



cDNA-PCR Sequencing V14 - Barcoding (SQK-PCB114.24)

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This document describes the fastest and simplest protocol for full-length cDNA sequencing.

The protocol:

- Offers the highest yield
- Has higher yields than traditional cDNA synthesis
- Enables analysis of splice variants and fusion transcripts
- Allows multiplexing of up to 24 different samples
- Is compatible with R10.4.1 flow cells only

For Research Use Only

FOR RESEARCH USE ONLY

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1. Overview of the protocol



This is an Early Access product.

For more information about our Early Access programmes, please see [this article on product release phases](#).

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

Please note: As of March 2026, the method used with our cDNA-PCR Barcoding Kit 24 V14 (SQK-PCB114.24) has been updated extensively, utilising new third-party reagents and new PCR conditions. We strongly recommend using this updated method for the best results. However, if you would like to continue using the old method, please refer to the **Document versions** section of this protocol.

Introduction to the cDNA-PCR Barcoding Kit 24 V14 protocol

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

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

Steps in the sequencing workflow:

Prepare for your experiment

You will need to:

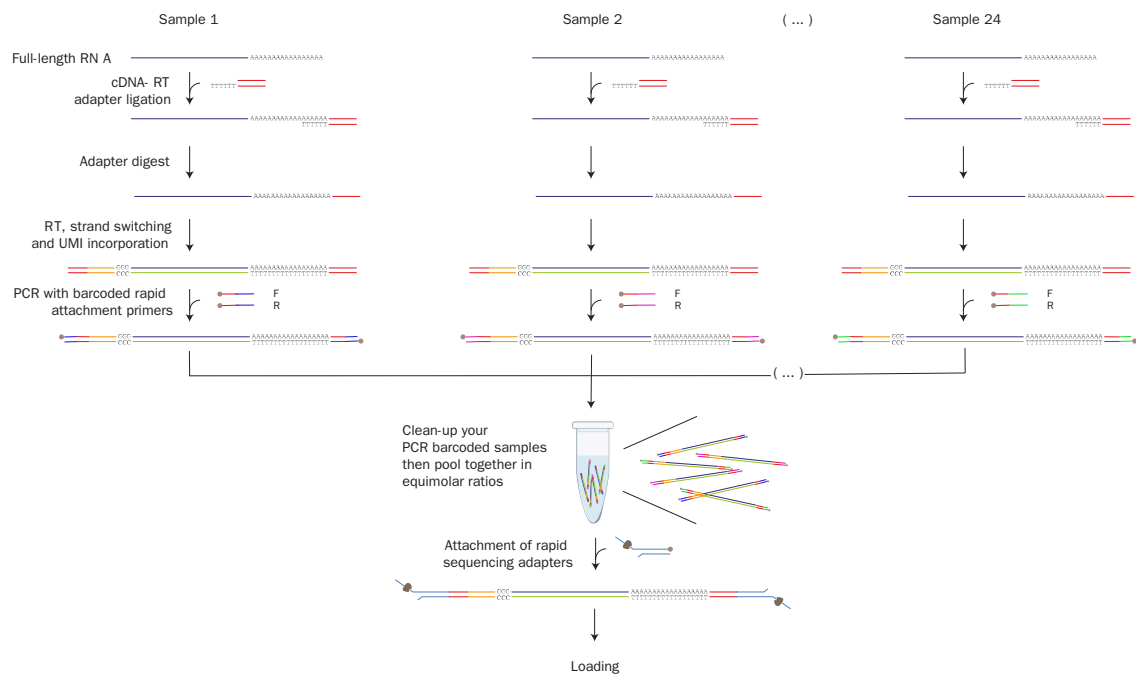
- Extract your RNA, and check its length, quantity and purity using the [Input DNA/RNA QC protocol](#). **The quality checks performed during the protocol are essential in ensuring experimental success.**
- Ensure you have your sequencing kit, the correct equipment and third-party reagents.
- Download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

Library preparation

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

Please note: Timings are indicative for an experienced user, utilising multichannel pipettes and magnetic separators suitable for PCR tubes or PCR plates. Timings may vary depending on user experience, number of samples multiplexed in your preparation, and the equipment available. Please ensure you have sufficient time to carry out the library preparation in one day, or pause your library experiment at an appropriate stage to continue the following day.

Library preparation step	Process	Time	Stop option
Reverse transcription and strand-switching	Prepare full-length cDNA from Poly(A)+ RNA (or total RNA) with the incorporation of the UMI.	170 minutes	-20°C overnight
Selecting for full-length transcripts by PCR	Amplify the cDNA by PCR using rapid attachment barcode primers during the PCR step.	170 minutes	4°C short-term storage or for repeated use, such as re-loading your flow cell. -80°C for single-use long-term storage.
Rapid adapter attachment	Attach the sequencing adapters to the PCR products.	10 minutes	We strongly recommend sequencing your library as soon as it is adapted.
Priming and loading the flow cell	Prime the flow cell and load the prepared cDNA 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 convert it into basecalled reads.
- **Optional:** Start the EPI2ME software and select a workflow for further analysis.



Compatibility of this protocol

This protocol should only be used in combination with:

- cDNA-PCR Barcoding Kit 24 V14 (SQK-PCB114.24)
- R10.4.1 flow cells (FLO-MIN114)
- Flow Cell Wash Kit (EXP-WSH004)
- RNA Control Expansion (EXP-RCS001)
- Rapid Adapter Auxiliary V14 (EXP-RAA114)
- Sequencing Auxiliary Vials V14 (EXP-AUX003)
- Flow Cell Priming Kit V14 (EXP-FLP004)
- MinION Mk1D - [MinION Mk1D IT requirements](#)
- GridION - [GridION IT requirements](#)

2. Equipment and consumables

Materials

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

Consumables

MinION/GridION Flow Cell
T3 DNA Ligase (NEB, M0317)
Lambda Exonuclease (NEB, M0262L)
USER (Uracil-Specific Excision Reagent) Enzyme (NEB, M5505L)
10 mM dNTP solution (e.g. NEB N0447)
TaKaRa Ex Premier DNA Polymerase (TaKaRa, RR370A/RR370B)
Maxima H Minus Reverse Transcriptase (200 U/μl) with 5x RT Buffer (ThermoFisher, EP0751)
RNaseOUT™, 40 U/μl (Life Technologies, 10777019)
Thermolabile Exonuclease I (NEB, M0568)
Agencourt RNAClean XP beads (Beckman Coulter®, A63987)
Agencourt AMPure XP beads (Beckman Coulter™, A63881)
Nuclease-free water (e.g. Thermo Scientific, AM9937)
Bovine Serum Albumin (BSA) (50 mg/ml) (e.g. Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
Freshly prepared 80% ethanol in nuclease-free water
1.5 ml Eppendorf DNA LoBind tubes
0.2 ml thin-walled PCR tubes
Qubit™ dsDNA HS Assay Kit (ThermoFisher, Q32851)
Qubit™ RNA HS Assay Kit (ThermoFisher, Q32852)
Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

MinION or GridION device
MinION/GridION Flow Cell Light Shield
Hula mixer (gentle rotator mixer)
Magnetic separation rack, suitable for 1.5 ml Eppendorf tubes
OR magnetic separator suitable for 0.2 ml PCR tube strips, e.g. DynaMag™-PCR Magnet (Thermo Fisher, 492025) or DynaMag™-96 Side Magnet (Thermo Fisher, 12331D)
Microfuge
Vortex mixer
Thermal cycler
Qubit™ fluorometer (or equivalent for QC check)
Agilent Bioanalyzer (or equivalent)
Multichannel pipettes suitable for dispensing 20–200 μl, and tips
P1000 pipette and tips
P200 pipette and tips
P100 pipette and tips
P20 pipette and tips

P10 pipette and tips
P2 pipette and tips
Ice bucket with ice
Timer

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

Third-party reagents

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

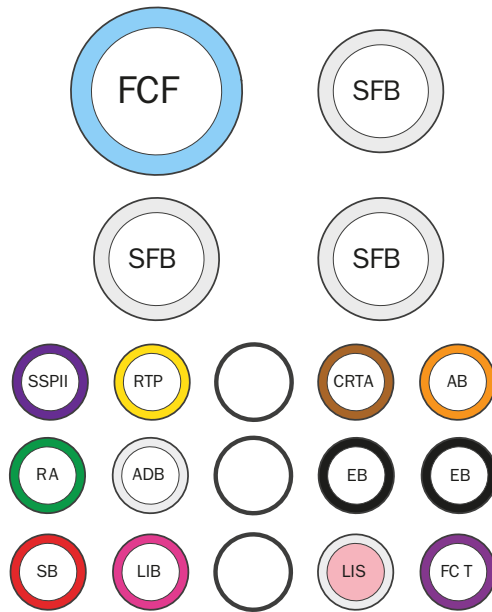
For all third-party reagents, we recommend following the manufacturer's instructions to prepare the reagents for use.

Check your flow cell

We highly recommend that you check the number of pores in your flow cell prior to starting a sequencing experiment. This should be done within 12 weeks of purchasing your MinION/GridION/PromethION Flow Cells. 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 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
MinION/GridION Flow Cell	800
PromethION Flow Cell	5000

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



	A	B	C	D	E	F	G	H
01	1	2	3	4	5	6	7	8
02	9	10	11	12	13	14	15	16
03	17	18	19	20	21	22	23	24
04	1	2	3	4	5	6	7	8
05	9	10	11	12	13	14	15	16
06	17	18	19	20	21	22	23	24
07	1	2	3	4	5	6	7	8
08	9	10	11	12	13	14	15	16
09	17	18	19	20	21	22	23	24
10	1	2	3	4	5	6	7	8
11	9	10	11	12	13	14	15	16
12	17	18	19	20	21	22	23	24

SSPII: Strand Switching Primer II
 RTP: RT Primer
 CRA: cDNA RT Adapter
 AB: Annealing Buffer
 RA: Rapid Adapter
 ADB: Adapter Buffer
 EB: Elution Buffer

SB: Sequencing Buffer
 LIB: Library Beads
 LIS: Library Solution
 SFB: Short Fragment Buffer
 FCF: Flow Cell Flush
 FCT: Flow Cell Tether
 Barcode Primer Plate: BP01-BP24

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Strand Switching Primer II	SSPII	Violet	1	350
RT Primer	RTP	Yellow	1	200
cDNA RT Adapter	CRTA	Amber	1	200
Annealing Buffer	AB	Orange	1	200
Rapid Adapter	RA	Green	1	15
Adapter Buffer	ADB	Clear	1	100
Elution Buffer	EB	Black	2	500
Short Fragment Buffer	SFB	Clear	3	13,000
Sequencing Buffer	SB	Red	1	700
Library Beads	LIB	Pink	1	600
Library Solution	LIS	White cap, pink label	1	600
Flow Cell Tether	FCT	Purple	1	200
Flow Cell Flush	FCF	Clear cap, light blue label	1	15,500
Barcode Primers 1-24 at 1 µM	BP01-24	-	2 plates, 3 sets of primer barcodes per plate	12 µl per well

3. Reverse transcription and strand-switching

Materials 10 ng enriched RNA (Poly(A)+ RNA or ribodepleted) or 500 ng total RNA per sample

cDNA RT Adapter (CRTA)
Annealing Buffer (AB)
S Fragment Buffer (SFB)
RT Primer (RTP)
Strand Switching Primer II (SSPII)

Consumables

T3 DNA Ligase (NEB, M0317)
RNaseOUT™, 40 U/μl (Life Technologies, 10777019)
Lambda Exonuclease (NEB, M0262L)
USER (Uracil-Specific Excision Reagent) Enzyme (NEB, M5505L)
10 mM dNTP solution (e.g. NEB, N0447)
Maxima H Minus Reverse Transcriptase (200 U/μl) with 5x RT Buffer (ThermoFisher, EP0752)
Nuclease-free water (e.g. ThermoFisher, AM9937)
Agencourt RNAClean XP beads (Beckman Coulter®, A63987)
Agencourt AMPure XP beads (Beckman Coulter, A63881)
Freshly prepared 80% 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™ Assay Tubes (Invitrogen, Q32856)

Equipment

Microfuge
Thermal cycler
Qubit™ fluorometer (or equivalent for QC check)
P1000 pipette and tips
P200 pipette and tips
P100 pipette and tips
P20 pipette and tips
P10 pipette and tips
P2 pipette and tips



Check your flow cell.

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

See the [flow cell check document](#) for more information.



Preparing the laboratory for handling RNA samples:

For optimal results, we recommend preparing your laboratory space and equipment prior to handling RNA to ensure the presence of RNase and contaminants is minimal:

- Clean the lab bench space where you will carry out the work with RNaseZap and tech wipes.
- Clean all equipment such as pipettes, tube racks, centrifuge and vortex with RNaseZap and tech wipes.
- Use fresh tip boxes and reagents to minimise risk of contamination.

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

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
cDNA RT Adapter (CRTA)	✓	✓	✓
Annealing Buffer (AB)	✓	✓	✓
Short Fragment Buffer (SFB)	✓	✓	✓
RT Primer (RTP)	✓	✓	✓
Strand Switching Primer II (SSPII)	✓	✓	✓
StickTogether DNA Ligase Buffer	✓	✓	Mix by vortexing
T3 DNA Ligase	Not frozen	✓	✓
RNaseOUT	Not frozen	✓	✓
Lambda Exonuclease	Not frozen	✓	✓
Uracil-Specific Excision Reagent (USER)	Not frozen	✓	✓
10 mM dNTP solution	✓	✓	✓
Maxima H Minus Reverse Transcriptase	Not frozen	✓	✓
Maxima H Minus 5x RT Buffer	✓	✓	Mix by vortexing



To run a control experiment, replace your sample input with 7 μ l diluted RNA Control Sample (RCS) from the RNA Control Expansion (EXP-RCS001) as follows:

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

Reagent	Volume
RNA Control Sample (RCS)	1 μ l
Nuclease-free water	41 μ l
Total	42 μl

Note: This will provide enough volume for 6 samples. Adjust your volumes accordingly for the number of samples you wish to run in your control experiment.

- Mix thoroughly by pipetting 10-20 times and briefly spin down.
- Use the 7 μ l of diluted RNA Control Sample (RCS) as your RNA input.

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

- Transfer 10 ng Poly(A)+ RNA, or 500 ng total RNA into a 0.2 ml thin-walled PCR tube.
- Adjust the volume to up to 7 μ l with nuclease-free water.
- Mix by flicking the tube to avoid unwanted shearing.
- Spin down briefly in a microfuge.

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

Reagent	Volume
RNA	7 µl
cDNA RT Adapter (CRTA)	1 µl
Annealing Buffer (AB)	1 µl
Total volume	9 µl



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

- 4 Mix gently by flicking the tubes, and spin down.
- 5 Incubate the reactions in the thermal cycler at 60°C for 5 minutes, then cool for 5 minutes at room temperature (18-23°C).
- 6 To each of the 0.2 ml PCR tubes containing you RNA sample(s), add the following:

Reaction	Volume
RNA sample (from previous step)	9 µl
StickTogether DNA Ligase Buffer	10 µl
T3 DNA Ligase 3 M U/ml	1 µl
RNaseOUT	1 µl
Total volume (including all reagents)	21 µl

- 7 Ensure the components are thoroughly mixed flicking the tube and spin down.

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

- 8 Incubate for 10 minutes at room temperature.

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

Reagent	Volume
RNA sample (from previous step)	21 µl
Lambda Exonuclease	1 µl
USER (Uracil-Specific Excision Reagent)	1 µl
Total volume (including all reagents)	23 µl



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

- 10 Ensure the components are thoroughly mixed by flicking the tubes and spin down.
- 11 Incubate for 5 minutes at 37°C in the thermal cycler. Then cool down to 4°C



The bead clean-up step can take place either in PCR tubes / PCR plates or 1.5 ml Eppendorf DNA LoBind tubes, depending on the number of samples you are multiplexing and the equipment you have available.

- For efficiency, the bead clean-up step can be performed in the PCR tubes / PCR plate from the previous reaction. You will require a magnetic separator suitable for PCR tubes or PCR plates and a suitable multichannel pipette.

Note: Please take care if using a multichannel pipette to avoid bead carry over or sample loss.

- If multiplexing fewer samples, limited by equipment, or if you would like more control over each bead clean-up; you can transfer each sample to clean 1.5 ml Eppendorf DNA LoBind tubes to carry out the following steps.

12 Resuspend the RNAClean XP beads by vortexing.

13 Add 42 µl of resuspended RNAClean XP beads to each reaction and mix gently by flicking the tubes.

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

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

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

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

17 Repeat the previous step.

- 18 Spin down and place the tubes back on the magnet. Pipette off any residual buffer. Briefly allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.
- 19 Remove the tubes from the magnetic rack and resuspend each pellet in 12 µl of nuclease-free water.
- 20 Incubate at room temperature for 10 minutes.
- 21 Pellet the beads on a magnet, for at least 5 minutes, until the eluate is clear and colourless.
- 22 Remove and retain 12 µl of eluate into a clean 0.2 ml thin-walled PCR tube per sample.
- 23 To each of the 0.2 ml PCR tubes, add the following:

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



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

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

25 Incubate the reaction for 5 minutes at room temperature.

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

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



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

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

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

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

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

31 Incubate using the following protocol in a thermal cycler:

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

- 32 Resuspend the AMPure XP Beads by vortexing.**
- 33 Add 40 µl of AMPure XP Beads (AXP) to each of the RT reaction tube, and mix by flicking.**
- 34 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.**
- 35 Prepare 5 ml of fresh 80% ethanol in nuclease-free water.**
- 36 Spin down the samples and pellet each tube on a magnet. Keep the tubes on the magnet, and pipette off the supernatant when clear and colourless.**
- 37 Keep the tubes on the magnet, and for each sample wash the beads with 100 µl of freshly prepared 80% ethanol as follows:**
 1. Wash the beads with 100 µl of freshly prepared 80% ethanol.
 2. Without disturbing the pellet, remove the 80% ethanol using a pipette and discard.
- 38 Repeat the previous step.**
- 39 Spin down and place each of the tubes back on the magnet. Pipette off any residual ethanol. Briefly allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.**

- 40 Remove the tubes from the magnetic rack and resuspend each pellet in 22.5 µl of nuclease-free water.
- 41 Incubate at room temperature for 10 minutes.
- 42 Pellet the beads on a magnet, for at least 5 minutes, until the eluate is clear and colourless.
- 43 Remove and retain 22.5 µl of each eluate into a separate clean 0.2 ml thin-walled PCR tube.

Note: You can dispose of the pelleted beads.



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

4. Selecting for full-length transcripts by PCR

Materials

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

Consumables

TaKaRa Ex Premier DNA Polymerase (TaKaRa, RR370A/RR370B)
Thermolabile Exonuclease I (NEB, M0568)
Agencourt AMPure XP beads (Beckman Coulter™, A63881)
Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
Freshly prepared 80% ethanol in nuclease-free water
Nuclease-free water (e.g. ThermoFisher, AM9937)
1.5 ml Eppendorf DNA LoBind tubes
Qubit™ Assay Tubes (Invitrogen, Q32856)
0.2 ml PCR tubes

Equipment

Microfuge
Microplate centrifuge
Thermal cycler
Vortex mixer
Hula mixer (gentle rotator mixer)

Magnetic separation rack, suitable for 1.5 ml Eppendorf tubes
Ice bucket with ice
P1000 pipette and tips
P200 pipette and tips
P100 pipette and tips
P20 pipette and tips
P10 pipette and tips
P2 pipette and tips
Qubit™ fluorometer (or equivalent for QC check)
Agilent Bioanalyzer (or equivalent)



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

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

Note: Use one set of Barcode Primers per sample.

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

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting	4. Place on ice until use
Barcode Primers (BP01 - BP24)	✓	✓	✓	✓
Elution Buffer (EB)	✓	✓	✓	✓
TaKaRa Ex Premier DNA Polymerase	Not frozen	✓	✓	✓
Thermolabile Exonuclease I	Not frozen	Not frozen	✓	✓



If using the barcode primers in plate format, the wells of the plate are intended for single use only. Please ensure your barcode primer well is sealed before use, and do not reuse the barcode primer well once pierced/opened.

2 Spin down the reverse-transcribed RNA samples in a microfuge.

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



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

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

Reagent	Volume
Reverse-transcribed sample (from previous step)	5 µl
Nuclease-free water	5 µl
Unique Barcode Primer (BP01-24)	2.5 µl
TaKaRa Ex Premier DNA Polymerase	12.5 µl
Total (including all reagents)	25 µl

5 Mix gently by pipetting.

6 Amplify using the following cycling conditions.

Cycle step	Temperature	Time	No. of cycles
Denaturation	98°C	10 seconds	5
Annealing	50°C	30 seconds	
Annealing and extension	68°C	3 minutes	
Denaturation	98°C	10 seconds	17
Annealing and extension	68°C	3 minutes	
Hold	4°C	∞	

7 Spin down the sample(s) briefly in a microfuge.

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



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

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



The bead clean-up step can take place either in PCR tubes / PCR plates or 1.5 ml Eppendorf DNA LoBind tubes, depending on the number of samples you are multiplexing and the equipment you have available.

- For efficiency, the bead clean-up step can be performed in the PCR tubes / PCR plate from the previous reaction. You will require a magnetic separator suitable for PCR tubes or PCR plates and a suitable multichannel pipette.
Note: Please take care if using a multichannel pipette to avoid bead carry over or sample loss.
- If multiplexing fewer samples, limited by equipment, or if you would like more control over each bead clean-up; you can transfer each sample to clean 1.5 ml Eppendorf DNA LoBind tubes to carry out the following steps.

- 10 Resuspend the AMPure XP beads by vortexing.
- 11 Add 18 µl of resuspended AMPure XP beads to each 1.5 ml Eppendorf DNA LoBind tube.
- 12 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
- 13 Prepare 5 ml of fresh 80% ethanol in nuclease-free water.
- 14 Spin down the samples and pellet on a magnet. Keep the tubes on the magnet, and pipette off the supernatant.

- 15** Keep the tubes on the magnet and wash the beads with 100 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
- 16** Repeat the previous step.
- 17** Spin down and place the tubes back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellets to the point of cracking.
- 18** Remove the tubes from the magnetic rack and resuspend each pellet in 15 µl of Elution Buffer (EB).
- 19** Incubate at room temperature for 10 minutes.
- 20** Pellet the beads on the magnet, for at least 5 minutes, until the eluate is clear and colourless.



At this stage your eluate might have a viscous consistency, please take care when pipetting to avoid aspirating beads.

Tip: Ensure you are using the correct volume pipette for this aspiration, and ensure you aspirate slowly to minimise bead displacement.

If you have resuspended your beads and are concerned about bead carryover, re-pellet on the magnet for a further 5 minutes.

- 21** Remove and retain 12 µl of each eluate into a separate clean 1.5 ml Eppendorf DNA LoBind tube.
 - Remove and retain the eluate which contains the cDNA library in a clean 1.5 ml Eppendorf DNA LoBind tube.
 - Dispose of the pelleted beads.
- 22** For each sample, analyse 1 µl of the amplified cDNA for size, quantity and quality using a Qubit fluorometer and Agilent Bioanalyzer (or equivalent)

for a QC check.



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

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

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

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



Library storage recommendations

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

5. Adapter addition

Materials

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

Consumables

- 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- Microfuge
- Ice bucket with ice
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips



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

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

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
Rapid Adapter (RA)	Not frozen	✓	✓
Adapter Buffer (ADB)	Not frozen	✓	✓

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

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

- 3 Add 1 µl of the diluted Rapid Adapter (RA) to the amplified cDNA library, making the total volume 12 µl.**
- 4 Mix gently by flicking the tube, and spin down.**
- 5 Incubate the reaction for 5 minutes at room temperature.**
- 6 Spin down briefly.**



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

6. Priming and loading the MinION and GridION Flow Cell

Materials

Flow Cell Flush (FCF)
Flow Cell Tether (FCT)
Library Solution (LIS)
Library Beads (LIB)
Sequencing Buffer (SB)

Consumables

MinION/GridION Flow Cell
Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
1.5 ml Eppendorf DNA LoBind tubes

Equipment

MinION or GridION device
MinION/GridION Flow Cell Light Shield
P1000 pipette and tips
P100 pipette and tips
P20 pipette and tips
P10 pipette and tips



Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).



Take the flow cell out of the fridge and leave it at room temperature for 20 minutes. This will improve visibility of the array during priming and sample loading.



Priming and loading a flow cell

We recommend all new users watch the '[Priming and loading your flow cell](#)' video before your first run.

Using the Library Solution

For most sequencing experiments, use the Library Beads (LIB) for loading your library onto the flow cell. However, for viscous libraries it may be difficult to load with the beads and may be appropriate to load using the Library Solution (LIS).

- 1 **Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.**



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

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

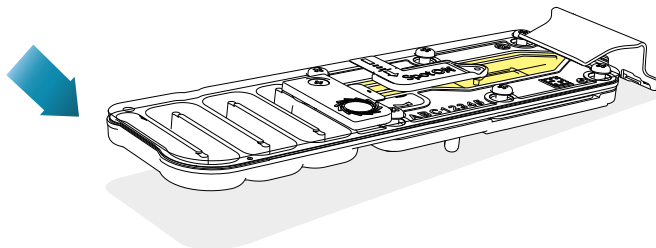
- 2 **To prepare the flow cell priming mix with BSA, combine the following reagents in a fresh 1.5 ml Eppendorf DNA LoBind tube. Mix by inverting the tube and pipette mix at room temperature:**

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

- 3 **Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the priming port cover to ensure correct thermal and electrical contact.**

1a

Insert the flow cell into the device under the clip and press down firmly.



Key:

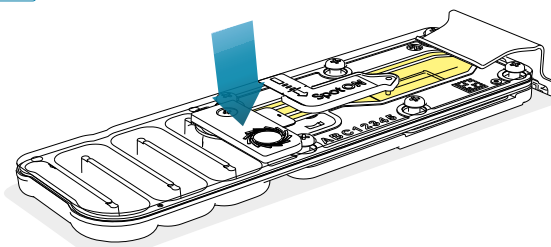
Storage buffer

Priming mix

DNA library

1b

Insert the flow cell into the device under the clip and press down firmly on the Priming port cover.





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

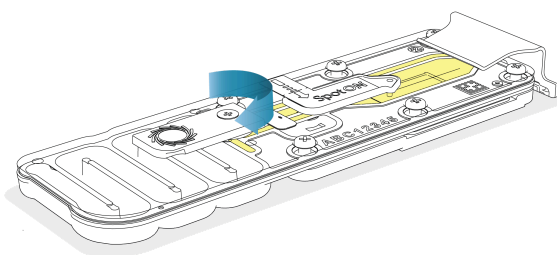
This step can be omitted if the flow cell has been checked previously.

See the [flow cell check document](#) for more information.

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

2

Slide open the **Priming port** cover.



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

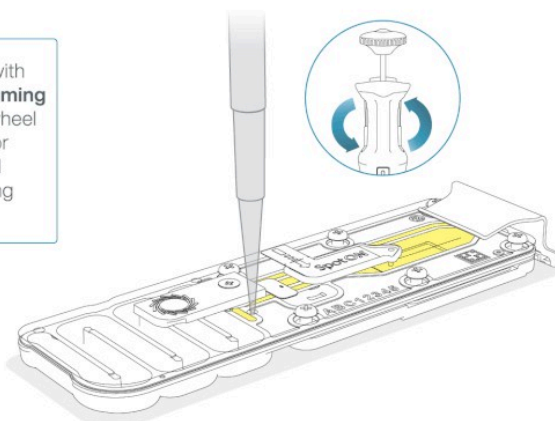
5 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:

1. Set a P1000 pipette to 200 μ l
2. Insert the tip into the priming port

3. Turn the wheel until the dial shows 220-230 μl , to draw back 20-30 μl , 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.

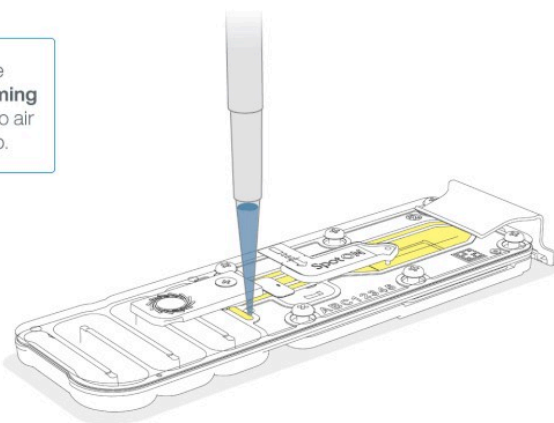
- 3 Insert a P1000 pipette with an empty tip into the **Priming port**. Turn the pipette wheel to draw back 20-30 μl or until you can see a small volume of buffer entering the pipette tip.



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

4

Slowly load 800 μ l of the priming mix into the **Priming port**. Ensure there are no air bubbles in the pipette tip.



Wait 5 minutes before proceeding to the next step.

7 Thoroughly mix the contents of the Library Beads (LIB) by pipetting.



The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

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

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

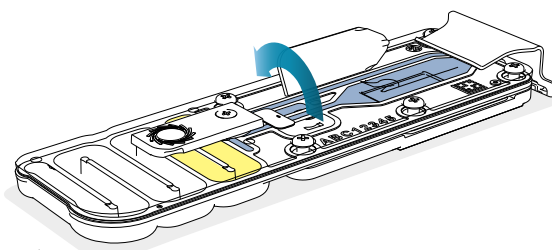
Reagent	Volume per flow cell
Sequencing Buffer (SB)	37.5 μ l
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using	25.5 μ l
DNA library	12 μ l
Total	75 μl

9 Complete the flow cell priming:

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

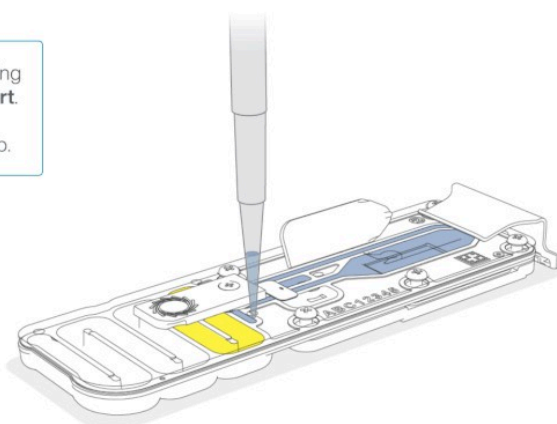
5

Gently flip open the SpotON sample port cover.



6

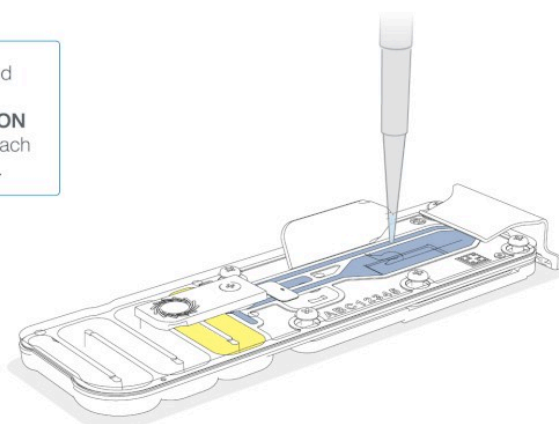
Load 200 μ l of the priming mix into the **Priming Port**. Ensure there are no air bubbles in the pipette tip.



- 10 Mix the prepared library gently by pipetting up and down just prior to loading.
- 11 Add 75 μ l of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

7

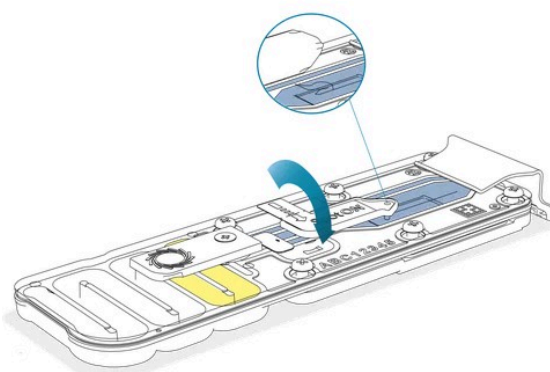
Pipette mix the prepared library and load 75 µl dropwise into the **SpotON** sample port, ensuring each drop flows into the port.



- 12** Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port.

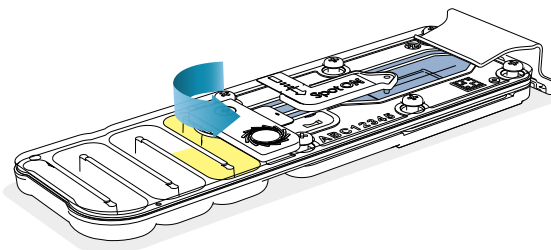
8

Gently replace the **SpotON** sample port cover.



9

Gently close the
Priming port.



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

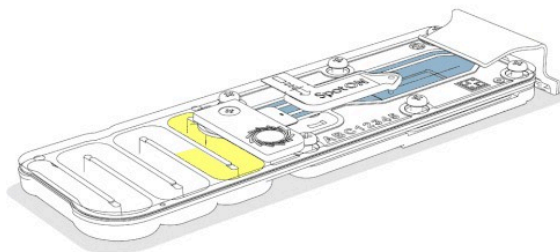
We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

13 Place the light shield onto the flow cell, as follows:

1. Carefully place the leading edge of the light shield against the clip. **Note:** Do not force the light shield underneath the clip.
2. Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.

10

Carefully align the **light shield** against the clip and lower onto the flow cell.



The MinION Flow Cell Light Shield is not secured to the flow cell and careful handling is required after installation.



Close the device lid and set up a sequencing run on MinKNOW.



When a flow cell is inserted into the MinION Mk1D, the device lid will sit on top of the flow cell, leaving a small gap around the sides. This is normal and has no impact on the performance of the device.

Please refer to this [FAQ](#) regarding the device lid.



7. Data acquisition and basecalling

How to start sequencing

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

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

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

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

- [MinION Mk1D user manual](#)
 - [GridION user manual](#)
-

To start a sequencing run on MinKNOW:

1. Navigate to the start page and click **Start sequencing**.
2. Fill in your experiment details, such as name and flow cell position and sample ID.
3. Select the **PCR cDNA Barcoding Kit 24 (SQK-PCB114-24)** on the Kit 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.

8. Flow cell reuse and returns

Materials Flow Cell Wash Kit (EXP-WSH004)

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

The [Flow Cell Wash Kit protocol](#) is available on the Nanopore Community.

- 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 Guide that can be found in this protocol.

9. 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, which are available in the [EPI2ME](#) 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.

Research analysis tools

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

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.

10. Issues during DNA/RNA extraction and library preparation

Below is a list of the most commonly encountered issues, with some suggested

causes and solutions.

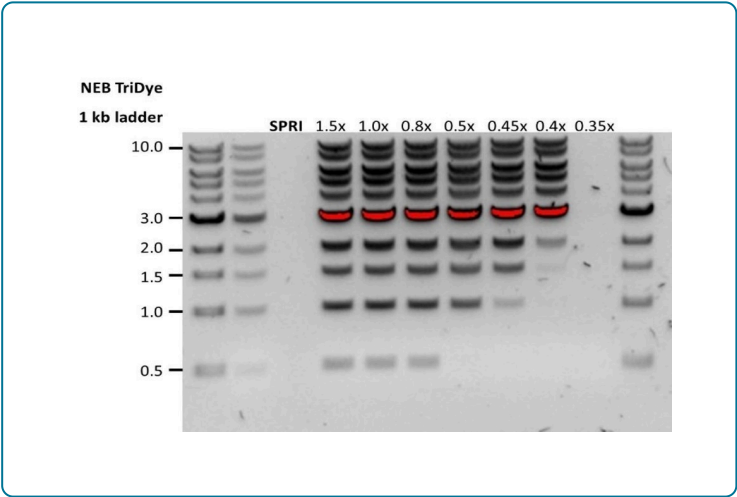
We also have an FAQ section available on the [Nanopore Community Support](#) section.

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

Low sample quality

Observation	Possible cause	Comments and actions
Low DNA purity (Nanodrop reading for DNA OD 260/280 is <1.8 and OD 260/230 is <2.0–2.2)	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the Contaminants document. Please try an alternative extraction method that does not result in contaminant carryover. Consider performing an additional SPRI clean-up step.
Low RNA integrity (RNA integrity number <9.5 RIN, or the rRNA band is shown as a smear on the gel)	The RNA degraded during extraction	Try a different RNA extraction method. For more info on RIN, please see the RNA Integrity Number document. Further information can be found in the DNA/RNA Handling page.
RNA has a shorter than expected fragment length	The RNA degraded during extraction	Try a different RNA extraction method. For more info on RIN, please see the RNA Integrity Number document. Further information can be found in the DNA/RNA Handling page. We recommend working in an RNase-free environment, and to keep your lab equipment RNase-free when working with RNA.

Low DNA recovery after AMPure bead clean-up

Observation	Possible cause	Comments and actions
Low recovery	DNA loss due to a lower than intended AMPure beads-to-sample ratio	1. AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample. 2. When the AMPure beads-to-sample ratio is lower than 0.4:1, DNA fragments of any size will be lost during the clean-up.
Low recovery	DNA fragments are shorter than expected	The lower the AMPure beads-to-sample ratio, the more stringent the selection against short fragments. Please always determine the input DNA length on an agarose gel (or other gel electrophoresis methods) and then calculate the appropriate amount of AMPure beads to use.
		
Low recovery after end-prep	The wash step used ethanol <70%	DNA will be eluted from the beads when using ethanol <70%. Make sure to use the correct percentage.

11. 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 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 DNA/RNA extraction or purification methods may be needed to improve the purity of the input material. The effects of contaminants are shown in the Contaminants Know-how piece . Please try an alternative extraction method that does not result in contaminant carryover.

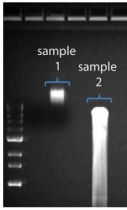
MinKNOW script failed

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

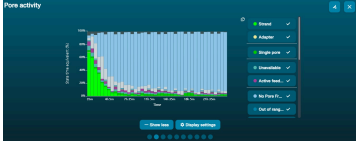
Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	Ensure you load the recommended amount of good quality library in the relevant library prep protocol onto your flow cell. Please quantify the library before loading and calculate mols using tools like the Promega Biomath Calculator , choosing "dsDNA: µg to pmol"
Pore occupancy close to 0	The Ligation Sequencing Kit was used, and sequencing adapters did not ligate to the DNA	Make sure to use the NEBNext Quick Ligation Module (E6056) and Oxford Nanopore Technologies Ligation Buffer (LNB, provided in the sequencing kit) at the sequencing adapter ligation step, and use the correct amount of each reagent. A Lambda control library can be prepared to test the integrity of the third-party reagents.
Pore occupancy close to 0	The Ligation Sequencing Kit was used, and ethanol was used instead of LFB or SFB at the wash step after sequencing adapter ligation	Ethanol can denature the motor protein on the sequencing adapters. Make sure the LFB or SFB buffer was used after ligation of sequencing adapters.
Pore occupancy close to 0	No tether on the flow cell	Tethers are adding during flow cell priming (FLT/FCT tube). Make sure FLT/FCT was added to FB/FCF before priming.

Shorter than expected read length

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

Large proportion of unavailable pores

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

Large proportion of inactive pores

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

Temperature fluctuation

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

Failed to reach target temperature

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

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