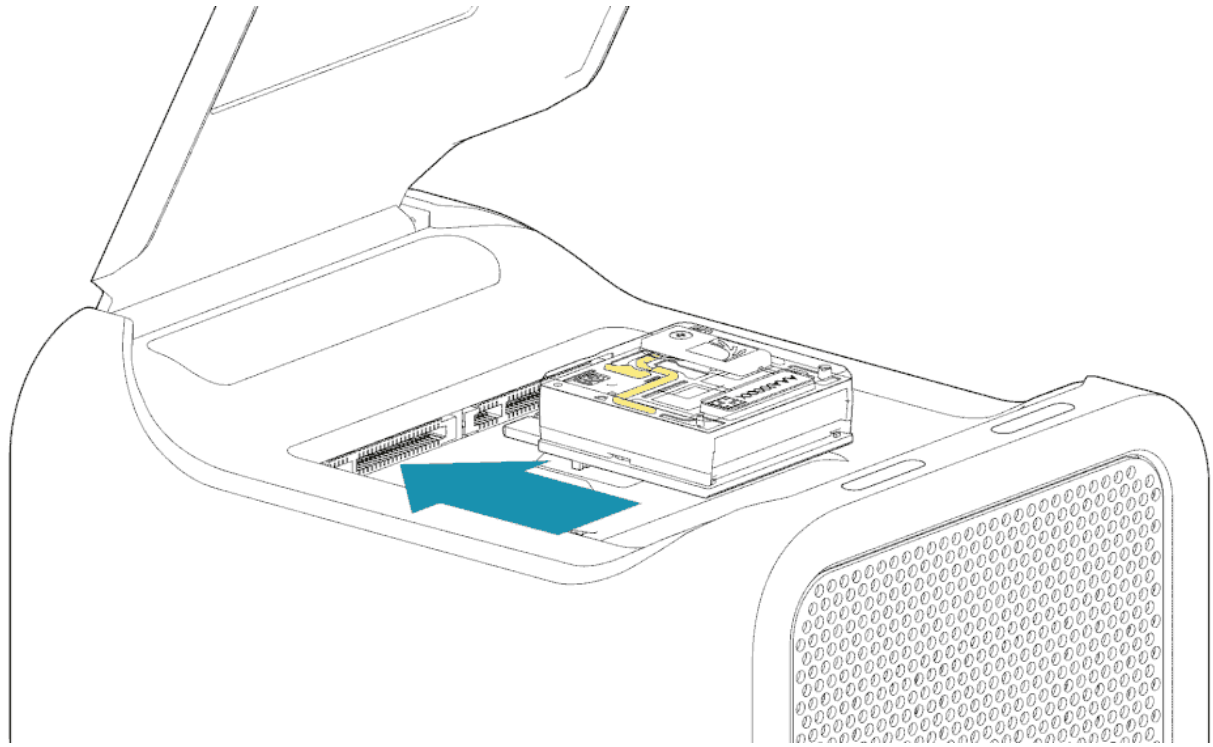
1. **Thaw the Sequencing Buffer (SB), Library Solution (LIS), RNA Flush Tether (RFT) and Flow Cell Flush (FCF) at room temperature. Mix by vortexing and spin down where applicable.**

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

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

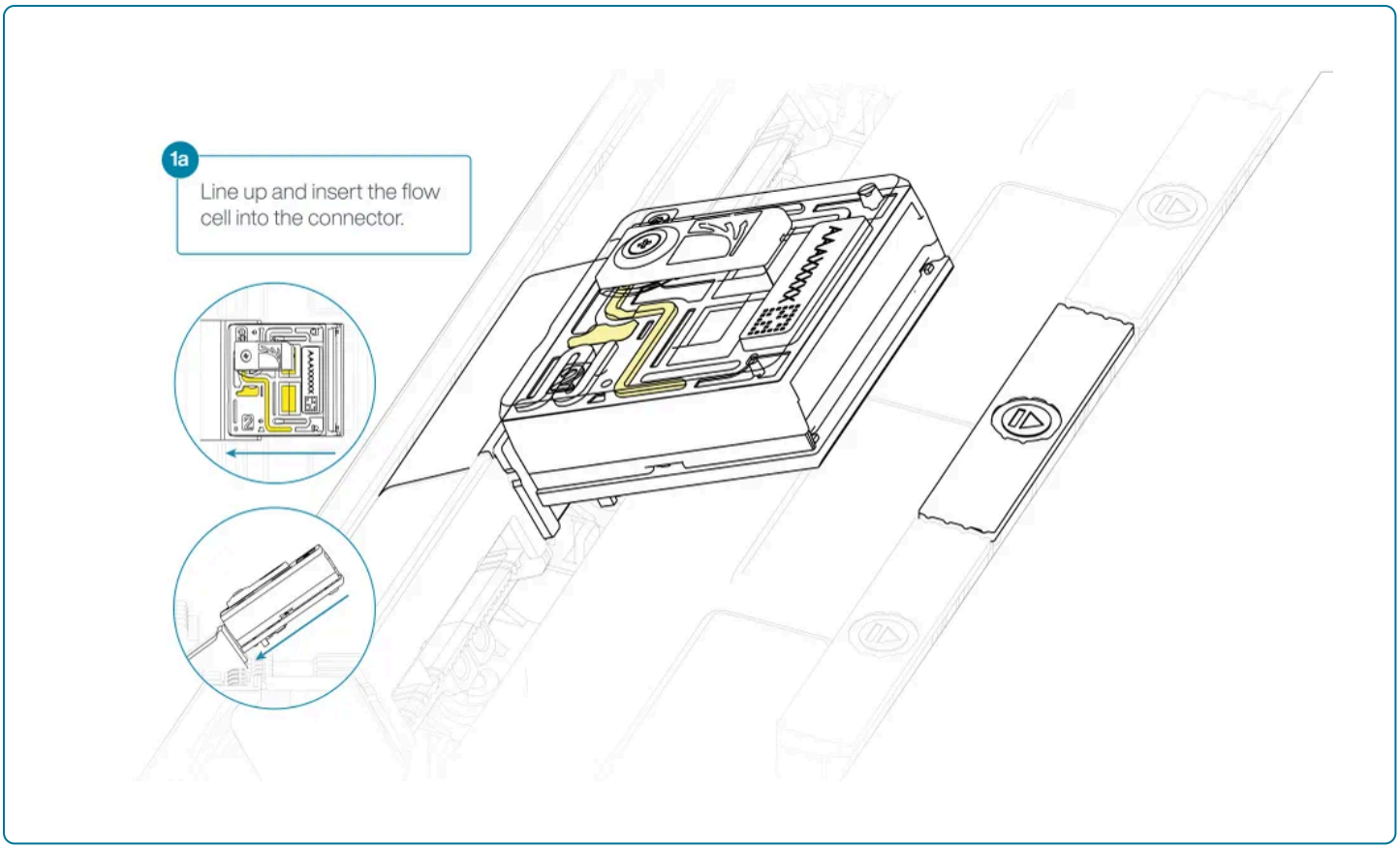
3 For the PromethION 2 Solo and PromethION 2 Integrated, load the flow cell(s) as follows:

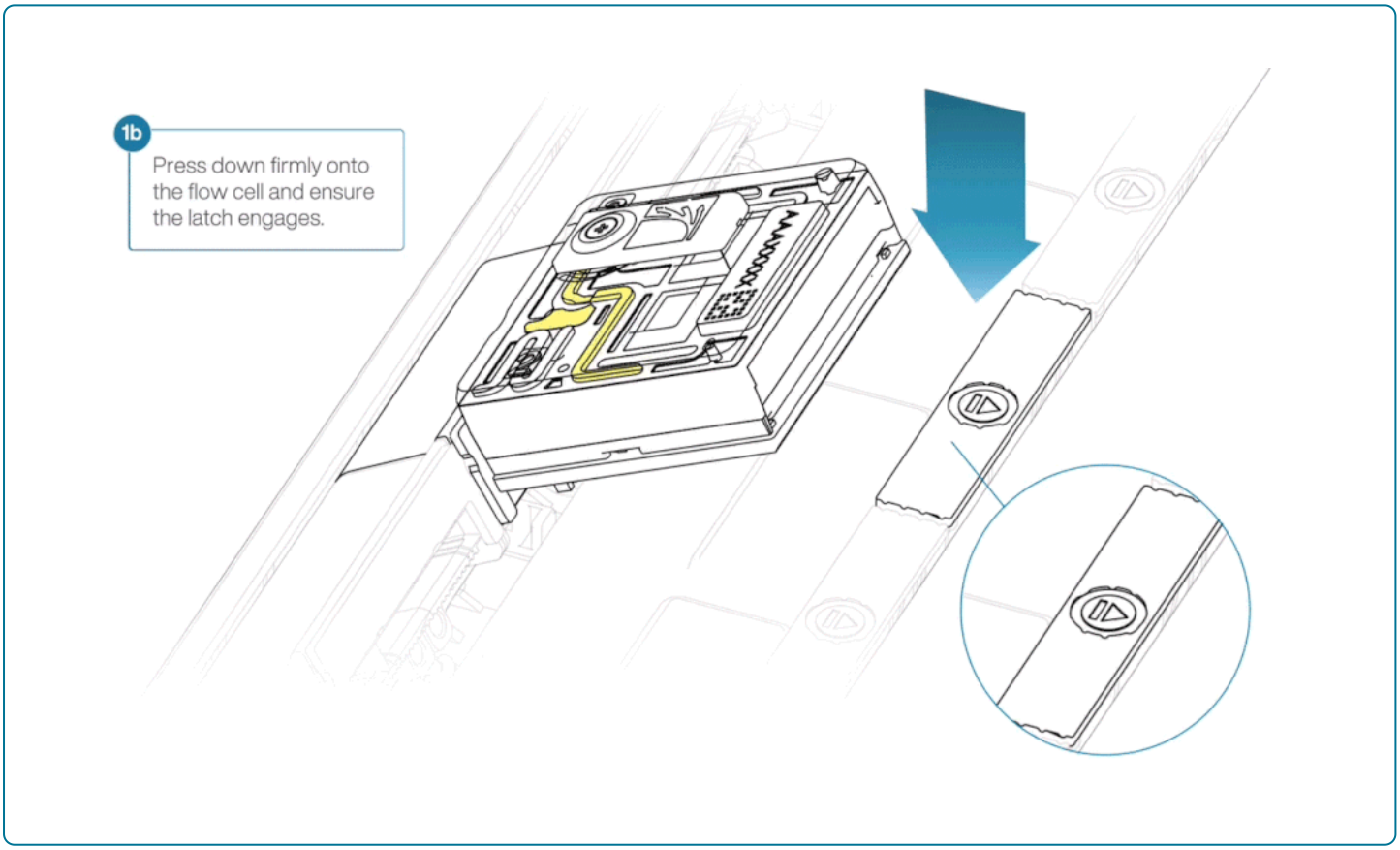
1. Place the flow cell flat on the metal plate.
2. Slide the flow cell into the docking port until the gold pins or green board cannot be seen.



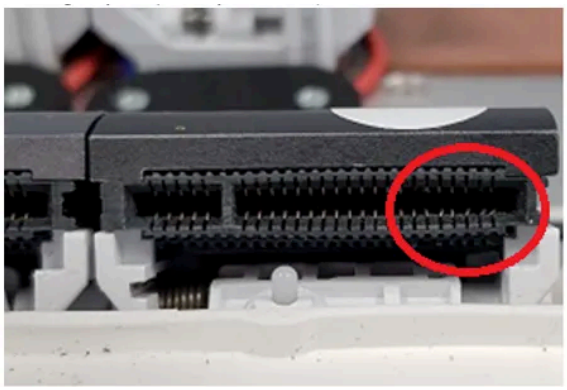
4 For the PromethION 24/48, load the flow cell(s) into the docking ports as follows:

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.


1a
Line up and insert the flow cell into the connector.


1b
Press down firmly onto the flow cell and ensure the latch engages.

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.

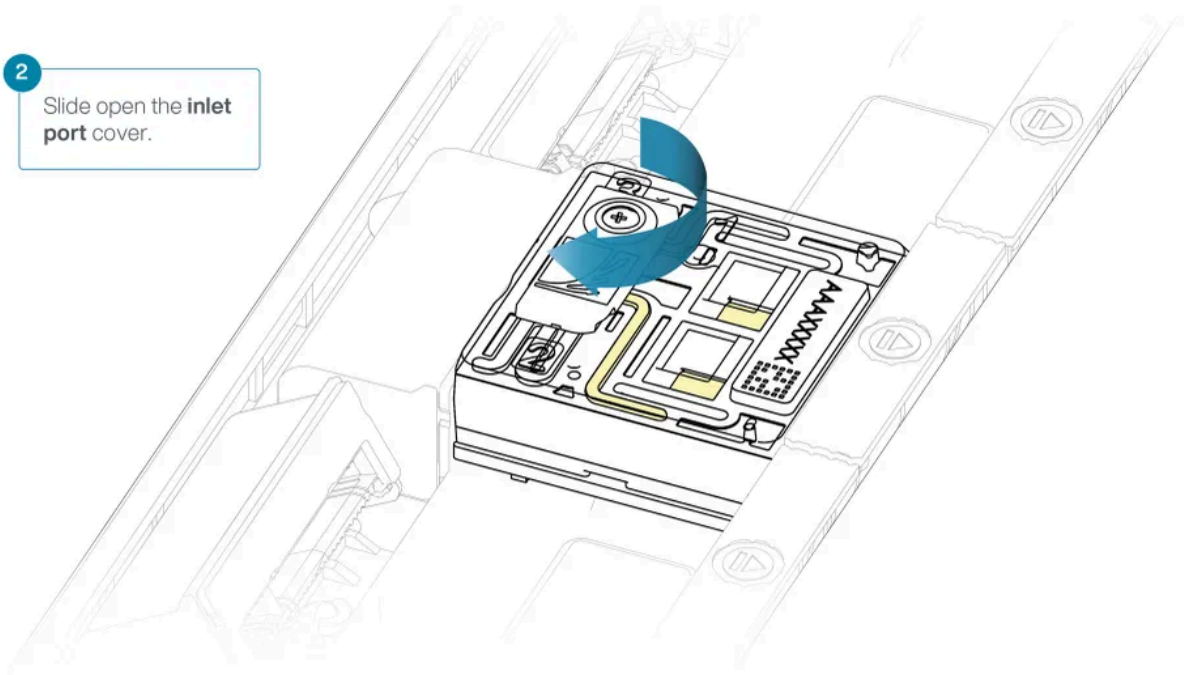


Complete a flow cell check to assess the number of pores available before loading the library.

This step can be omitted if the flow cell has been checked previously.

See the flow cell check document for more information.

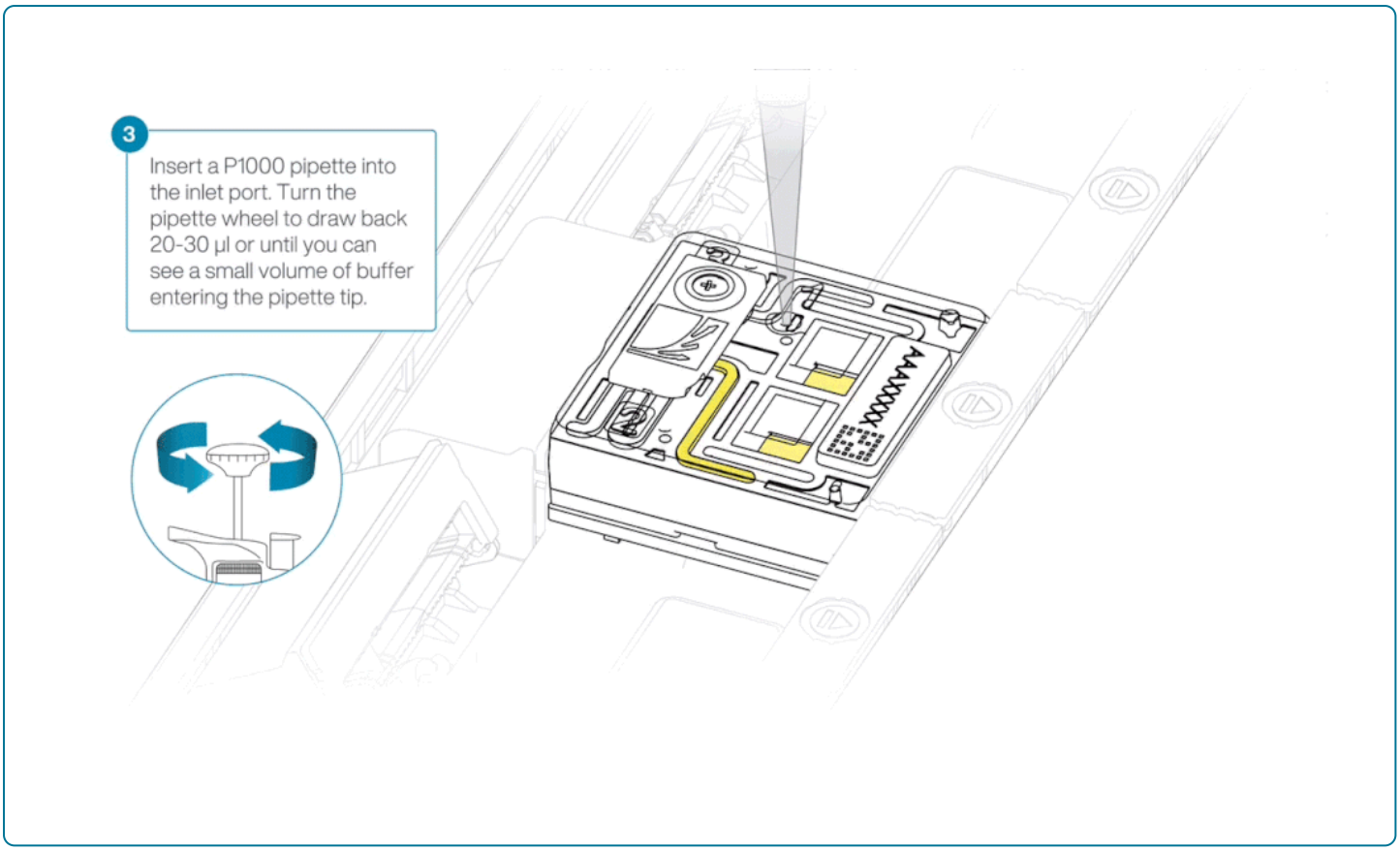
5 Slide the inlet port cover clockwise to open.



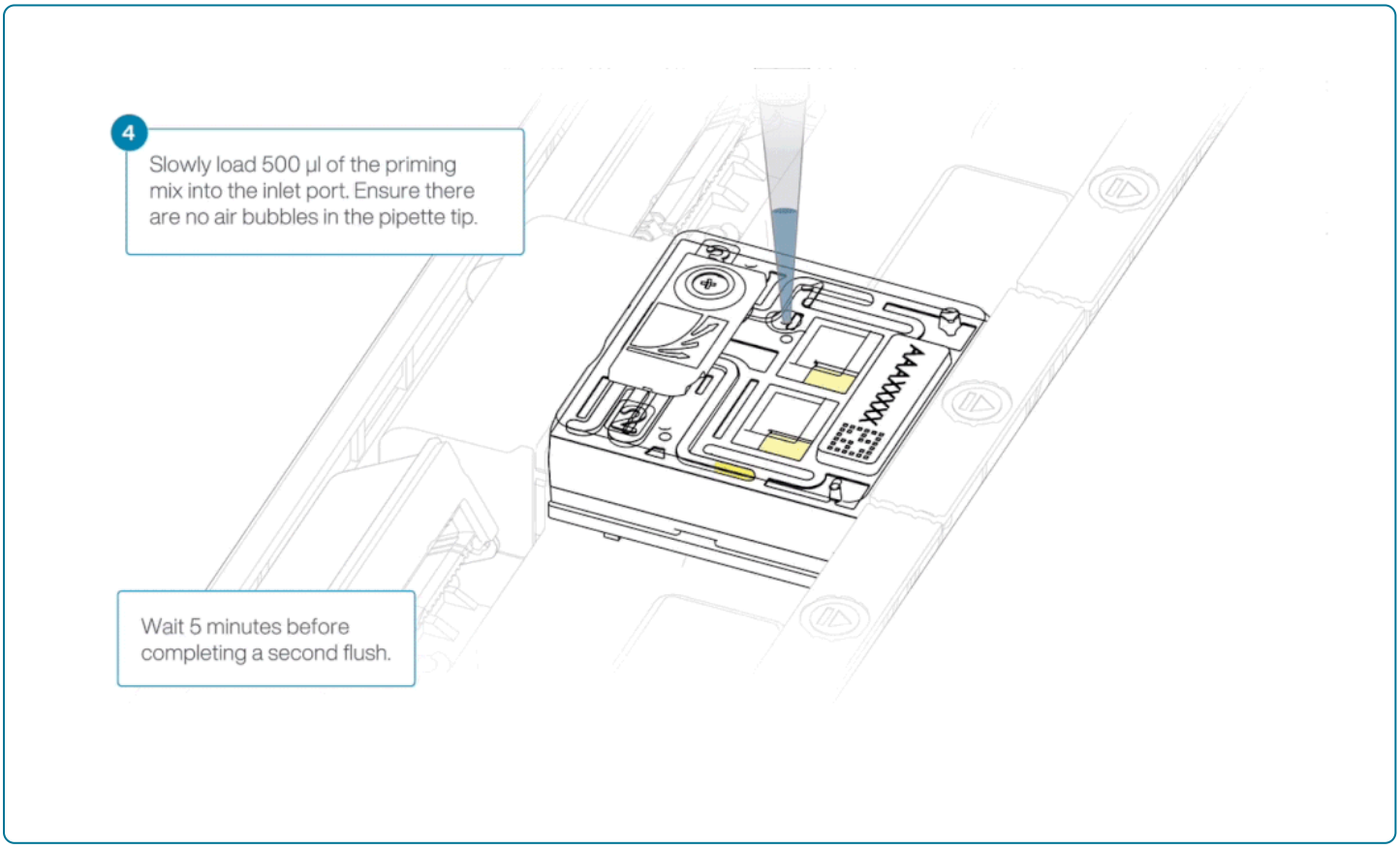
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage the pores.

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

1. Set a P1000 pipette with 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.



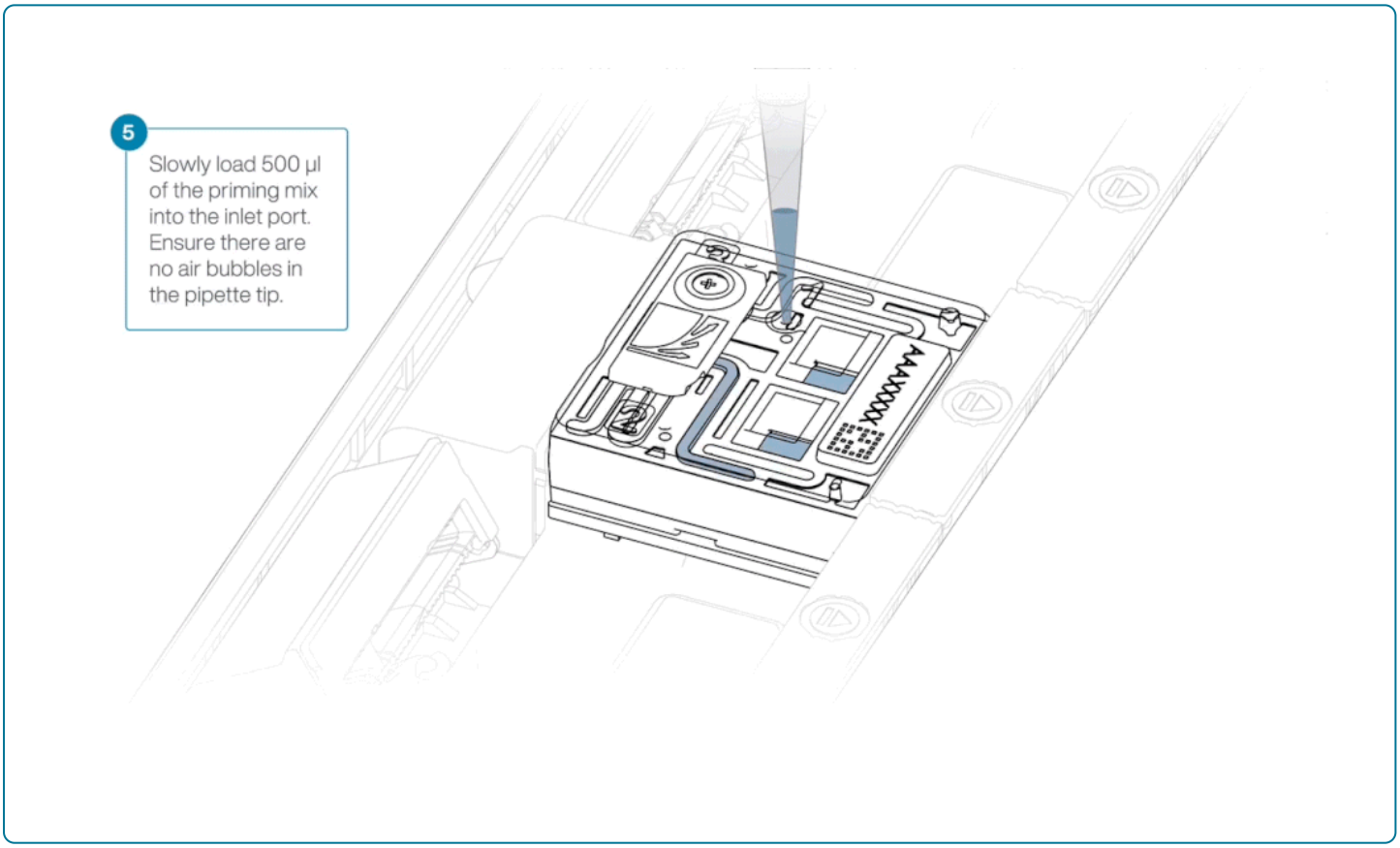
7 Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes. During this time, prepare the library for loading using the next steps in the protocol.



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

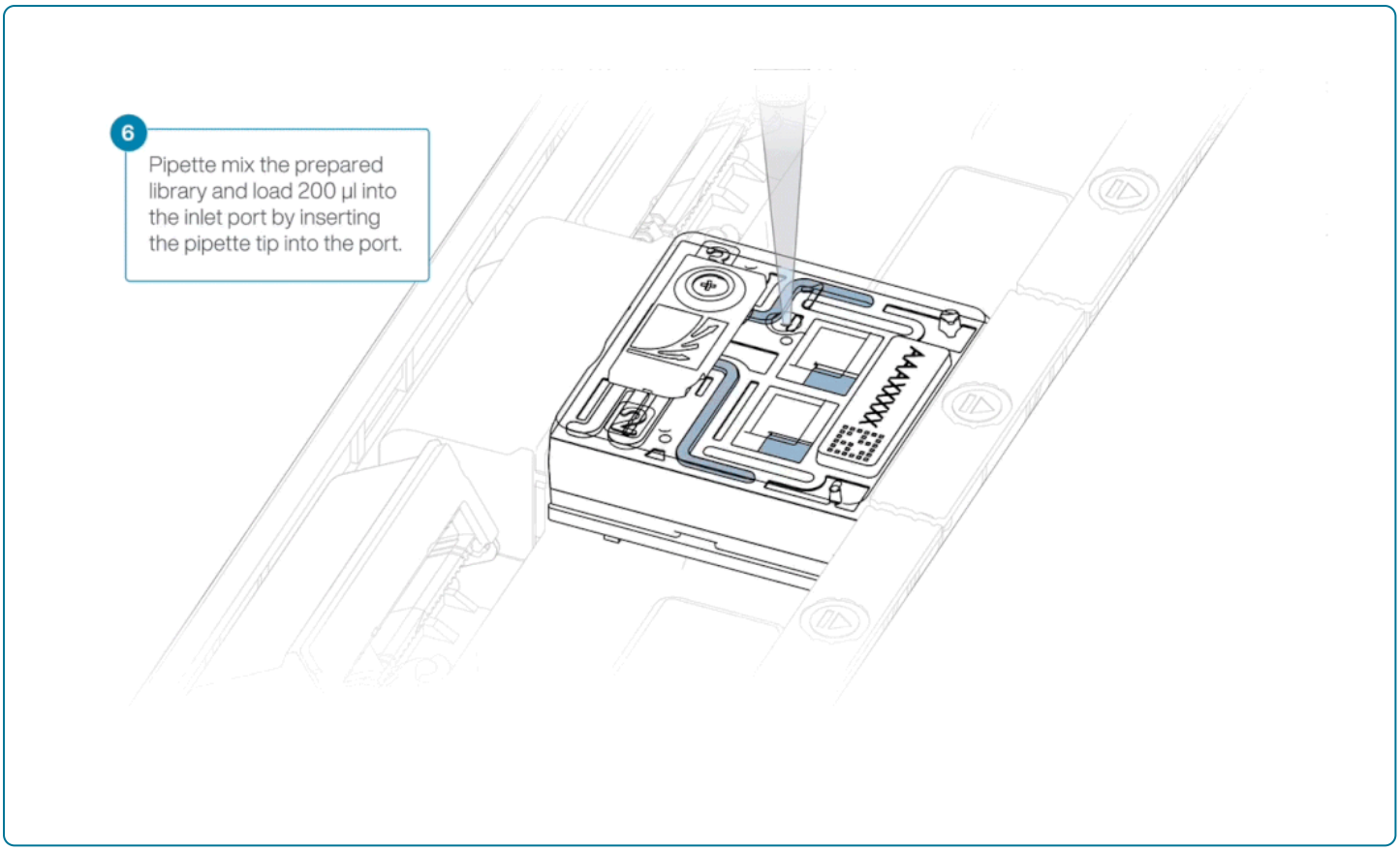
Reagent	Volume per flow cell
Sequencing Buffer (SB)	100 µl
Library Solution (LIS)	68 µl
RNA library	32 µl
Total	**200 µl**

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

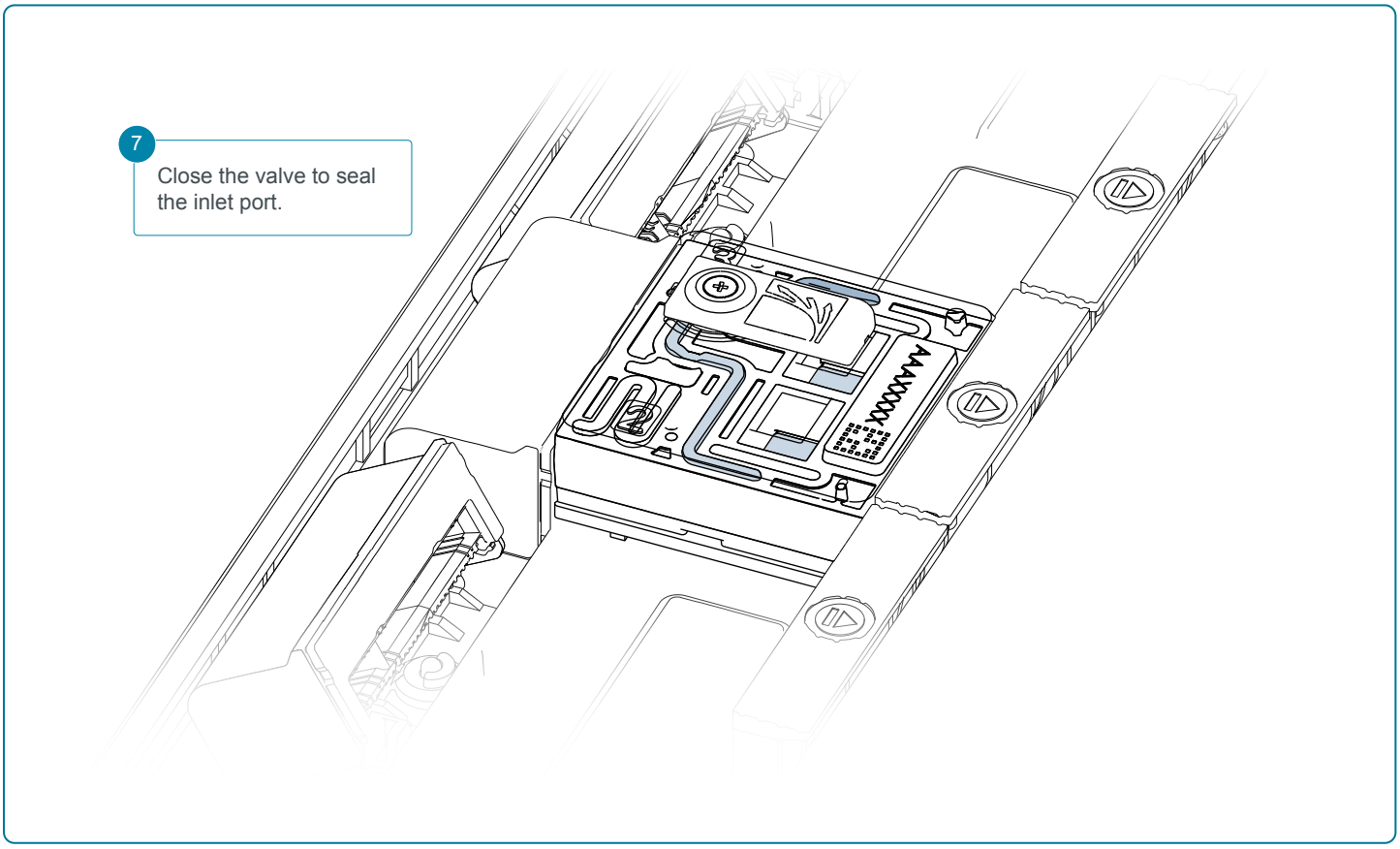


10 Mix the prepared library by gently pipetting up and down, just prior to loading.

11 Load 200 µl of library into the inlet port using a P1000 pipette.



12 Close the valve to seal the inlet port.

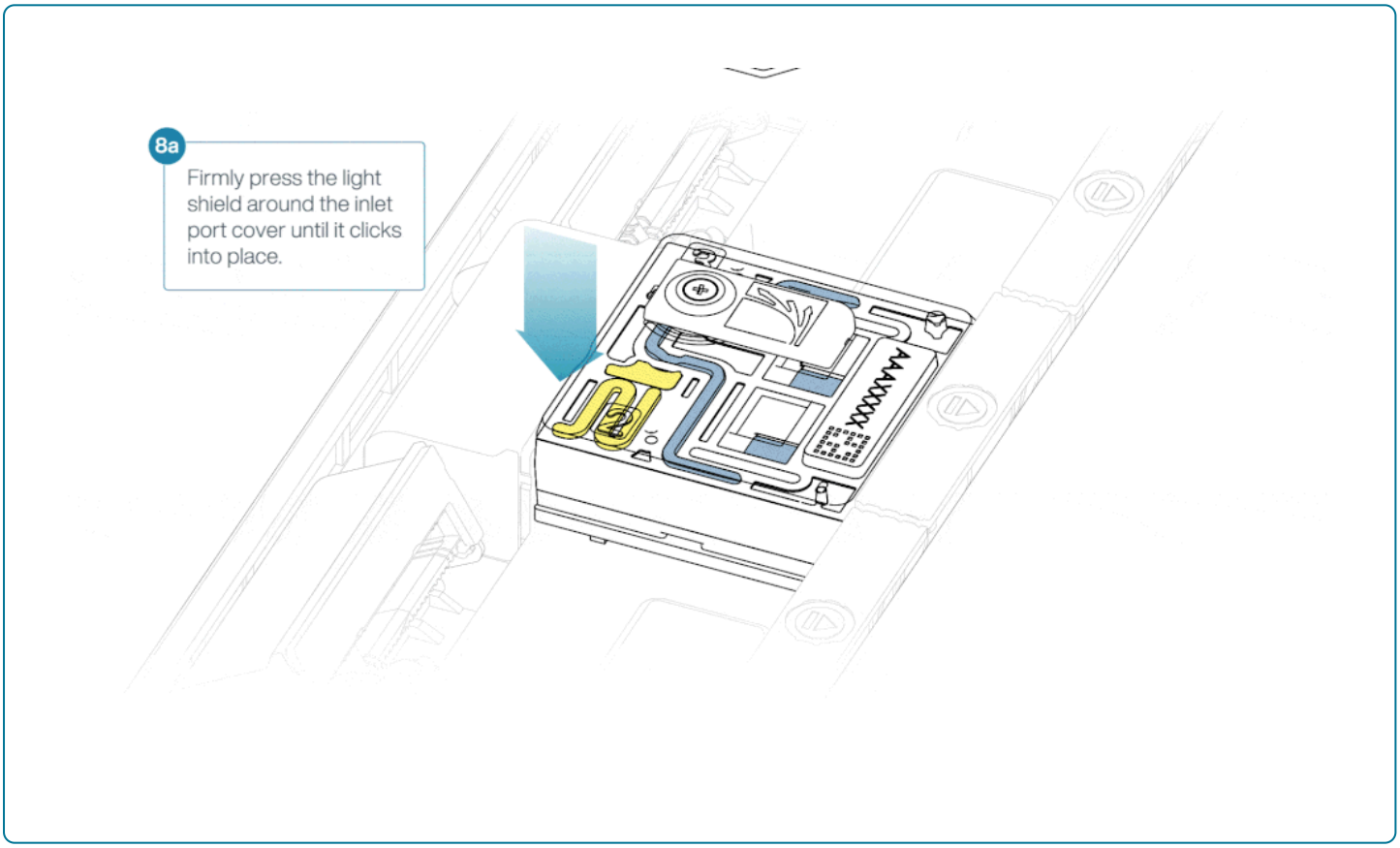


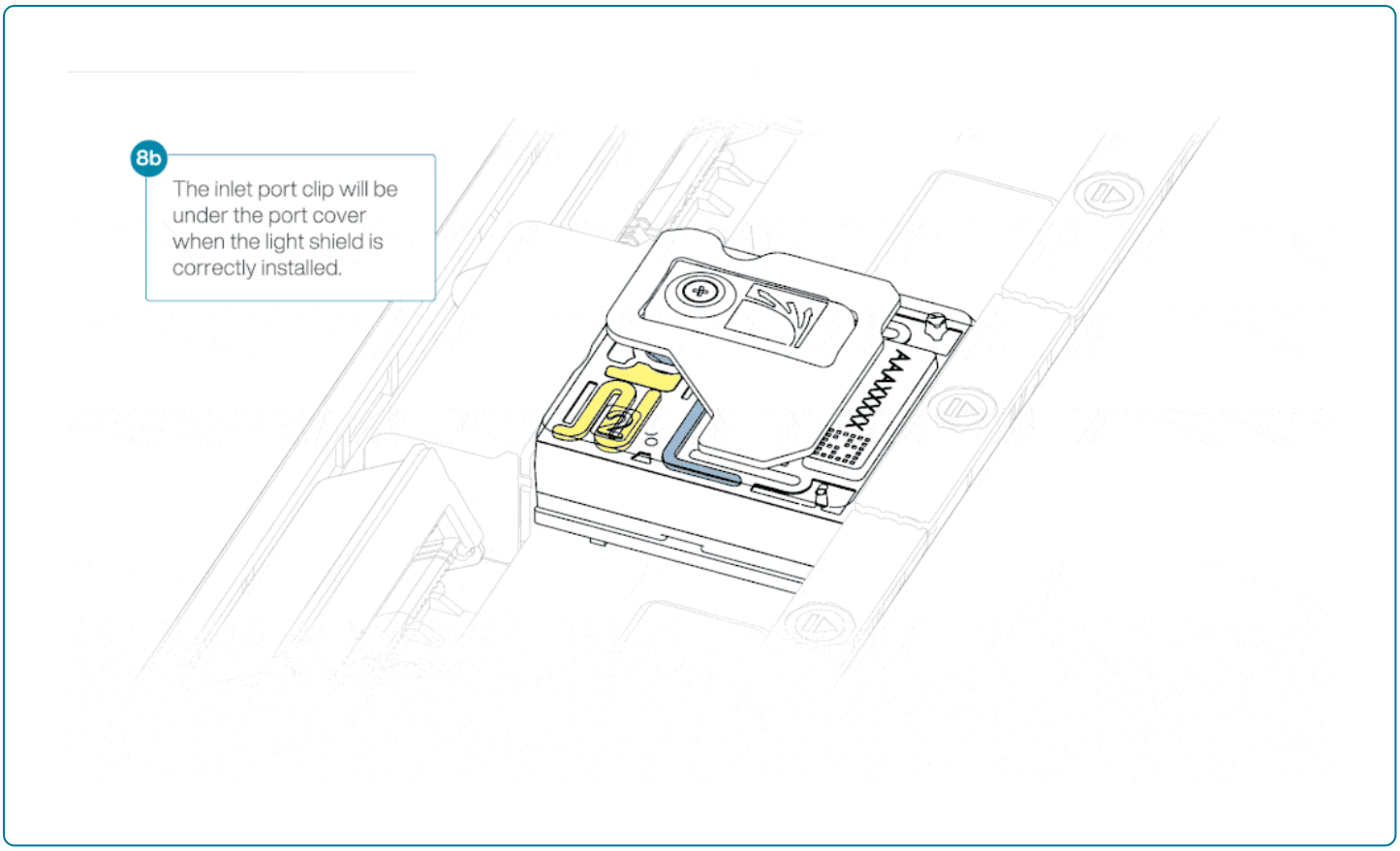
For optimal sequencing output, install the light shield on your flow cell as soon as the library has been loaded.

We recommend leaving the light shield on the flow cell after the 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.





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.

5. 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 direcly or remotely connected to a sequencing device.
- Directly on a GridION or PromethION 24/48 sequencing device.

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

- PromethION 24/48 user manual
- PromethION 2 Solo user manual
- PromethION 2 Integrated 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 **Direct RNA Sequencing Kit (SQK-RNA004)** on the Kit selection page.

4. Configure the sequencing and output parameters for your sequencing run or keep to the default settings on the Run configuration tab.

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. Click **Start** to initiate 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.

6. Flow cell reuse and returns

Materials Flow Cell Wash Kit (EXP-WSH004) or Flow Cell Wash Kit XL (EXP-WSH004-XL)

> **Our Flow Cell Wash Kit (EXP-WSH004 or EXP-WSH004-XL) is compatible with RNA Flow Cells and the Direct RNA Sequencing Kit (SQK-RNA004).**
>
> However, please be aware that:
>
> - The DNase I present in the wash kit will not help to recover blocked pores in your flow cell following Direct RNA sequencing.
> - Instead, it will wash off most of the library from the array and remove all adapter from the remaining sample, preventing it from being recaptured and sequenced. This will allow a subsequent library to be loaded.

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°C to +8°C.**

The Flow Cell Wash Kit protocol is available on the Nanopore Community.

> **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 send the flow cell back to Oxford Nanopore.**

Instructions for returning flow cells can be found here.

> **If you encounter issues or have questions about your sequencing experiment, please refer to the Troubleshooting Section in this protocol.**

7. Downstream analysis

Post-basecalling analysis

There are several options for further analysing your basecalled data:

EPI2ME workflows

For in-depth data analysis, Oxford Nanopore Technologies offers a range of bioinformatics tutorials and workflows available in EPI2ME. 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.

Research analysis tools

The Research division at Oxford Nanopore Technologies 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.

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 resource centre and search for bioinformatics tools for your application. 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.

8. 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.

9. 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 videos for how to load a MinION Flow Cell and how to load a PromethION Flow Cell.
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"
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 how to load a MinION Flow Cell or how to load a PromethION Flow Cell videos 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 link for more information on MinION temperature control.

English

ONT plc is a member of the producer compliance scheme run by ERP UK Ltd, who manage the submission of documentation in support of WEEE compliance for ONT plc's manufacture and supply of Electrical and Electronic equipment in the UK.

ONT's WEEE PRN is WEE/MM3828AA. See attached certificate for the submission period 2022.

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