PROTOCOL

Single-cell sequencing on GridION

Overview of the protocol

PCR-cDNA Sequencing Kit features

This kit is highly recommended for users who:

- would like to identify and quantify full-length transcripts
- want to explore isoforms, splice variants and fusion transcripts using full-length cDNAs
- would like to generate a large number of cDNA reads

Introduction to the single-cell transcriptomics protocol

This protocol describes how to carry out sequencing of cDNA from single cells using the PCR-cDNA Sequencing Kit (SQK-PCS111). You will need to have reverse-transcribed single cell mRNA into cDNA using the 10X Genomics Next GEM Single Cell 3' Kit (V3.1).

Steps in the sequencing workflow

Prepare for your experiment

You will need to:

- Have previously-prepared single-cell barcoded cDNA using the 10X Genomics Next GEM Single Cell 3' Kit (V3.1).

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

You will need to:

- Biotin tag your cDNAs and amplify by PCR
- Pull down the amplicons on streptavidin beads, and amplify again by PCR
- Attach sequencing adapters to the PCR products
- Prime the flow cell, and load your cDNA library into the flow cell

Sequencing and analysis

You will need to:

- Start a sequencing run using the MinKNOW™ software, which will collect raw data from the device and convert it into basecalled reads
- Analyse the data further using a pipeline of your choice

Contents

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- PCR-cDNA Sequencing Kit (SQK-PCS111)
- R9.4.1 flow cells (FLO-MIN106)
- Flow Cell Wash Kit (EXP-WSH004)

Equipment and consumables

Materials	10 ng of cDNA amplicons prepared using 10X Genomics Next GEM Single Cell 3' Kits (V3.1) cDNA-PCR Sequencing Kit (SQK-PCS111) Custom ordered-oligo at 10 µM: [Btn]Fwd_3580_partial_read1_defined (sequence provided below) Custom-ordered oligo at 10 µM: Rev_PR2_partial_TSO_defined (sequence provided below)
Consumables	M280 streptavidin, 10 µg/µl (Invitrogen, cat # 11205D) LongAmp Hot Start Taq 2X Master Mix (NEB, M0533S) Agencourt AMPure XP beads 1 M Tris-HCl, pH 7.5 5 M NaCl (Sigma, 71386) 0.5 M EDTA, pH 8 Nuclease-free water (e.g. ThermoFisher, cat # AM9937) Freshly prepared 70% ethanol in nuclease-free water Agilent Technologies DNA 12000 Kit 1.5 ml Eppendorf DNA LoBind tubes 0.2 ml thin-walled PCR tubes 15 ml Falcon tubes
Equipment	Hula mixer (gentle rotator mixer) Magnetic separator, suitable for 1.5 ml Eppendorf tubes Microfuge Vortex mixer Thermal cycler P1000 pipette and tips P200 pipette and tips P100 pipette and tips P20 pipette and tips P10 pipette and tips P2 pipette and tips Ice bucket with ice Timer Qubit fluorometer (or equivalent for QC check) Agilent Bioanalyzer (or equivalent)

For this protocol, you will need 10 ng amplified cDNA.

Input DNA

How to QC your input DNA

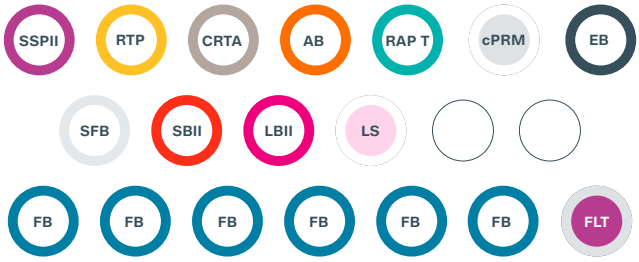
It is important that the input DNA meets the quantity and quality requirements. Using too little or too much DNA, or DNA of poor quality (e.g. highly fragmented or containing RNA or chemical contaminants) can affect your library preparation.

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

Chemical contaminants

Depending on how the DNA is extracted from the raw sample, certain chemical contaminants may remain in the purified DNA, which can affect library preparation efficiency and sequencing quality. Read more about contaminants on the Contaminants page of the Community.

cDNA-PCR Sequencing Kit (SQK-PCS111) contents



SSPII: Strand Switching Primer II
RTP: RT Primer
CRTA: cDNA RT Adapter
AB: Annealing Buffer
RAP T: Rapid Adapter T
cPRM: cDNA Primers
SBII: Sequencing Buffer II
SFB: Short Fragment Buffer
EB: Elution Buffer
LBII: Loading Beads II
LS: Loading Solution F
B: Flush Buffer
FLT: Flush Tether

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Strand Switching Primer II	SSPII	Violet	1	20 µl
RT Primer	RTP	Yellow	1	10 µl
cDNA RT Adapter	CRTA	Amber	1	10 µl
Rapid Adapter T	RAP T	Green	1	10 µl
Annealing Buffer	AB	Orange	1	10 µl
cDNA Primer	cPRM	White cap, grey label	1	40 µl
Elution Buffer	EB	Black	1	500 µl
Short Fragment Buffer	SFB	Clear	1	1,800 µl
Sequencing Buffer II	SBII	Red	1	500 µl
Loading Beads II	LBII	Pink	1	360 µl
Loading Solution	LS	White cap, pink label	1	400 µl
Flush Buffer	FB	Blue	6	1,170 µl
Flush Tether	FLT	White cap, purple label	1	200 µl

Custom-ordered oligo sequences

Order the following HPLC-purified oligos at 100 µM, and dilute to 10 µM in TE buffer:

[Btn]Fwd_3580_partial_read1_defined
5'-/5Biosg/CAGCACTTGCCTGTCGCTCTATCTTCCTACACGACGCTCTTCCGATCT-3'

Rev_PR2_partial_TSO_defined
5'-CAGCTTTCTGTTGGTGCTGATATTGCAAGCAGTGGTATCAACGCAGAG-3'

Computer requirements and software

GridION IT requirements

The GridION™ device contains all the hardware required to control up to five flow cells and acquire the data. The device is further enhanced with high performance GPU technology for real-time basecalling. Read more in the GridION IT requirements document.

Software for nanopore sequencing

MinKNOW

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

MinKNOW use

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

EPI2ME (optional)

The EPI2ME™ cloud-based platform performs further analysis of basecalled data, for example alignment to the Lambda genome, barcoding, or taxonomic classification. You can the EPI2ME platform if you would like further analysis of your data post-basecalling. Please note that EPI2ME does not currently offer a workflow for single-cell transcriptomics analysis.

EPI2ME installation and use

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

Guppy (optional)

The Guppy command-line software can be used instead of MinKNOW for basecalling and demultiplexing reads into folders for each barcode found in Oxford Nanopore library preparation kits. You can use it if you would like to re-analyse old data, or integrate basecalling into your analysis pipeline.

Guppy installation and use

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

Check your flow cell

We highly recommend that you check the number of pores in your flow cell prior to starting a sequencing experiment. This should be done within three months of purchasing for MinION™/GridION™/PromethION™ Flow Cells, or within four weeks of purchasing for Flongle™ Flow Cells. Oxford Nanopore Technologies will replace any flow cell with fewer than the number of pores in the table below, when the result is reported within two days of performing the flow cell check, and when the storage recommendations have been followed. To do the flow cell check, please follow the instructions in the Flow Cell Check document.

Flow cell	Minimum number of active pores covered by warranty
Flongle Flow Cell	50
MinION/GridION Flow Cell	800
PromethION Flow Cell	5000

Pre-pull-down PCR

Materials

10 ng of cDNA amplicons prepared using 10X Genomics Next GEM Single Cell 3' Kits (V3.1)

Custom ordered-oligo at 10 µM: [Btn]Fwd_3580_partial_read1_defined (sequence provided in Equipment and Consumables)

Custom-ordered oligo at 10 µM: Rev_PR2_partial_TSO_defined (sequence provided in Equipment and Consumables)

Consumables

LongAmp Hot Start Taq 2X Master Mix (NEB, M0533S)

Agencourt AMPure XP beads

Nuclease-free water (e.g. ThermoFisher, cat #AM9937)

Freshly prepared 70% ethanol in nuclease-free water

1.5 ml Eppendorf DNA LoBind tubes

0.2 ml thin-walled PCR tubes

Equipment

Thermal cycler

Microfuge

Hula mixer (gentle rotator mixer)

Magnetic rack

Ice bucket with ice

P1000 pipette with tips

P200 pipette and tips

P100 pipette and tips

P20 pipette and tips

P2 pipette and tips

1 Set up the following biotin tagging reaction in a 0.2 ml thin-walled PCR tube:

Reagent	Volume
cDNA template	10 ng, x µl
[Btn]Fwd_3580_partial_read1_defined, 10 µM	2 µl
Rev_PR2_partial_TSO_defined, 10 µM	2 µl
Nuclease-free water	21-x µl
LongAmp Hot Start Taq 2X Master Mix	25 µl
Total	**50 µl**

2 **Amplify using the following cycling conditions:**

Cycle step	Temperature	Ramp rate	Time	No. of cycles
Initial denaturation	94°C	max	3 min	1
Denaturation Annealing Extension	94°C 66°C down to 58°C 65°C	max 0.2°C/s max	30 s 90 s 6 mins	4
Final extension	65°C	max	10 min	1
Hold	4°C	-	∞	-

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

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

5 **Add 40 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube.**

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

7 **Prepare 500 µl of fresh 70% ethanol in nuclease-free water.**

8 **Spin down the samples and pellet the beads on a magnet until the eluate is clear and colourless. Keep the tubes on the magnet and pipette off the supernatant.**

9 **Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

10 **Repeat the previous step.**

11 **Briefly 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 pellet to the point of cracking.**

12 **Remove the tube from the magnetic rack and resuspend the pellet in 10 µl nuclease-free water. Spin down and incubate for 2 minutes at room temperature.**

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

14 **Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Pull-down

Consumables

1 M Tris-HCl, pH 7.5
5 M NaCl (Sigma, 71386)
0.5 M EDTA, pH 8
M280 streptavidin, 10 µg/µl (Invitrogen, cat # 11205D)
15 ml Falcon tubes
1.5 ml Eppendorf DNA LoBind tubes
0.2 ml thin-walled PCR tubes

Equipment

Vortex mixer
Microfuge
Hula mixer (gentle rotator mixer)
Magnetic rack
Ice bucket with ice
P1000 pipette with tips
P200 pipette and tips
P100 pipette and tips
P20 pipette and tips
P2 pipette and tips

1 **Prepare 4 ml of 2X wash/bind buffer (10 mM Tris-HCl pH 7.5, 2 M NaCl, 1 mM EDTA).**

Reagent	Stock concentration	Final concentration	Volume
Tris-HCl pH 7.5	1 M	10 mM	40 µl
NaCl	5 M	2 M	1600 µl
EDTA	0.5 M	1 mM	8 µl
Nuclease-free water	-	-	2352 µl
Total	**-**	**-**	**4 ml**

2 **Transfer 3.5 ml of the 2X wash/bind buffer to a fresh 15 ml Falcon tube and add 3.5 ml of nuclease-free water to make 7 ml of 1X wash/bind buffer (5 mM Tris-HCl pH 7.5, 1 M NaCl, 0.5 mM EDTA).**

3 **Resuspend the M280 streptavidin beads (10 µg/µl) by vortexing.**

4 **Transfer 5 µl of the streptavidin beads to a clean 1.5 ml Eppendorf DNA LoBind tube.**

5 **Add 1 ml of 1X wash/bind buffer and vortex the beads with buffer for 5 s. Pellet the beads on a magnet for two minutes, then pipette off the supernatant.**

6 **Repeat the previous step two more times.**

7 **Resuspend the beads in 10 µl of 2X wash/bind buffer to achieve a final bead concentration of 5 µg/µl.**

IMPORTANT

It is critical that 2X buffer is used for this step. Using 1X buffer will result in inefficient binding.

8 **Add 10 µl of 5 µg/µl prepared beads (50 µg beads total) to the tube with biotinylated cDNA.**

9 **Incubate on a Hula mixer (rotator mixer) for 20 mins at room temperature.**

10 **Add 1 ml of 1X wash/bind buffer and vortex the DNA and beads with buffer for 5 s. Pellet the beads on a magnet for two minutes, then pipette off the supernatant. Take care to not aspirate any of the beads.**

11 **Repeat the previous step two more times.**

12 **Add 200 µl of 10 mM Tris-HCl pH 7.5 and vortex the beads for 5 s.**

13 **Spin down and place the tube back on the magnet for 3 minutes. Pipette off the supernatant.**

14 **Remove the tube from the magnetic rack and resuspend the pellet in 20 µl of nuclease-free water. Vortex for 5 s and briefly spin down to collect the amplicon-bead conjugate.**

Post-pull-down PCR

Materials	cDNA Primer (cPRM) Elution Buffer from the Oxford Nanopore kit (EB)
Consumables	LongAmp Hot Start Taq 2X Master Mix (NEB, M0533S) Nuclease-free water (e.g. ThermoFisher, cat #AM9937) Agencourt AMPure XP beads Freshly prepared 70% ethanol in nuclease-free water 0.2 ml thin-walled PCR tubes 1.5 ml Eppendorf DNA LoBind tubes
Equipment	Thermal cycler Vortex mixer Hula mixer 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)

1 **In a 0.2 ml thin-walled PCR tube, prepare the following PCR reaction:**

Reagent	Volume
cPRM	1 µl
Nuclease-free water	4 µl
LongAmp Hot Start Taq 2X Master Mix	25 µl
Total	**30 µl**

2 **Resuspend the amplicon-bead conjugate by pipetting and then transfer 20 µl of the conjugate into the tube containing the PCR reaction. Mix by pipetting.**

3 **Do not spin down the tube; transfer immediately to the thermal cycler and amplify using the following cycling conditions:**

Cycle step	Temperature	Time	No. of cycles
Initial denaturation	94°C	3 min	1
Denaturation Annealing Extension	94°C 62°C 65°C	15 s 15 s 6 min	4
Final extension	65°C	10 min	1
Hold	4°C	∞	-

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

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

6 **Add 40 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube.**

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

8 **Prepare 500 µl of fresh 70% ethanol in nuclease-free water.**

9 **Spin down the sample and pellet on a magnet until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant.**

10 **Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

11 **Repeat the previous step.**

12 **Briefly spin down and place the tubes back on the magnet for the beads to pellet. Pipette off any residual ethanol. Allow to dry for 30 seconds, but do not dry the pellets to the point of cracking.**

13 **Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB).**

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

15 **Remove and retain 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**
Dispose of the pelleted beads.

16 **Quantify 1 µl of eluted sample using a Qubit fluorometer - recovery aim >50 ng total.**

Adapter addition

~5 minutes

Materials	Elution Buffer from the Oxford Nanopore kit (EB) Rapid Adapter T (RAP T)
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

1 **Analyse 1 µl of sample using the Agilent Bioanalyzer. Determine the average amplicon size from this data, and use this to calculate the input sample volume for the next step.**

2 **Calculate the required sample volume for 35 fmol and dilute this into 12 µl of EB.**

3 **Add 0.5 µl of Rapid Adapter T (RAP T) to the amplified cDNA library.**

4 **Mix well by pipetting and spin down.**

5 **Incubate the reaction for 5 minutes at room temperature.**

END OF STEP

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

Priming and loading the SpotON Flow Cell for GridION

~10 minutes

Materials	Sequencing Buffer II (SBII) Loading Beads II (LBII) Flush Buffer (FB) Flush Tether (FLT)
Consumables	1.5 ml Eppendorf DNA LoBind tubes Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
Equipment	GridION device SpotON Flow Cell P1000 pipette and tips P100 pipette and tips P20 pipette and tips P10 pipette and tips

TIP

Priming and loading a flow cell

We recommend all new users watch the 'Priming and loading your flow cell' video before your first run.

Using the Loading Solution

We recommend using the Loading Beads II (LBII) for loading your library onto the flow cell for most sequencing experiments. However, if you have previously used water to load your library, you must use Loading Solution (LS) instead of water.

Note: some customers have noticed that viscous libraries can be loaded more easily when not using Loading Beads.

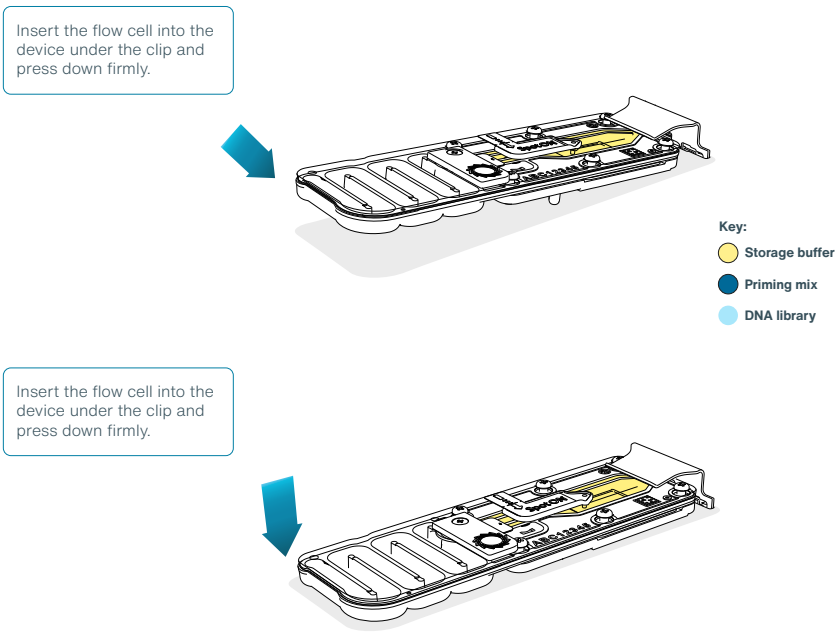
1 **Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) or Loading Solution (LS, if using), Flush Tether (FLT) and Flush Buffer (FB) at room temperature before mixing the reagents by vortexing, and spin down the SBII and FLT at room temperature.**

2 **Mix the Sequencing Buffer II (SBII), Flush Buffer (FB), Flush Tether (FLT) and Loading Solution (LS, if using) tubes by vortexing. Spin down the SBII and FLT at room temperature.**

3 **Prepare the flow cell priming mix: Add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing.**

4 Slide open the GridION lid and insert the flow cell.

Press down firmly on the flow cell to ensure correct thermal and electrical contact.

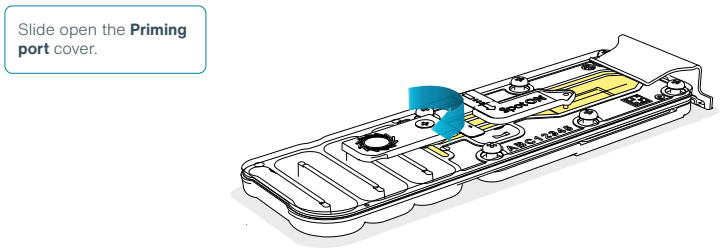


Optional action

Complete a flow cell check to assess the number of pores available before loading the library. This step can be omitted if the flow cell has been checked previously.

Please see the MinKNOW flow cell check protocol for more information

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



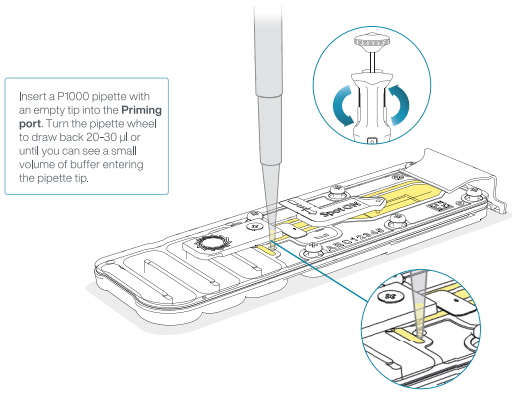
IMPORTANT

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

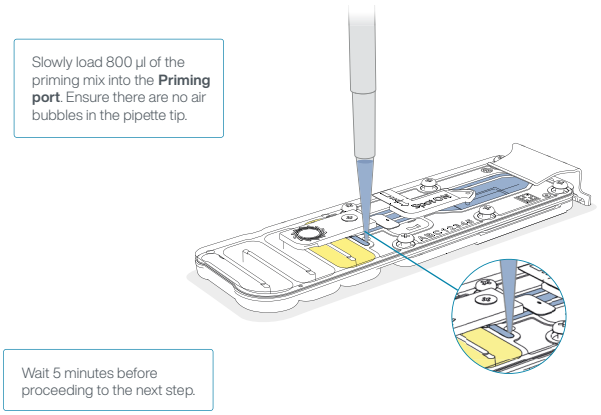
6 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µl):

1. Set a P1000 pipette to 200 µl
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 ul, to draw back 20-30 ul, or until you can see a small volume of buffer entering the pipette tip

Note: Visually check that there is continuous buffer from the priming port across the sensor array.



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



8 Thoroughly mix the contents of the Loading Beads II (LBII) by pipetting.

IMPORTANT

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

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

Reagent	Volume per flow cell
Sequencing Buffer II (SBII)	37.5 µl
Loading Beads II (LBII) mixed immediately before use, or Loading Solution (LS), if using	25.5 µl
DNA library	12 µl
Total	**75 µl**

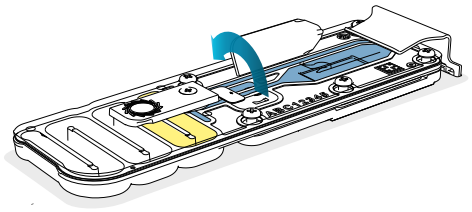
Note: Load the library onto the flow cell immediately after adding the Sequencing Buffer II (SBII) and Loading Beads II (LBII) because the fuel in the buffer will start to be consumed by the adapter.

10 Complete the flow cell priming:

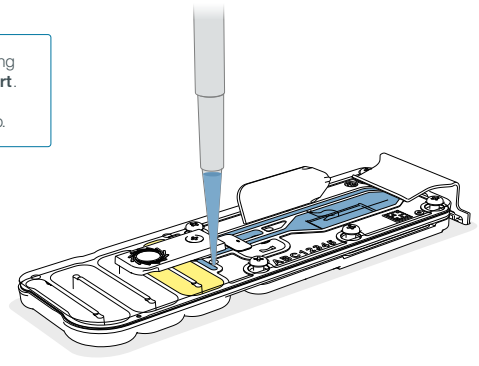
1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load **200 µl** of the priming mix into the flow cell via the priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.

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

Gently flip open the **SpotON** sample port cover.



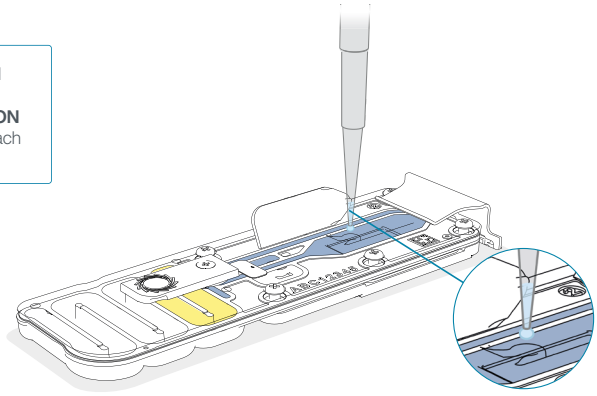
Load 200 µl of the priming mix into the **Priming port**. Ensure there are no air bubbles in the pipette tip.



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

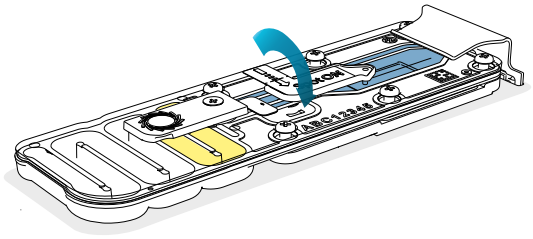
12 Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

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

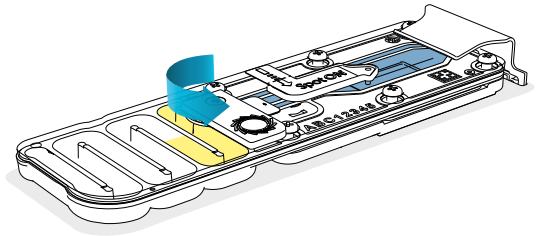


13 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the GridION lid.

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



Gently close the **Priming port**.



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 three options for how to carry out sequencing:

1. **Data acquisition and basecalling in real-time using MinKNOW on a computer**

   Follow the instructions in the MinKNOW protocol beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section.

2. **Data acquisition and basecalling in real-time using the GridION device**

   Follow the instructions in the GridION user manual.

3. **Data acquisition and basecalling in real-time using the MinION Mk1C device**

   Follow the instructions in the MinION Mk1C user manual.

4. **Data acquisition and basecalling in real-time using the 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 or Guppy**

   Follow the instructions in the MinKNOW protocol beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section. When setting your experiment parameters, set the Basecalling tab to OFF. After the sequencing experiment has completed, follow the instructions in the Post-run analysis section of the MinKNOW protocol or the Guppy protocol starting from the "Quick Start Guide for Guppy" section.

Downstream analysis

EPI2ME Labs provides a Nextflow-based workflow for the analysis of single-cell sequencing data.

The workflow, wf-single-cell, processes the FASTQ format sequence data prepared by the MinKNOW software. The workflow screens each sequence read for 10X cell barcode information and assigns reads to a cell of origin. A subset of sequences from "true" cells are dynamically filtered on the basis of the number of assigned sequence reads. These sequences are mapped to the reference genome and tables of both gene and transcript abundance are prepared for each cell. These "cell barcode x gene" or transcript abundance information are used to prepare the familiar UMAP plots that may show the stratification of the cell types present within the sample.

Ending the experiment

Materials Flow Cell Wash Kit (EXP-WSH004)

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

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

2 **Follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore.**

Instructions for returning flow cells can be found here.
All flow cells must be flushed with deionised water before returning the product.

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.

Troubleshooting

IMPORTANT

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

Issues during DNA/RNA extraction and library preparation

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

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

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

Low sample quality

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

Low DNA recovery after AMPure bead clean-up

Observation	Possible cause	Comments and actions
Low recovery	DNA loss due to a lower than intended AMPure beads-to- sample ratio	1. AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample. 2. When the AMPure beads-to-sample ratio is lower than 0.4:1, DNA fragments of any size will be lost during the clean-up.

Low recovery	DNA fragments are shorter than expected	The lower the AMPure beads-to-sample ratio, the more stringent the selection against short fragments. Please always determine the input DNA length on an agarose gel (or other gel electrophoresis methods) and then calculate the appropriate amount of AMPure beads to use. 
Low recovery after end-prep	The wash step used ethanol <70%	DNA will be eluted from the beads when using ethanol <70%. Make sure to use the correct percentage.

Issues during the sequencing run

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

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

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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 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	The flow cell is not correctly inserted into the device	Stop the sequencing run, remove the flow cell from the sequencing device and insert it again, checking that the flow cell is firmly seated in the device and that it has reached the target temperature. If applicable, try a different position on the device (GridION/PromethION).
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	Contaminations in the library damaged or blocked the pores	The pore count during the Flow Cell Check is performed using the QC DNA molecules present in the flow cell storage buffer. At the start of sequencing, the library itself is used to estimate the number of active pores. Because of this, variability of about 10% in the number of pores is expected. A significantly lower pore count reported at the start of sequencing can be due to contaminants in the library that have damaged the membranes or blocked the pores. Alternative DNA/RNA extraction or purification methods may be needed to improve the purity of the input material. The effects of contaminants are shown in the Contaminants Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover.

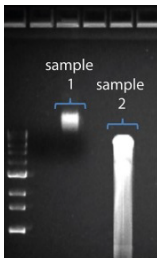
MinKNOW script failed

Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support.

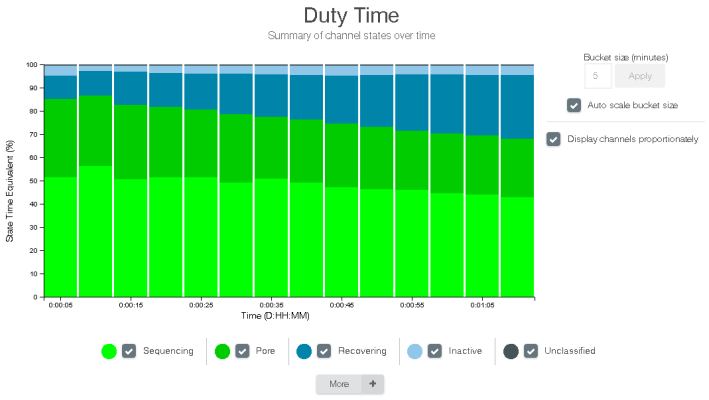
Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	5–50 fmol of good quality library can be loaded on to a MinION Mk1B/GridION Flow Cell. Please quantify the library before loading and calculate mols using tools like the Promega Biomath Calculator, choosing "dsDNA: µg to pmol"
Pore occupancy close to 0	The Ligation Sequencing Kit was used, and sequencing adapters did not ligate to the DNA	Make sure to use the NEBNext Quick Ligation Module (E6056) and Oxford Nanopore Technologies Ligation Buffer (LNB, provided in the SQK-LSK109 kit) at the sequencing adapter ligation step, and use the correct amount of each reagent. A Lambda control library can be prepared to test the integrity of the third-party reagents.
Pore occupancy close to 0	The Ligation Sequencing Kit was used, and ethanol was used instead of LFB or SFB at the wash step after sequencing adapter ligation	Ethanol can denature the motor protein on the sequencing adapters. Make sure the LFB or SFB buffer was used after ligation of sequencing adapters.
Pore occupancy close to 0	No tether on the flow cell	Tethers are adding during flow cell priming (FLT tube). Make sure FLT was added to FB before priming.

Shorter than expected read length

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

Large proportion of recovering pores

Observation	Possible cause	Comments and actions
Large proportion of recovering pores (shown as dark blue in the channels panel and duty time plot)	Contaminants are present in the sample	Some contaminants can be cleared from the pores by the unblocking function built into MinKNOW. If this is successful, the pore status will change to “single pores”. If the portion of recovering pores (unavailable pores in the extended view) stays large or increases: 1. A flow cell wash can be performed, or 2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.  The duty time plot above shows an increasing proportion of “recovering” pores over the course of a sequencing experiment.

Large proportion of inactive pores

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

Reduction in sequencing speed and q-score later into the run

Observation	Possible cause	Comments and actions
Reduction in sequencing speed and q-score later into the run	Fast fuel consumption is typically seen when the flow cell is overloaded with library (~5–10 fmol of library is recommended)	Add more fuel to the flow cell by following the instructions in the MinKNOW protocol. In future experiments, load lower amounts of library to the flow cell.

Temperature fluctuation

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

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 Mk1B temperature control.

Guppy – no input .fast5 was found or basecalled

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

Guppy – no Pass or Fail folders were generated after basecalling

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

Guppy – unusually slow processing on a GPU computer

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

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

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

MinIT – the MinIT software cannot be updated

Observation	Possible cause	Comments and actions
The MinIT software cannot be updated	The firewall is blocking IPs for update	Please consult your IT department, as the MinIT software requires access to the following AWS IP ranges. Access to the following IP addresses is also needed: 178.79.175.200 96.126.99.215
The MinIT device software cannot be updated	The device already has the latest version of the software	Occasionally, the MinIT software admin page displays "updates available" even when the software is already up-to-date. Please compare the version listed on the admin page with the one on the Software Downloads page. Alternatively, SSH into the MinIT through a SSH Client (e.g. Bitvise or Putty, as described in the MinIT protocol) on a Windows computer or the terminal window on a Mac, run the command, dpkg -l | grep minit, to find out the version of the MinIT software and sudo apt update if an update is needed. If the issue still persists, please contact Technical Support with details of the error.