Overview of the protocol

Ligation Sequencing Kit V14 features

This kit is recommended for users who:

- Want to achieve median raw read accuracy of Q20+ (99%) and above.
- Want to optimise their sequencing experiment for output.
- Require control over read length.
- Would like to utilise upstream processes such as size selection, whole genome amplification, or enrichment for long reads.

IMPORTANT

Adaptive sampling in Kit 14 chemistry

While using Kit 14 chemistry, this workflow has been optimised to enrich specific regions of interest (ROIs) with Adaptive sampling rather than duplex basecalling, ensuring highest output and the best sequencing results.

For more background information about designing an adaptive sampling experiment, please refer to the Adaptive sampling best practice document: Adaptive sampling best practice

Reduced representation methylation sequencing (RRMS)

Nanopore sequencing enables direct detection of methylated cytosines (e.g., at CpG sites), without the need for bisulphite conversion. CpG sites frequently occur in high density clusters called CpG islands (CGI) and >60% of human genes have their promoters embedded within CGIs.

Changes in methylation patterns within promoters is associated with changes in gene expression and disease states such as cancer: exploring methylation differences between tumour samples and normal samples can help to uncover mechanisms associated with tumour formation and development.

Adaptive sampling (AS) offers a fast, flexible and precise method to enrich for regions of interest (e.g. CGIs) by depleting off-target regions during sequencing itself with no requirement for upfront sample manipulation. Here we introduce Reduced Representation Methylation Sequencing (RRMS): Oxford Nanopore's methylation detection is combined with AS to target 310 Mb of the human genome which are highly enriched for CpGs including ~28,000 CpG islands, ~50,600 shores and ~42,700 shelves as well as ~21,600 promotor regions.

To benchmark, we performed RRMS on five replicates of a metastatic melanoma cell line and its normal pair for a male individual (COLO829/COLO829_BL) and a triple negative breast cancer cell-line pair (HCC1395/HCC1935_BL). Each sample was run on a single MinION flow cell: RRMS resulted in high-confidence methylation calls (>10 overlapping reads) for 7.3-8.5 million CpGs per sample.

Introduction to the DNA extraction and Ligation Sequencing protocol for RRMS

This protocol describes how to carry out DNA extraction and reduced representation methylation sequencing (RRMS) of human samples using the Ligation Sequencing Kit V14 (SQK-LSK114) and the Adaptive Sampling feature in MinKNOW.

Steps in the sequencing workflow:

Prepare for your experiment

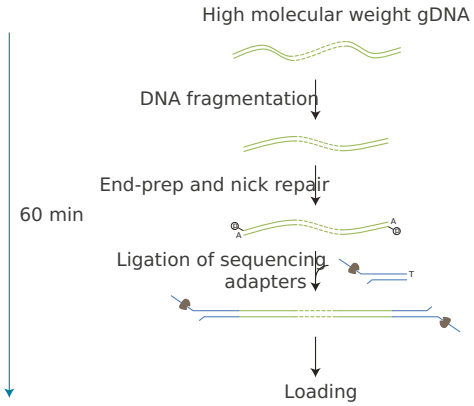
You will need to:

- Extract your DNA, fragment it using the Covaris g-TUBE, and check its length, quantity and purity. **The quality checks performed during the protocol are essential in ensuring experimental success.**
- Ensure you have your sequencing kit, the correct equipment and third-party reagents
- Download the software for acquiring and analysing your data
- Ensure that you have the correct .bed file for Adaptive Sampling
- Check your flow cell to ensure it has enough pores for a good sequencing run

Library preparation

You will need to:

- Repair the DNA, and prepare the DNA ends for adapter attachment
- Attach sequencing adapters supplied in the kit to the DNA ends
- Prime the flow cell, and load your DNA 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. While configuring the run, turn on the Adaptive Sampling setting and import a pre-prepared .bed file with your regions of interest, along with a FASTA reference file.
- Sequence the sample for a total of 96 hours, with two flow cell washes when the available pore count drops to around 40% of the starting pore count (typically after ~30 hours and the second time after ~64 hours).
- Use the Guppy protocol to call modified bases, and then the commands recommended at the end of this protocol to aggregate the modified bases and perform CpG island annotation.

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Ligation Sequencing Kit V14 (SQK-LSK114)
- R10.4.1 flow cells (FLO-MIN114)
- Flow Cell Wash Kit (EXP-WSH004)

Equipment and consumables

Materials	• 2 µg extracted genomic DNA (e.g. from cell culture or tissue sample) • Ligation Sequencing Kit V14 (SQK-LSK114) • Flow Cell Wash Kit (EXP-WSH004)
Consumables	• NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L). Alternatively, you can use the NEBNext® products below: • NEBNext FFPE Repair Mix (NEB, M6630) • NEBNext Ultra II End repair/dA-tailing Module (NEB, cat # E7546) • NEBNext Quick Ligation Module (NEB, cat # E6056) • Freshly prepared 80% ethanol in nuclease-free water • Nuclease-free water (e.g. ThermoFisher, AM9937) • 1.5 ml Eppendorf DNA LoBind tubes • 0.2 ml thin-walled PCR tubes • Qubit™ Assay Tubes (Invitrogen, Q32856) • Qubit dsDNA HS Assay Kit (Invitrogen, Q32851) • (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
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
Optional Equipment	• Agilent Bioanalyzer (or equivalent) • Qubit fluorometer (or equivalent for QC check) • Eppendorf 5424 centrifuge (or equivalent)

After performing DNA extraction and DNA fragmentation, you will need 2 µg genomic DNA to take forward into the library preparation.

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.

NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing

For customers new to nanopore sequencing, we recommend buying the NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (catalogue number E7180S or E7180L), which contains all the NEB reagents needed for use with the Ligation Sequencing Kit.

Please note, for our amplicon protocols, NEBNext FFPE DNA Repair Mix and NEBNext FFPE DNA Repair Buffer are not required.

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.

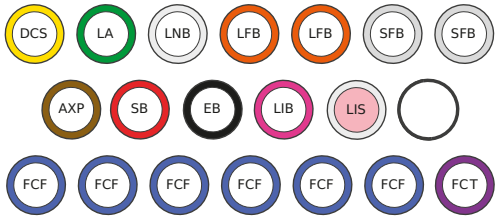
IMPORTANT

We strongly recommend using the Ligation Buffer (LNB) supplied in the Ligation Sequencing Kit V14 rather than the third-party ligase buffer supplied in the NEBNext Quick Ligation Module to ensure high ligation efficiency of the Ligation Adapter (LA).

IMPORTANT

Ligation Adapter (LA) used in this kit and protocol is not interchangeable with other sequencing adapters.

Ligation Sequencing Kit V14 (SQK-LSK114) contents



DCS : DNA Control Strand
LA : Ligation Adapter
LNB : Ligation Buffer
LFB : Long Fragment Buffer
SFB : Short Fragment Buffer
AXP : AMPure XP Beads
SB : Sequencing Buffer
EB : Elution Buffer
LIB : Library Beads
LIS : Library Solution
FCF : Flow Cell Flush
FCT : Flow Cell Tether

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
DNA Control Strand	DCS	Yellow	1	35
Ligation Adapter	LA	Green	2	40
AMPure XP Beads	AXP	Amber	1	1,200
Ligation Buffer	LNB	White	1	200
Long Fragment Buffer	LFB	Orange	2	1,800
Short Fragment Buffer	SFB	Clear	2	1,800
Sequencing Buffer	SB	Red	1	700
Elution Buffer	EB	Black	1	1,200
Library Beads	LIB	Pink	1	600
Library Solution	LIS	White cap, pink sticker on label	1	600
Flow Cell Flush	FCF	Blue	6	1,170
Flow Cell Tether	FCT	Purple	1	200

Note: This product contains AMPure XP Reagent manufactured by Beckman Coulter, Inc. and can be stored at -20°C with the kit without detriment to reagent stability.

.bed file

1 **Download the .bed file from the Adaptive Sampling catalogue.**

The Adaptive Sampling catalogue provides a way for both the Oxford Nanopore team and Community members to share .bed files with genomic target regions used for Adaptive Sampling experiments. The .bed files along with a reference genome can be uploaded into MinKNOW.

For RRMS experiments, download the Reduced representation methylation sequencing (RRMS) file.

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 by barcode, and basecall/demultiplex data after a sequencing run has completed.

MinKNOW use

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

EPI2ME (optional)

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

EPI2ME installation and use

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

Guppy (optional)

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

Guppy installation and use

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

Check your flow cell

We highly recommend that you check the number of pores in your flow cell prior to starting a sequencing experiment. This should be done within three months of purchasing for MinION/GridION/PromethION flow cells, or within four weeks of purchasing for Flongle flow

cells. Oxford Nanopore Technologies will replace any flow cell with fewer than the number of pores in the table below, when the result is reported within two days of performing the flow cell check, and when the storage recommendations have been followed. To do the flow cell check, please follow the instructions in the Flow Cell Check document.

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

DNA extraction

DNA extraction

Extract DNA from your sample using one of our recommended extraction protocols. For the benchmarking of this method, the Oxford Nanopore team extracted DNA from ~5 million cells using the protocol: Human cell line DNA – QIAGEN Puregene Cell Kit. We also offer multiple mammalian sample extraction protocols, which you can use for other sample types.

DNA fragmentation

The DNA was sheared using the protocol forCovaris g-TUBE fragmentation, with the following modifications:

- The input was 2 µg of DNA in 50 µl.
- The DNA was fragmented in the g-TUBE at 11,000 rpm in 30 sec pulses. The resulting fragment length should be ~6-7 kb.

Take forward 48 µl of your fragmented DNA into the next step.

DNA repair and end-prep

~35 minutes

Materials

- gDNA in 48 µl nuclease-free water
- AMPure XP Beads (AXP)

Consumables

- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- NEBNext FFPE DNA Repair Mix (NEB, cat # M6630)
- NEBNext Ultra II End Repair / dA-tailing Module (NEB, cat # E7546)

	• Freshly prepared 80% ethanol in nuclease-free water • Qubit™ Assay Tubes (Invitrogen, Q32856) • Qubit dsDNA HS Assay Kit (ThermoFisher, cat # Q32851)
Equipment	• P1000 pipette and tips • P100 pipette and tips • P10 pipette and tips • Thermal cycler at 20°C and 65°C • Microfuge • Hula mixer (gentle rotator mixer) • Magnetic rack • Ice bucket with ice
Optional Equipment	• Qubit fluorometer (or equivalent for QC check)

1 Prepare the NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice.

For optimal performance, NEB recommend the following:

1. Thaw all reagents on ice.
2. Flick and/or invert the reagent tubes to ensure they are well mixed.
   Note: Do not vortex the FFPE DNA Repair Mix or Ultra II End Prep Enzyme Mix.
3. Always spin down tubes before opening for the first time each day.
4. The Ultra II End Prep Buffer and FFPE DNA Repair Buffer may have a little precipitate. Allow the mixture to come to room temperature and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for 30 seconds to solubilise any precipitate.
   Note: It is important the buffers are mixed well by vortexing.
5. The FFPE DNA Repair Buffer may have a yellow tinge and is fine to use if yellow.

2 Prepare the DNA in nuclease-free water.

- Transfer 2 µg of the fragmented DNA into a 1.5 ml Eppendorf DNA LoBind tube
- Adjust the volume to 48 µl with nuclease-free water
- Mix thoroughly by flicking the tube
- Spin down briefly in a microfuge

3 **In a 0.2 ml thin-walled PCR tube, mix the following:**

Between each addition, pipette mix 10-20 times.

Reagent	Volume
DNA from the previous step	48 µl
NEBNext FFPE DNA Repair Buffer	3.5 µl
NEBNext FFPE DNA Repair Mix	2 µl
Ultra II End-prep Reaction Buffer	3.5 µl
Ultra II End-prep Enzyme Mix	3 µl
Total	**60 µl**

4 **Ensure the components are thoroughly mixed by pipetting, and spin down.**

5 **Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.**

IMPORTANT

AMPure XP bead clean-up

It is recommended that the repaired/end-prepped DNA sample is subjected to the following clean-up with AMPure XP beads. This clean-up can be omitted for simplicity and to reduce library preparation time. However, it has been observed that omission of this clean-up can: reduce subsequent adapter ligation efficiency, increase the prevalence of chimeric reads, and lead to an increase in pores being unavailable for sequencing. If omitting the clean-up step, proceed to the next section.

6 **Resuspend the AMPure XP Beads (AXP) by vortexing.**

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

8 **Add 60 µl of resuspended the AMPure XP Beads (AXP) to the end-prep reaction and mix by flicking the tube.**

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

10 **Prepare 500 µl of fresh 80% ethanol in nuclease-free water.**

11 **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.**

12 **Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

13 **Repeat the previous step.**

14 **Spin down and place the tube 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.**

15 **Remove the tube from the magnetic rack and resuspend the pellet in 61 µl nuclease-free water. Incubate for 2 minutes at room temperature.**

16 **Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.**

17 **Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Quantify 1 µl of eluted sample using a Qubit fluorometer.

END OF STEP

Take forward the repaired and end-prepped DNA into the adapter ligation step. However, at this point it is also possible to store the sample at 4°C overnight.

Adapter ligation and clean-up

~30 minutes

Materials

- Ligation Adapter (LA)
- Ligation Buffer (LNB) from the Ligation Sequencing Kit
- Long Fragment Buffer (LFB)
- AMPure XP Beads (AXP)
- Elution Buffer (EB) from the Oxford Nanopore sequencing kit

Consumables

- NEBNext Quick Ligation Module (NEB, cat # E6056)
- 1.5 ml Eppendorf DNA LoBind tubes
- Qubit™ Assay Tubes (Invitrogen, Q32856)
- Qubit dsDNA HS Assay Kit (ThermoFisher, cat # Q32851)

Equipment

- Magnetic rack
- Microfuge
- Vortex mixer
- P1000 pipette and tips
- P100 pipette and tips

- P20 pipette and tips
- P10 pipette and tips
- Qubit fluorometer (or equivalent for QC check)

IMPORTANT

Although the recommended third-party ligase is supplied with its own buffer, the ligation efficiency of the Ligation Adapter (LA) is higher when using the Ligation Buffer (LNB) supplied in the Ligation Sequencing Kit.

1 **Spin down the Ligation Adapter (LA) and Quick T4 Ligase, and place on ice.**

2 **Thaw Ligation Buffer (LNB) at room temperature, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.**

3 **Thaw the Elution Buffer (EB) at room temperature, mix by vortexing, spin down and place on ice.**

4 **Thaw the Long Fragment Buffer (LFB) at room temperature, mix by vortexing, spin down and place on ice.**

5 **In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order:**

Between each addition, pipette mix 10-20 times.

Reagent	Volume
DNA sample from the previous step	60 µl
Ligation Buffer (LNB)	25 µl
NEBNext Quick T4 DNA Ligase	10 µl
Ligation Adapter (LA)	5 µl
Total	**100 µl**

6 **Ensure the components are thoroughly mixed by pipetting, and spin down.**

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

IMPORTANT

If you have omitted the AMPure purification step after DNA repair and end-prep, do not incubate the reaction for longer than 10 minutes.

8 **Resuspend the AMPure XP Beads (AXP) by vortexing.**

9 **Add 40 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by flicking the tube.**

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

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

12 **Wash the beads by adding 250 µl Long Fragment Buffer (LFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.**

13 **Repeat the previous step.**

14 **Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.**

15 **Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB). Spin down and incubate for 10 minutes at room temperature. For high molecular weight DNA, incubating at 37°C can improve the recovery of long fragments.**

16 **Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.**

17 **Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Dispose of the pelleted beads

Quantify 1 µl of eluted sample using a Qubit fluorometer.

IMPORTANT

We recommend loading 150 ng of the final prepared library onto the flow cell.

The loading recommendation has been optimised for the sample preparation and sequencing output of this protocol. The loading quantity differs from the standard Kit 14 ligation protocols due to a higher input requirement in the adaptive sampling.

18 **Take forward 150 ng of the final prepared library in 12 µl of Elution Buffer (EB).**

END OF STEP

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

TIP

Library storage recommendations

We recommend storing libraries in Eppendorf DNA LoBind tubes at**4°C for short term** storage or repeated use, for example, reloading 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.

IMPORTANT

Sequencing and flow cell washes

Sequence the sample for a total of 96 hours, with two flow cell washes.
After ~30 hours, or when the pore count drops to 40-50% of the initial number at the start of the experiment, pause the run and wash the flow cell using the Flow Cell Wash Kit. Load another 150 ng of library and sequence for another ~23 hours. After this, repeat the flow cell wash for the second time, load another 150 ng of library and sequence for the remaining ~43 hours.

Note: To avoid pore numbers falling too low before performing the flow cell wash, it may be necessary to pause the experiment overnight.

Priming and loading the SpotON flow cell

~10 minutes

Materials	• Flow Cell Flush (FCF) • Flow Cell Tether (FCT) • Library Solution (LIS) • Library Beads (LIB) • Sequencing Buffer (SB)
Consumables	• 1.5 ml Eppendorf DNA LoBind tubes • SpotON Flow Cell • Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
Equipment	• MinION or GridION device • P1000 pipette and tips • P100 pipette and tips • P20 pipette and tips • P10 pipette and tips

IMPORTANT

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

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 Library Solution

We recommend using the Library Beads (LIB) 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 Library Solution (LIS) instead of water.
Note: Some customers have noticed that viscous libraries can be loaded more easily when not using Library Beads (LIB).

1 Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and one tube of Flow Cell Flush (FCF) at room temperature. Mix by vortexing and spin down.

IMPORTANT

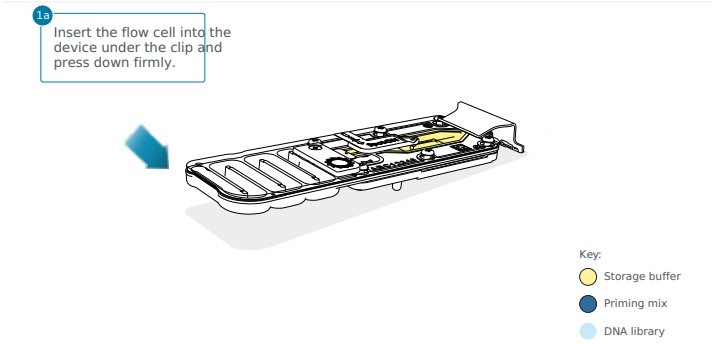
For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), we recommend adding Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.

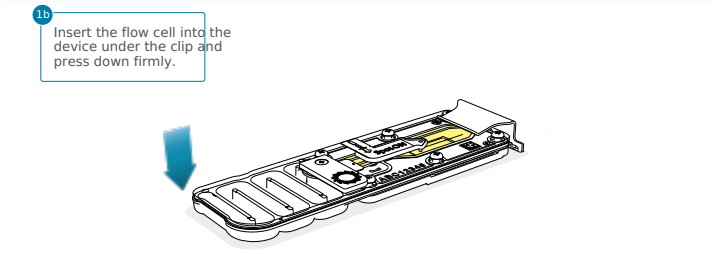
Note: We do not recommend using any other albumin type (e.g. recombinant human serum albumin).

2 To prepare the flow cell priming mix with BSA, add the following reagents directly to the tube of Flow Cell Flush (FCF), and mix by inverting the tube and pipette mix at room temperature:

Reagent	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 Flow Cell Flush (FCF) 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 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.

See the flow cell check instructions in the MinKNOW protocol for more information.

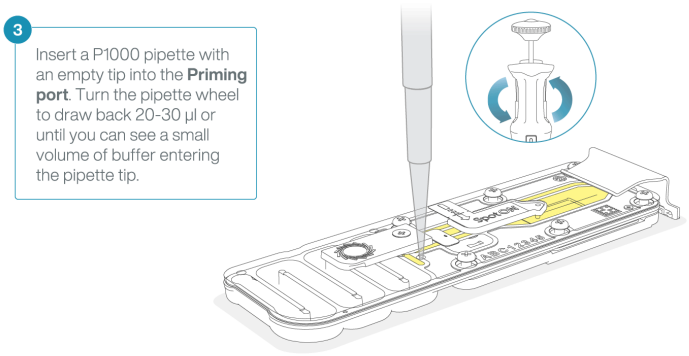
4 Slide the flow cell priming port cover clockwise to open the priming port.

IMPORTANT

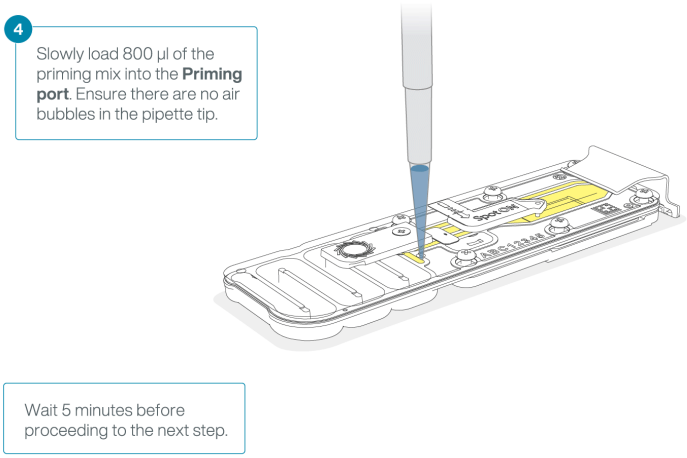
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

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 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.



6 **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.**



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

IMPORTANT

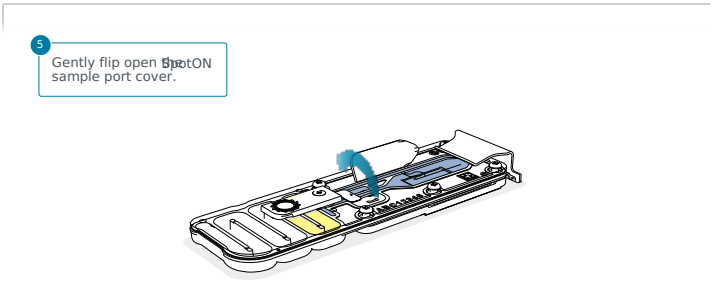
The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

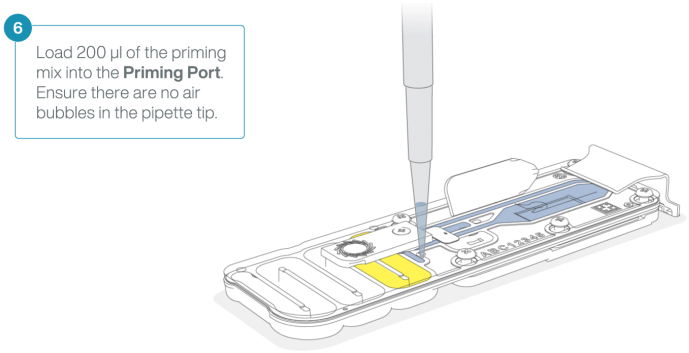
8 **In a new tube, prepare the library for loading as follows:**

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

9 Complete the flow cell priming:

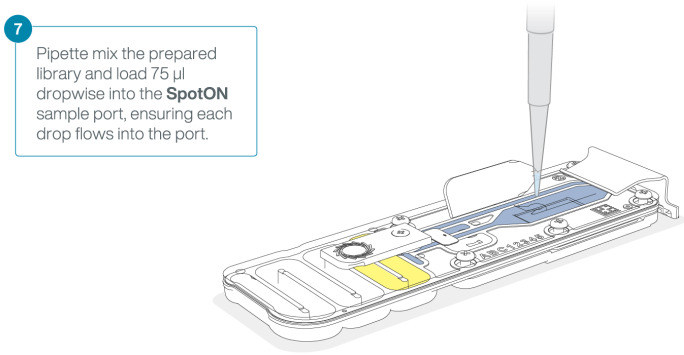
1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load **200 µl** of the priming mix into the flow cell priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.



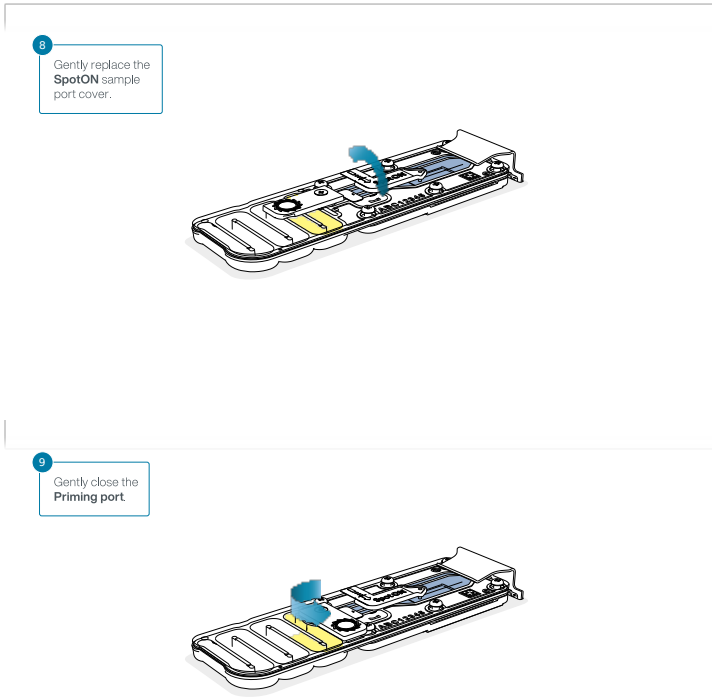


10 Mix the prepared library gently by pipetting up and down just prior to loading.

11 Add 75 µl 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.



12 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION or GridION device lid.



Data acquisition and basecalling

Overview of nanopore data analysis

For a full overview of nanopore data analysis, which includes options for basecalling and post-basecalling analysis, please refer to the Data Analysis document.

How to start sequencing

The sequencing device control and data acquisition are carried out by the MinKNOW software. It is assumed you have already installed MinKNOW on your computer. Further instructions for setting up your sequencing run can be found in the MinKNOW protocol.

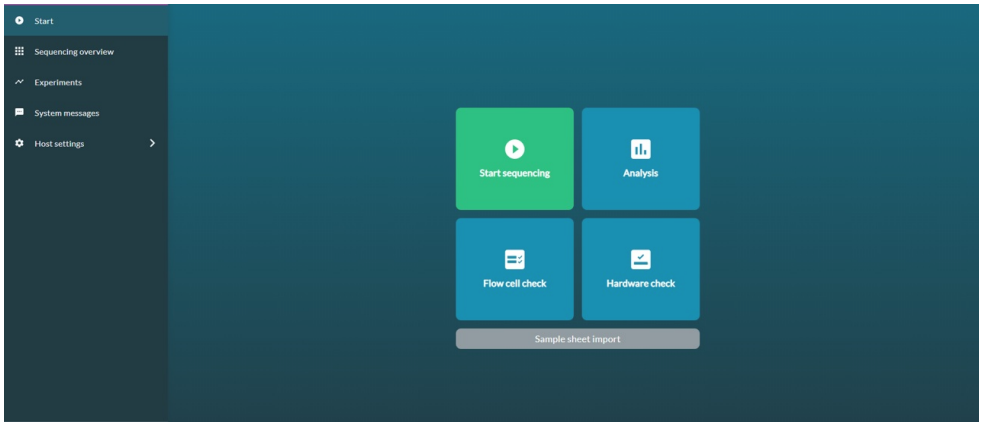
Basecalling will be carried out post-sequencing in the Downstream analysis section of the protocol.

1 **Open the MinKNOW software using the desktop shortcut and log into the MinKNOW software using your Community credentials.**

2 **Select your connected device.**

3 Sequencing set-up

Select **Start sequencing**.



4 Select positions

Enter your **Experiment name** and **sample ID** as prompted.

5 Kit selection

The kit selection tab will provide a dropdown of available kits. Check the SQK-LSK114 kit.

6 Run options

The run options tab provides variables for run time and starting voltage. Leave these at the default values.

Under **Adaptive sampling**, check **Enrich or deplete sequences**.

In the expanded menu, upload reference FASTA file and the .bed file.

7 Basecalling

In the analysis tab check **Basecalling OFF**.

Note: Data basecalling will be carried out post-sequencing in the Downstream analysis section of the protocol.

8 Output

Select where you would like to save the data.

9 **Review**

Review your sequencing set-up and click **Start**.

Downstream analysis

1 **Ensure you have installed the latest version of Guppy. To perform basecalling and methylation calling using Remora, open a terminal and enter the following commands:**

```
guppy_basecaller \
-i {input_fast5s} -s {output_folder} \
-c dna_r10.4.1_e8.2_400bps_modbases_5mc_cg_hac.cfg \
--align_ref {reference_fasta} \
--device auto \
--compress_fastq --bam_out --recursive \
--num_callers 5 --cpu_threads_per_caller 4
```

2 **Concatenate all BAM files output by Guppy into one:**

```
samtools cat {output_folder}/pass/*bam | \
samtools sort -@ 8 -o {out_bam} -
```

3 Index the merged BAM file:

```
samtools index -@ 8 {out_bam}
```

This will create a single sorted and indexed BAM file ({out_bam}) that contains canonical bases as well as per-read modifications and can be loaded into IGV. To visualise the per-read modification calls in IGV, load the BAM file and set "colour reads as" to "modifications".

This BAM file can be used to check the on-target coverage achieved during the Reduced Representation Methylation Sequencing (RRMS) run:

```
mosdepth -x -t 8 -n -b {target_bed} {prefix} {out_bam}
```

4 To create strand-specific and strand-aggregated methylation frequencies for all genomic positions (CpGs), run:

```
modbam2bed -m 5mC -e --cpg -t 8 \
--aggregate --prefix {prefix} \
{reference_fasta} \
{out_bam} > {out_mod_bed}
```

This will create two BEDMETHYL files: one will report methylation frequencies per genomic position and per strand, the second file will include the prefix specified and will report methylation frequencies by aggregating calls from the forward and reverse strand. The tool can be found in the following repository: https://github.com/epi2me-labs/modbam2bed

5 Filter reference CpG positions without canonical or modified calls (e.g. deletions from the reference) and genomic positions without calls from both strands:

```
cat {prefix}_cpg.acc.bed | csvtk filter2 -H -t \
-f '$11 != "nan" && $6 != "+" && $6 != "-"' > {out_mod_bed_agg_filt}
```

6 Convert the BEDMETHYL file to a TSV file that is compatible with the DMR tool DSS:

```
awk -v OFS='\t' 'BEGIN{print "chr","pos","N","X"}{print $1,$2,($12+$13),$13}' {out_mod_bed_agg_filt} > {out_mod_bed_agg_filt_DSS}
```

7 Convert the BEDMETHYL file to a BEDGRAPH file that will be used for obtaining the BIGWIG format useful for IGV visualisation:

```
awk -v OFS='\t' '{print $1,$2,$3,$11}' {out_mod_bed_agg_filt} | \
sort -k1,1 -k2,2n > {out_mod_bed_agg_filt_bedgraph}
bedGraphToBigWig {out_mod_bed_agg_filt_bedgraph} {reference_chrSize} {out_mod_bed_agg_filt_bigwig}
```

8 **Repeat the above steps for all your samples.**

For detection of differentially methylated regions use DSS as described here: https://bioconductor.org/packages/release/bioc/vignettes/DSS/inst/doc/DSS.html

Benchmarking results

For information about benchmarking the performance of RRMS, please see ourRRMS performance document.

Ending the experiment

Materials

- Flow Cell Wash Kit (EXP-WSH004)

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

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

TIP

We recommend you to wash the flow cell as soon as possible after you stop the run. However, if this is not possible, leave the flow cell on the device and wash it the next day.

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

Instructions for returning flow cells can be foundhere.

All flow cells must be flushed with deionised water before returning the product.

IMPORTANT

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

Issues during DNA/RNA extraction and library preparation

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

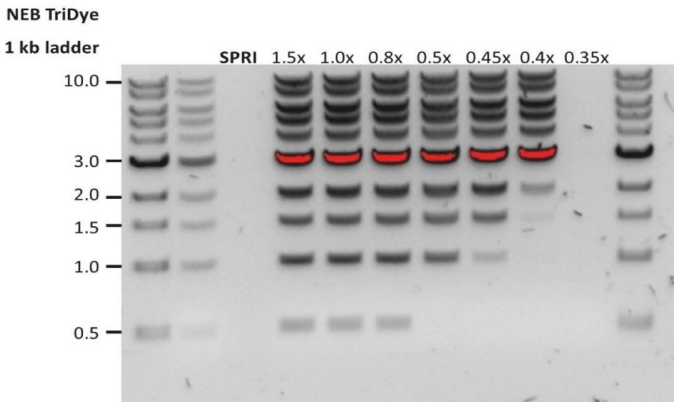
We also have an FAQ section available on the Nanopore Community Support section.

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. 

Observation	Possible cause	Comments and actions
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.

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

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

Observation	Possible cause	Comments and actions
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	An air bubble was introduced into the nanopore array	After the Flow Cell Check it is essential to remove any air bubbles near the priming port before priming the flow cell. If not removed, the air bubble can travel to the nanopore array and irreversibly damage the nanopores that have been exposed to air. The best practice to prevent this from happening is demonstrated in this video.
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	The flow cell is not correctly inserted into the device	Stop the sequencing run, remove the flow cell from the sequencing device and insert it again, checking that the flow cell is firmly seated in the device and that it has reached the target temperature. If applicable, try a different position on the device (GridION/PromethION).

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	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

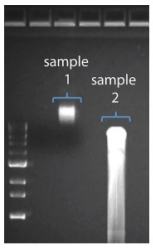
Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support.

Pore occupancy below 40%

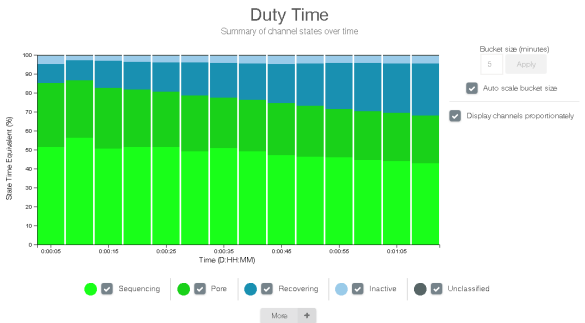
Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	5–50 fmol of good quality library can be loaded on to a MinION Mk1B/GridION flow cell. Please quantify the library before loading and calculate mols using tools like the Promega Biomath 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

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

Large proportion of recovering pores

Observation	Possible cause	Comments and actions
Large proportion of recovering pores (shown as dark blue in the channels panel and duty time plot)	Contaminants are present in the sample	Some contaminants can be cleared from the pores by the unblocking function built into MinKNOW. If this is successful, the pore status will change to "single pores". If the portion of recovering pores (unavailable pores in the extended view) stays large or increases: 1. A nuclease flush 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.  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 (please see the appropriate protocol for your DNA library to see the recommendation).	Add more fuel to the flow cell by following the instructions in the MinKNOW protocol. In future experiments, load lower amounts of library to the flow cell.

Temperature fluctuation

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

Failed to reach target temperature

Observation	Possible cause	Comments and actions
MinKNOW shows "Failed to reach target temperature"	The instrument was placed in a location that is colder than normal room temperature, or a location with poor ventilation (which leads to the flow cells overheating)	MinKNOW has a default timeframe for the flow cell to reach the target temperature. Once the timeframe is exceeded, an error message will appear and the sequencing experiment will continue. However, sequencing at an incorrect temperature may lead to a decrease in throughput and lower q-scores. Please adjust the location of the sequencing device to ensure that it is placed at room temperature with good ventilation, then re-start the process in MinKNOW. Please refer to this FAQ for more information on MinION Mk 1B temperature control.

Guppy – no input .fast5 was found or basecalled

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

Guppy – no Pass or Fail folders were generated after basecalling

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