Overview of the protocol

PCR Barcoding Expansion features:

We have two PCR barcoding expansions available depending on the number of barcodes required:

- PCR Barcoding Expansion 1-12 (EXP-PBC001): up to 12 unique barcodes are available
- PCR Barcoding Expansion 1-96 (EXP-PBC096): up to 96 unique barcodes are available

These expansions are used with the Ligation Sequencing Kit V14 and recommended for users who:

- Want to multiplex up to 12 or 96 barcodes, depending on the expansion they are using.
- Would like to achieve raw read sequencing modal accuracy of Q20+ (99%) or above.
- Want to optimise their sequencing experiment for output.
- Require control over read length.
- Would like to utilise upstream processes such as size selection or whole genome amplification.

Introduction to the PCR Barcoding protocol

This protocol describes how to carry out PCR barcoding of gDNA using the Ligation Sequencing Kit V14 (SQK-LSK114) with the PCR Barcoding Expansion Pack 1-12 (EXP-PBC001) or 1-96 (EXP-PBC096). This protocol also outlines recommendations to PCR barcode amplicons. Using the PCR Barcoding expansions allows for up to either 12 or 96 samples to be combined and loaded onto a single flow cell. It is highly recommended that a Lambda control experiment is completed first to become familiar with the technology.

Note: For amplicon inputs, first-round PCR product with the following tailed primers are required. Please see the equipment and consumables page for further information.
5’ TTTCTGTTGGTGCTGATATTGC-[project-specific forward primer sequence] 3’
5’ ACTTGCCTGTCGCTCTATCTTC-[project-specific reverse primer sequence] 3’

Steps in the sequencing workflow:

Prepare for your experiment
You will need to:

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

Experiment workflow
Prepare your gDNA input:

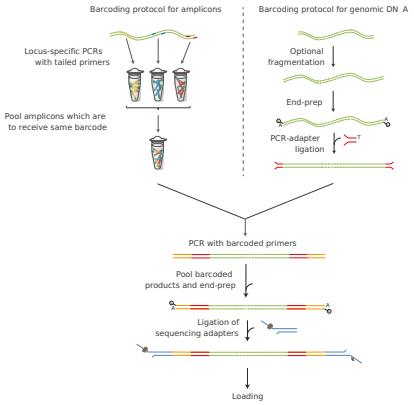
- Prepare the DNA ends for adapter attachment
- Attach barcoding adapters to the DNA ends
- Amplify each barcoded sample by PCR, then pool the samples together

OR prepare your amplicon input:

- Perform a round of PCR to incorporate tailed primers
- Complete a second round of PCR to incorporate the Oxford Nanopore barcode sequences and amplify each barcoded sample, then pool the samples together

Library preparation:

- Prepare the DNA ends for adapter attachment
- Attach sequencing adapters to the DNA ends
- Prime the flow cell, and load your DNA library into the flow cell



Sequencing and analysis

You will need to:

- In the current MinKNOW software version, the PCR Barcoding Expansions are not available in "Kit selection" when setting up a sequencing run. These will be included in the next software update. For the meantime, we recommend the following:
  - Start a sequencing run in the MinKNOW software using SQK-LSK114, which will collect raw data from the device and convert it into basecalled reads.
  - Demultiplex your data post-run on MinKNOW using the barcoding option, choosing either EXP-PBC001 or EXP-PBC096 as your barcoding kit. Further information is available in the "Post-run barcoding" of the MinKNOW protocol.
- Start the EPI2ME software and select the barcoding workflow for further analysis (this step is optional).

IMPORTANT

We do not recommend mixing barcoded libraries with non-barcoded libraries prior to sequencing.

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- PCR Barcoding Expansion Pack 1-12 (EXP-PBC001)
- PCR Barcoding Expansion Pack 1-96 (EXP-PBC096)
- Ligation Sequencing Kit V14 (SQK-LSK114)
- R10.4.1 MinION Flow Cells (FLO-MIN114)
- Flow Cell Wash Kit (EXP-WSH004)
- Control Expansion (EXP-CTL001)
- Flow Cell Priming Kit V14 (EXP-FLP004)

Equipment and consumables

Materials	• <100–200 fmol of each DNA sample to be barcoded in 45 µl • OR <100–200 fmol first-round PCR product (with tailed primers) per sample • PCR Barcoding Expansion 1-12 (EXP-PBC001) • PCR Barcoding Expansion 1-96 (EXP-PBC096) • Ligation Sequencing Kit V14 (SQK-LSK114)
Consumables	• NEBNext Ultra II End repair/dA-tailing Module (NEB, cat # E7546) • NEBNext Quick Ligation Module (NEB, cat # E6056) • NEB Blunt/TA Ligase Master Mix (NEB, cat # M0367) • LongAmp Taq 2X Master Mix (e.g. NEB, cat # M0287) • 1.5 ml Eppendorf DNA LoBind tubes • 0.2 ml thin-walled PCR tubes or 0.2 ml 96-well PCR plate • Freshly prepared 80% ethanol in nuclease-free water • Agencourt AMPure XP beads (Beckman Coulter™, cat # A63881) • Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • Qubit™ Assay Tubes (Invitrogen, cat # Q32856) • Qubit dsDNA HS Assay Kit (Invitrogen, cat # Q32851) • (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
Equipment	• Hula mixer (gentle rotator mixer) • 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 • Magnetic rack • Timer • Multichannel pipette • Qubit fluorometer (or equivalent for QC check)
Optional Equipment	• Agilent Bioanalyzer (or equivalent) • Eppendorf 5424 centrifuge (or equivalent)

For gDNA input, <100–200 fmol of each DNA sample to be barcoded in 45 µl is required.

For amplicon input, start from the "Barcoding PCR" step with <100–200 fmol first-round PCR product with tailed primers in the following volumes per sample:

- 48 µl for up to 12 barcodes
- 24 µl for 13 barcodes or more

Start from the "Barcoding PCR" step after the amplicons have undergone a first round of PCR to incorporate the following 5' tail sequences:

5’ TTTCTGTTGGTGCTGATATTGC-[project-specific forward primer sequence] 3’
5’ ACTTGCCTGTCGCTCTATCTTC-[project-specific reverse primer sequence] 3’

The sequences above are required for the Barcoding PCR step to incorporate the Oxford Nanopore barcode sequences into your amplicon. Multiple first-round PCR products can be pooled together and we recommend all amplicon samples to receive the same barcode should be quantified and pooled in the desired ratios before the barcoding PCR step is performed.

After the first round of PCR to incorporate the 5' tail is complete, a purification step is required to remove any proteins, salts, dNTPs and primers. We recommend using AMPure XP Beads but other methods suitable for the amplicon size may also be used.

If you do not want to redesign your primers, the Barcode Adapter (BCA) from the PCR Barcoding Expansion 1-12 or 1-96 (EXP-PBC001 or EXP-PBC096) can be ligated onto the ends of the amplicons and the protocol started from the first end-prep step.

IMPORTANT

Optional fragmentation and size selection

By default, the protocol contains no DNA fragmentation step, however in some cases it may be advantageous to fragment your sample. For example, when working with lower amounts of input gDNA (25 ng–125 ng), fragmentation will increase the number of DNA molecules and therefore increase throughput. Instructions are available in the DNA Fragmentation section of Extraction methods.

Additionally, we offer several options for size-selecting your DNA sample to enrich for long fragments - instructions are available in the Size Selection section of Extraction methods.

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.

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.

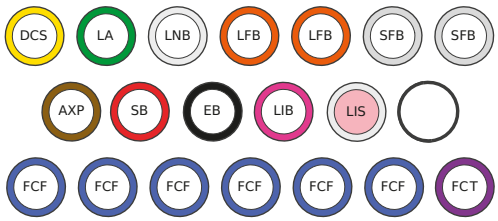
For all third-party reagents, we recommend following the manufacturer's instructions to prepare the reagents for use.

IMPORTANT

AMPure XP Beads

Within the Ligation Sequencing Kit V14 (SQK-LSK114), AMPure XP Beads (AXP) are supplied at the volume needed to complete the "End-prep" and "Adapter ligation and clean up" steps of the protocol. However, for initially preparing the sample ends and the PCR steps, extra AMPure XP Beads are required for purification and clean-up. Please note, other purification methods are available.

Ligation Sequencing Kit V14 (SQK-LSK114) contents

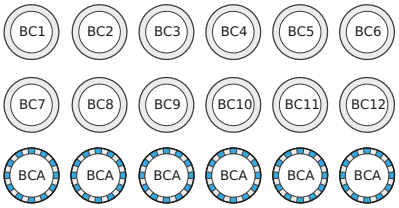


DCS : DNA Control Strand
LA : Ligation Adapter
LNB : Ligation Buffer
LFB : Long Fragment Buffer
SFB : Short Fragment Buffer
AXP : AMPure XP Beads
SB : Sequencing Buffer
EB : Elution Buffer
LIB : Library Beads
LIS : Library Solution
FCF : Flow Cell Flush
FCT : Flow Cell Tether

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
DNA CS	DCS	Yellow	1	35
Ligation Adapter	LA	Green	1	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.

PCR Barcoding Expansion 1-12 (EXP-PBC001) contents



BC1: PCR barcode 1
BC2: PCR barcode 2
BC3: PCR barcode 3
BC4: PCR barcode 4
BC5: PCR barcode 5
BC6: PCR barcode 6
BC7: PCR barcode 7
BC8: PCR barcode 8
BC9: PCR barcode 9
BC10: PCR barcode 10
BC11: PCR barcode 11
BC12: PCR barcode 12
BCA: Barcode adapter

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
PCR Barcode 1-12	BC1-12	Clear	12	20
Barcode Adapter	BCA	Blue stripe	1	260

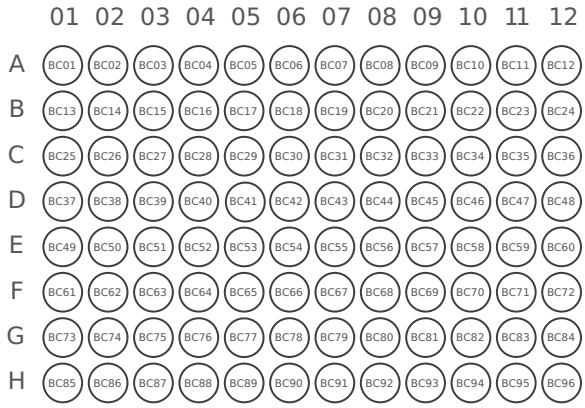
PCR Barcoding Expansion Pack 1-96 (EXP-PBC096)

Name	Acronym	Cap colour	No. of vials/plates	Fill volume per well (µl)
PCR Barcode Primer Mix plate	BC01-96	White	1 plate	24
Barcode Adapter plate	BCA	Blue	1 plate	240

Layout of barcodes in the 96 tube plate

The wells of the 96 tube plate correspond to the barcodes in the following way. All barcodes are supplied at 10 µM concentration and

to be used at a final concentration of 0.2 µM.



Capping and decapping the 96 well format

96 barcode sequences

Component	Sequence
BC01 / RB01	AAGAAAGTTGTCGGTGTCTTTGTG
BC02 / RB02	TCGATTCCGTTTGTAGTCGTCTGT
BC03 / RB03	GAGTCTTGTGTCCCAGTTACCAGG
BC04 / RB04	TTCGGATTCTATCGTGTTTCCCTA
BC05 / RB05	CTTGTCCAGGGTTTGTGTAACCTT
BC06 / RB06	TTCTCGCAAAGGCAGAAAGTAGTC

Component	Sequence
BC07 / RB07	GTGTTACCGTGGGAATGAATCCTT
BC08 / RB08	TTCAGGGAACAAACCAAGTTACGT
BC09 / RB09	AACTAGGCACAGCGAGTCTTGGTT
BC10 / RB10	AAGCGTTGAAACCTTTGTCCTCTC
BC11 / RB11	GTTTCATCTATCGGAGGGAATGGA
BC12 / RB12	CAGGTAGAAAGAAGCAGAATCGGA
BC13 / 16S13 / RB13	AGAACGACTTCCATACTCGTGTGA
BC14 / 16S14 / RB14	AACGAGTCTCTTGGGACCCATAGA
BC15 / 16S15 / RB15	AGGTCTACCTCGCTAACACCACTG
BC16 / 16S16 / RB16	CGTCAACTGACAGTGGTTCGTACT
BC17 / 16S17 / RB17	ACCCTCCAGGAAAGTACCTCTGAT
BC18 / 16S18 / RB18	CCAAACCCAACAACCTAGATAGGC
BC19 / 16S19 / RB19	GTTCCTCGTGCAGTGTCAAGAGAT
BC20 / 16S20 / RB20	TTGCGTCCTGTTACGAGAACTCAT
BC21 / 16S21 / RB21	GAGCCTCTCATTGTCCGTTCTCTA
BC22 / 16S22 / RB22	ACCACTGCCATGTATCAAAGTACG
BC23 / 16S23 / RB23	CTTACTACCCAGTGAACCTCCTCG
BC24 / 16S24 / RB24	GCATAGTTCTGCATGATGGGTTAG
BC25 / RB25	GTAAGTTGGGTATGCAACGCAATG
BC26 / RB26	CATACAGCGACTACGCATTCTCAT
BC27 / RB27	CGACGGTTAGATTCACCTCTTACA
BC28 / RB28	TGAAACCTAAGAAGGCACCGTATC
BC29 / RB29	CTAGACACCTTGGGTTGACAGACC
BC30 / RB30	TCAGTGAGGATCTACTTCGACCCA
BC31 / RB31	TGCGTACAGCAATCAGTTACATTG

Component	Sequence
BC32 / RB32	CCAGTAGAAGTCCGACAACGTCAT
BC33 / RB33	CAGACTTGGTACGGTTGGGTAACT
BC34 / RB34	GGACGAAGAACTCAAGTCAAAGGC
BC35 / RB35	CTACTTACGAAGCTGAGGGACTGC
BC36 / RB36	ATGTCCCAGTTAGAGGAGGAAACA
BC37 / RB37	GCTTGCGATTGATGCTTAGTATCA
BC38 / RB38	ACCACAGGAGGACGATACAGAGAA
BC39 / RB39	CCACAGTGTCAACTAGAGCCTCTC
BC40 / RB40	TAGTTTGGATGACCAAGGATAGCC
BC41 / RB41	GGAGTTCGTCCAGAGAAGTACACG
BC42 / RB42	CTACGTGTAAGGCATACCTGCCAG
BC43 / RB43	CTTTCGTTGTTGACTCGACGGTAG
BC44 / RB44	AGTAGAAAGGGTTCCTTCCCACTC
BC45 / RB45	GATCCAACAGAGATGCCTTCAGTG
BC46 / RB46	GCTGTGTTCCACTTCATTCTCCTG
BC47 / RB47	GTGCAACTTTCCCACAGGTAGTTC
BC48 / RB48	CATCTGGAACGTGGTACACCTGTA
BC49 / RB49	ACTGGTGCAGCTTTGAACATCTAG
BC50 / RB50	ATGGACTTTGGTAACTTCCTGCGT
BC51 / RB51	GTTGAATGAGCCTACTGGGTCCTC
BC52 / RB52	TGAGAGACAAGATTGTTCGTGGAC
BC53 / RB53	AGATTCAGACCGTCTCATGCAAAG
BC54 / RB54	CAAGAGCTTTGACTAAGGAGCATG
BC55 / RB55	TGGAAGATGAGACCCTGATCTACG
BC56 / RB56	TCACTACTCAACAGGTGGCATGAA
BC57 / RB57	GCTAGGTCAATCTCCTTCGGAAGT
BC58 / RB58	CAGGTTACTCCTCCGTGAGTCTGA
BC59 / RB59	TCAATCAAGAAGGGAAAGCAAGGT
BC60 / RB60	CATGTTCAACCAAGGCTTCTATGG
BC61 / RB61	AGAGGGTACTATGTGCCTCAGCAC
BC62 / RB62	CACCCACACTTACTTCAGGACGTA
BC63 / RB63	TTCTGAAGTTCCTGGGTCTTGAAC

Component	Sequence
BC64 / RB64	GACAGACACCGTTCATCGACTTTC
BC65 / RB65	TTCTCAGTCTTCCTCCAGACAAGG
BC66 / RB66	CCGATCCTTGTGGCTTCTAACTTC
BC67 / RB67	GTTTGTCATACTCGTGTGCTCACC
BC68 / RB68	GAATCTAAGCAAACACGAAGGTGG
BC69 / RB69	TACAGTCCGAGCCTCATGTGATCT
BC70 / RB70	ACCGAGATCCTACGAATGGAGTGT
BC71 / RB71	CCTGGGAGCATCAGGTAGTAACAG
BC72 / RB72	TAGCTGACTGTCTTCCATACCGAC
BC73 / RB73	AAGAAACAGGATGACAGAACCCTC
BC74 / RB74	TACAAGCATCCCAACACTTCCACT
BC75 / RB75	GACCATTGTGATGAACCCTGTTGT
BC76 / RB76	ATGCTTGTTACATCAACCCTGGAC
BC77 / RB77	CGACCTGTTTCTCAGGGATACAAC
BC78 / RB78	AACAACCGAACCTTTGAATCAGAA
BC79 / RB79	TCTCGGAGATAGTTCTCACTGCTG
BC80 / RB80	CGGATGAACATAGGATAGCGATTC
BC81 / RB81	CCTCATCTTGTGAAGTTGTTTCGG
BC82 / RB82	ACGGTATGTCGAGTTCCAGGACTA
BC83 / RB83	TGGCTTGATCTAGGTAAGGTCGAA
BC84 / RB84	GTAGTGGACCTAGAACCTGTGCCA
BC85 / RB85	AACGGAGGAGTTAGTTGGATGATC
BC86 / RB86	AGGTGATCCCAACAAGCGTAAGTA
BC87 / RB87	TACATGCTCCTGTTGTTAGGGAGG
BC88 / RB88	TCTTCTACTACCGATCCGAAGCAG
BC89 / RB89	ACAGCATCAATGTTTGGCTAGTTG
BC90 / RB90	GATGTAGAGGGTACGGTTTGAGGC
BC91 / RB91	GGCTCCATAGGAACTCACGCTACT
BC92 / RB92	TTGTGAGTGGAAAGATACAGGACC
BC93 / RB93	AGTTTCCATCACTTCAGACTTGGG
BC94 / RB94	GATTGTCCTCAAACTGCCACCTAC
BC95 / RB95	CCTGTCTGGAAGAAGAATGGACTT

Component	Sequence
BC96 / RB96	CTGAACGGTCATAGAGTCCACCAT

Computer requirements and software

MinION Mk1C IT requirements

The MinION Mk1C contains fully-integrated compute and screen, removing the need for any accessories to generate and analyse nanopore data. Read more in the MinION Mk1C IT requirements document.

MinION Mk1B IT requirements

Sequencing on a MinION Mk1B requires a high-spec computer or laptop to keep up with the rate of data acquisition. Read more in the MinION IT Requirements document.

Software for nanopore sequencing

MinKNOW

The MinKNOW software controls the nanopore sequencing device, collects sequencing data and basecalls in real time. You will be using MinKNOW for every sequencing experiment to sequence and demultiplex if samples are barcoded. Please note that live demultiplexing in MinKNOW is not enabled for the Amplicon Barcoding Kit 24. Post-run demultiplexing using the standalone Guppy software is required.

MinKNOW use

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

Guppy

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.

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.

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

End-prep

~25 minutes

Materials

- <100–200 fmol of each DNA sample to be barcoded in 45 µl

Consumables

- NEBNext Ultra II End repair/dA-tailing Module (NEB, cat # E7546)
- Freshly prepared 80% ethanol in nuclease-free water
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Agencourt AMPure XP beads (Beckman Coulter™, cat # A63881)
- 0.2 ml thin-walled PCR tubes or 0.2 ml 96-well PCR plate
- Qubit™ Assay Tubes (Invitrogen, cat # Q32856)
- Qubit dsDNA HS Assay Kit (Invitrogen, cat # Q32851)

Equipment

- P1000 pipette and tips
- P100 pipette and tips
- P10 pipette and tips
- Thermal cycler
- Ice bucket with ice
- Microfuge
- Magnetic rack suitable for 0.2 ml thin-walled PCR tubes or 96-well plates
- Vortex mixer

Optional Equipment

- Qubit fluorometer (or equivalent for QC check)

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

For optimal perfomance, 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 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 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.

2 **Prepare the DNA in nuclease-free water.**

1. Transfer <100-200 fmol DNA of each sample into a fresh 0.2 ml PCR tube or plate
2. Adjust the volume to 45 µl with nuclease-free water
3. Mix thoroughly by flicking the tube to avoid unwanted shearing
4. Spin down briefly in a microfuge

3 **Set up the end-repair reaction as follows for each library:**

Between each addition, pipette mix 10-20 times.

Reagent	Volume per sample
<100-200 fmol DNA	45 µl
Ultra II End-prep reaction buffer	7 µl
Ultra II End-prep enzyme mix	3 µl
Nuclease-free water	5 µl
Total	**60 µl**

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

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

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

7 **Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting.**

8 **Incubate at room temperature for 5 minutes.**

9 **Prepare sufficient fresh 80% ethanol in nuclease-free water for all of your samples. Allow enough for 400 µl per sample, with some excess.**

10 **Spin down the samples and pellet on a magnet until supernatant is clear and colourless. Keep the samples on the magnet, and pipette off the supernatant.**

11 **Keep the samples 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.**

12 **Repeat the previous step.**

13 **Spin down and place the samples back on the magnetic rack. Pipette off any residual ethanol. Allow the pellet to dry for ~30 seconds, but do not dry the pellet to the point of cracking.**

14 **Remove the samples from the magnet and resuspend each pellet in 16 µl nuclease-free water. Incubate for 2 minutes at room temperature.**

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

16 **Remove eluate once it is clear and colourless. Transfer each eluted sample to a new tube or plate well.**

17 **Quantify 1 µl of end-prepped DNA using a Qubit fluorometer.**

END OF STEP

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

Ligation of Barcode Adapter

~40 minutes

Materials

- Barcode Adapter (BCA)

Consumables

- NEB Blunt/TA Ligase Master Mix (NEB, cat # M0367)
- Agencourt AMPure XP beads (Beckman Coulter™, cat # A63881)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 80% ethanol in nuclease-free water
- 0.2 ml thin-walled PCR tubes or 0.2 ml 96-well PCR plate
- Qubit™ Assay Tubes (Invitrogen, cat # Q32856)

- Qubit dsDNA HS Assay Kit (Invitrogen, cat # Q32851)

Equipment

- Microfuge
- Hula mixer (gentle rotator mixer)
- Vortex mixer
- Ice bucket with ice
- Multichannel pipette
- Magnetic rack
- P1000 pipette and tips
- P100 pipette and tips
- P10 pipette and tips
- Qubit fluorometer (or equivalent for QC check)

1 **Prepare the NEB Blunt/TA Ligase Master Mix according to the manufacturer's instructions, and place on ice:**

1. Thaw the reagents at room temperature.
2. Spin down the reagent tubes for 5 seconds.
3. Ensure the reagents are fully mixed by performing 10 full volume pipette mixes.

2 **Spin down the Barcode Adapter (BCA), pipette mix and place on ice.**

3 **Add the reagents in the order given below, into fresh 0.2 ml PCR tubes or 96-well plate:**

Between each addition, pipette mix 10-20 times.

Reagent	Volume
End-prepped DNA	15 µl
Barcode Adapter	10 µl
Blunt/TA Ligase Master Mix	25 µl
Total	**50 µl**

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

5 **Incubate the samples for 10 minutes at room temperature.**

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

7 **Add 20 µl of resuspended AMPure XP beads to each sample for a 0.4X clean and mix by pipetting up and down ten times.**

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

9 **Prepare sufficient fresh 80% ethanol in nuclease-free water for all of your samples. Allow enough for 400 µl per sample, with some excess.**

10 **Place on a magnetic rack, allow beads to pellet and pipette off supernatant.**

11 **Keep the samples 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.**

12 **Repeat the previous step.**

13 **Place the samples back on the magnet. Pipette off any residual 80% ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.**

14 **Remove the samples from the magnet and resuspend pellet in 25 µl nuclease-free water. Incubate for 2 minutes at room temperature.**

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

16 **Remove and retain the eluate once it is clear and colourless. Transfer each eluted sample to a fresh 0.2 ml PCR tube or plate.**

- Dispose of the pelleted beads.

17 **Quantify 1 µl of the adapter ligated DNA using a Qubit fluorometer.**

END OF STEP

Take forward the adapter ligated samples into the Barcoding PCR step. However, at this point it is also possible to store the sample at 4°C overnight.

Barcoding PCR

~15 minutes

Materials

- 100-200 fmol of each adapter-ligated sample
- OR 100-200 fmol of each amplicon sample
- PCR Barcodes (BC01-96, at 10 µM)

Consumables	• LongAmp Taq 2X Master Mix (e.g. NEB, cat # M0287) • Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • Agencourt AMPure XP Beads (Beckman Coulter™, cat # A63881) • Freshly prepared 80% ethanol in nuclease-free water • 1.5 ml Eppendorf DNA LoBind tubes • 0.2 ml thin-walled PCR tubes or 0.2 ml 96-well PCR plate • Qubit™ Assay Tubes (Invitrogen, cat # Q32856) • Qubit dsDNA HS Assay Kit (Invitrogen, cat # Q32851)
Equipment	• Thermal cycler • Magnetic rack • Microfuge • P1000 pipette and tips • P100 pipette and tips • P10 pipette and tips • Qubit fluorometer (or equivalent for QC check)

Please note, this protocol is written for a template input of 100–200 fmol with PCR Barcodes (BC01-96) used at a final concentration of 0.2 µM. However, the input mass and the number of PCR cycles may be adjusted as appropriate depending on the requirements of the experiment.

1 Thaw the PCR Barcodes (BC01-96) required for your number of samples at room temperature. Individually mix the barcodes by pipetting, spin down, and place on ice.

> **IMPORTANT**
>
> **If using amplicon samples, ensure the samples have undergone a round of PCR with tailed primers before commencing with the protocol.**

2 Prepare the samples in nuclease-free water:

1. Transfer 100-200 fmol of each sample to a clear 0.2 ml PCR tube or plate
    - **For 1–12 samples:** Adjust the volume to **48 µl** with nuclease-free water
    - **For 13–96 samples:** Adjust the volume to **24 µl** with nuclease-free water
2. Mix thoroughly by flicking the tube or plate to avoid unwanted shearing
3. Spin down briefly in a microfuge

3 Select a unique barcode for each sample to be run together on the same flow cell.

Note: Only use one barcode per sample.

4 Set up a barcoding PCR reaction as follows for each library in fresh 0.2 ml PCR tubes or plate.

Between each addition, pipette mix 10-20 times.

Reagent	Volume per sample for using 1–12 barcodes	Volume per sample for using 13 barcodes or more
PCR Barcode (one of BC1-BC96, at 10 µM)	2 µl	1 µl
Adapter ligated DNA or amplicons with tailed primers	48 µl	24 µl
LongAmp Taq 2X master mix	50 µl	25 µl
Total volume	**100 µl**	**50 µl**

If the amount of input material is altered, the number of PCR cycles may need to be adjusted to produce the same yield.

5 Mix by pipetting and briefly spin down.

6 Amplify using the following cycling conditions:

Cycle step	Temperature	Time	No. of cycles
Initial denaturation	95 °C	3 mins	1
Denaturation	95 °C	15 secs	12-15 (b)
Annealing	62 °C (a)	15 secs (a)	12-15 (b)
Extension	65 °C (c)	dependent on length of target fragment (d)	12-15 (b)
Final extension	65 °C	dependent on length of target fragment (d)	1
Hold	4 °C	∞	

a. This is specific to the Oxford Nanopore primer and should be maintained

b. Adjust accordingly if input quantities are altered

c. This temperature is determined by the type of polymerase that is being used (given here for LongAmp Taq polymerase)

d. Adjust accordingly for different lengths of amplicons and the type of polymerase that is being used. During the development of this kit, 8 minutes was used as standard for DNA fragmented to 8 kb.

7 Resuspend the AMPure XP beads by vortexing.

8 **Add 0.4X volume of resuspended AMPure XP Beads to each reaction and mix by flicking the tube.**

Reagent	Volume for 100 µl samples	Volume for 50 µl samples
AMPure XP Beads	40 µl	20 µl

9 **Incubate at room temperature for 5 minutes.**

10 **Prepare sufficient fresh 80% ethanol in nuclease-free water for all of your samples. Allow enough for 400 µl per sample, with some excess.**

11 **Place on a magnetic rack, allow beads to pellet and pipette off supernatant.**

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

13 **Repeat the previous step.**

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

15 **Remove the samples from the magnetic rack and resuspend each pellet in 25 µl nuclease-free water. Incubate for 2 minutes at room temperature.**

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

17 **Remove and retain 25 µl of each eluate into clean 0.2 ml PCR tubes or plate.**

Dispose of the pelleted beads

18 **Quantify the barcoded library using a Qubit fluorometer and pool all barcoded libraries in the desired ratios in a 1.5 ml DNA LoBind Eppendorf tube.**

Gel analysis of amplified and ligated DNA

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.

19 **Prepare 1 µg of pooled barcoded libraries in 49 µl nuclease-free water.**

If the volume of your pool exceeds the 49 µl required for the end-prep reaction, consider a 2.5X AMPure XP Bead purification of the pool to concentrate your sample.

END OF STEP

This pooled library is now ready to be end-repaired and adapted for sequencing. However, at this point it is also possible to store the sample at 4°C overnight.

End-prep

~25 minutes

Materials	• DNA Control Sample (DCS) • 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 Ultra II End Repair / dA-tailing Module (NEB, cat # E7546) • Freshly prepared 80% ethanol in nuclease-free water • Qubit™ Assay Tubes (Invitrogen, cat # Q32856) • Qubit dsDNA HS Assay Kit (Invitrogen, 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 **Thaw the AMPure XP Beads (AXP) and DNA Control Sample (DCS) at room temperature and mix by vortexing. Keep the beads at room temperature and store the DNA Control Sample (DCS) on ice.**

TIP

We recommend using the DNA Control Sample (DCS) in your library prep for troubleshooting purposes. However, you can omit this step and make up the extra 1 µl with your sample DNA.

2 Prepare the NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice:

For optimal perfomance, 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 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 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.

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

Reagent	Volume
DNA Control Sample (DCS)	1 µl
DNA	49 µl
Ultra II End-prep Reaction Buffer	7 µl
Ultra II End-prep Enzyme Mix	3 µl
Total	**60 µl**

4 Ensure the reaction is thoroughly mixed by gently pipetting and spin down briefly.

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

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

~35 minutes

Materials	• Ligation Adapter (LA) • Ligation Buffer (LNB) from the Ligation Sequencing Kit • Long Fragment Buffer (LFB) • Short Fragment Buffer (SFB) • 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 dsDNA HS Assay Kit (Invitrogen, cat # Q32851) • Qubit™ Assay Tubes (Invitrogen, cat # Q32856)
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 Prepare the NEBNext Quick Ligation Reaction Module according to the manufacturer's instructions, and place on ice:

1. Thaw the reagents at room temperature.
2. Spin down the reagent tubes for 5 seconds.
3. Ensure the reagents are fully mixed by performing 10 full volume pipette mixes.

The NEBNext Quick Ligation Reaction Buffer (5x) may have a little precipitate. Allow the mixture to come to room temperature and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for several seconds to ensure the reagent is thoroughly mixed.

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

3 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.

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

IMPORTANT

Depending on the wash buffer (LFB or SFB) used, the clean-up step after adapter ligation is designed to either enrich for DNA fragments of >3 kb, or purify all fragments equally.

- To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB)
- To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB)

5 Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at room temperature, mix by vortexing, spin down and place on ice.

6 **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**

7 **Ensure the reaction is thoroughly mixed by gently pipetting and spin down briefly.**

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

9 **Resuspend the AMPure XP beads (AXP) by vortexing.**

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

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

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

13 **Wash the beads by adding either 250 µl Long Fragment Buffer (LFB) or 250 µl Short Fragment Buffer (SFB). 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.**

14 **Repeat the previous step.**

15 **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.**

16 **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.**

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

18 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.

19 Make up your library to 12 µl at 10-20 fmol, using Elution Buffer (EB).

Optional Action
If quantities allow, the library may be diluted in Elution Buffer (EB) for splitting across multiple flow cells.

Additional buffer for doing this can be found in the Sequencing Auxiliary Vials expansion (EXP-AUX003), available to purchase separately. This expansion also contains additional vials of Sequencing Buffer (SB) and Library Beads (LIB), required for loading the libraries onto flow cells.

IMPORTANT

We recommend loading 10-20 fmol of this final prepared library onto the R10.4.1 flow cell.

Loading more than 20 fmol of DNA can reduce the rate of duplex read capture. Dilute the library in Elution Buffer if required.

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, 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.

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

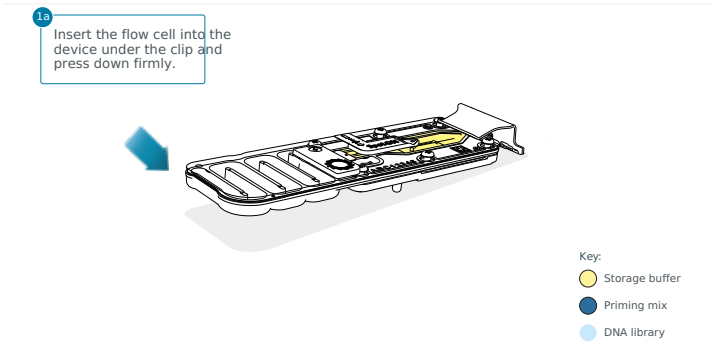
For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), we recommend adding Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.

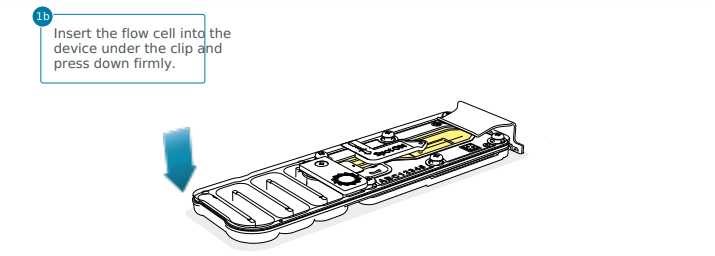
Note: We do not recommend using any other albumin type (e.g. recombinant human serum albumin).

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 pipetting 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
Total volume	**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.

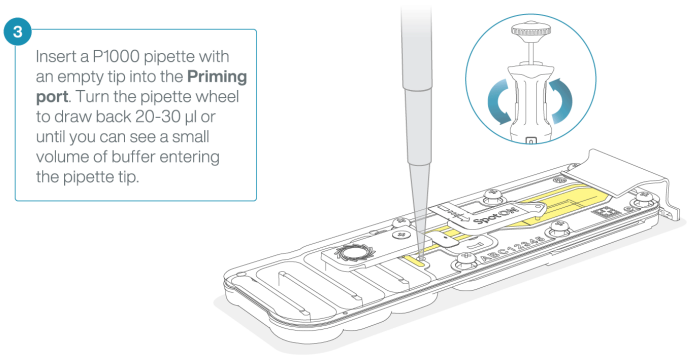
4 **Slide the flow cell priming port cover clockwise to open the priming port.**

IMPORTANT

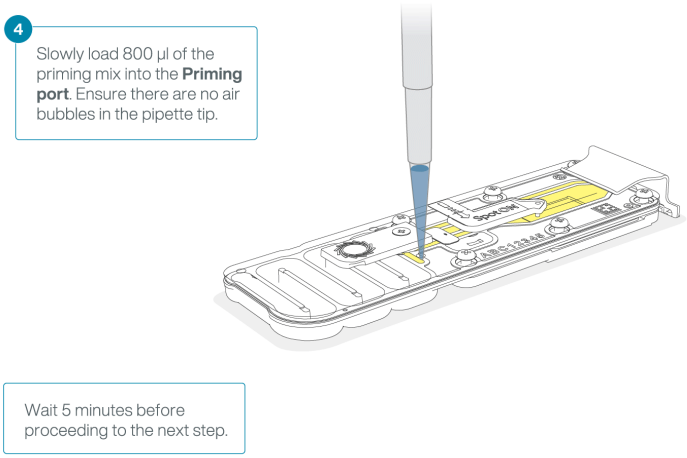
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

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

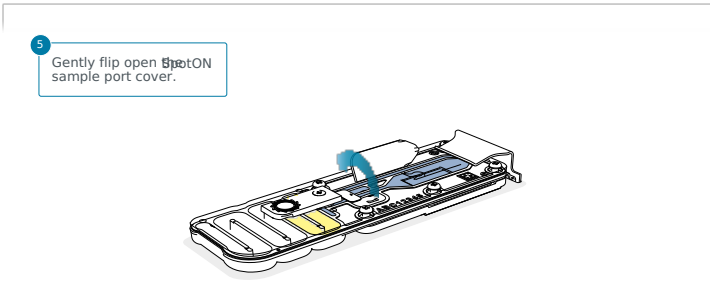
The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

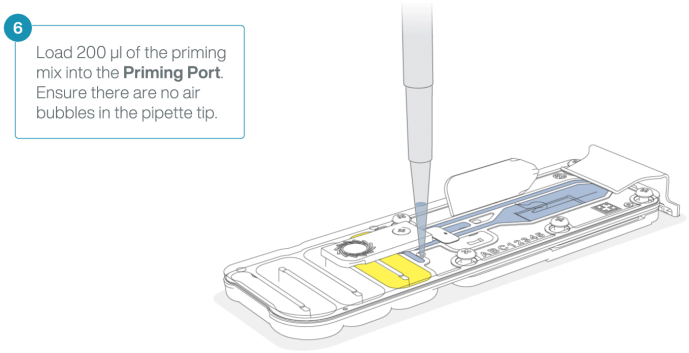
8 **In a new tube, prepare the library for loading as follows:**

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:

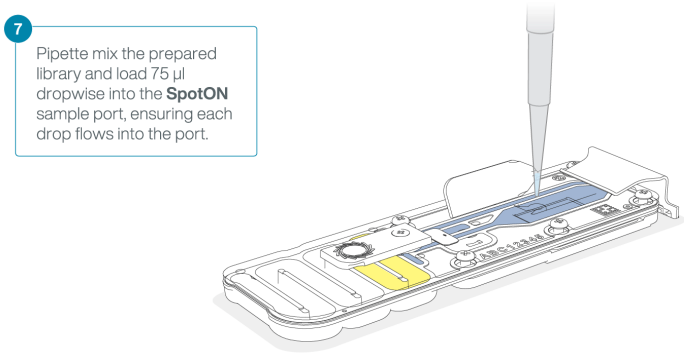
1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load **200 µl** of the priming mix into the flow cell priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.



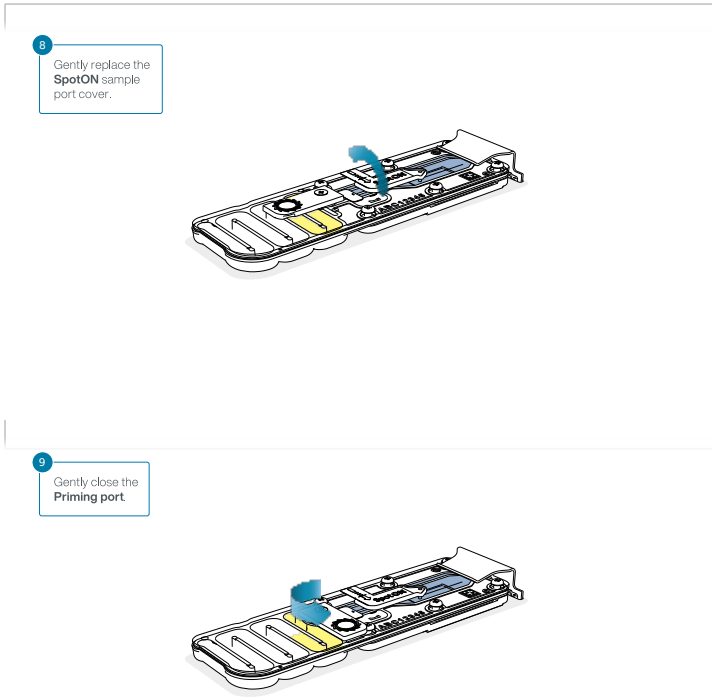


10 Mix the prepared library gently by pipetting up and down just prior to loading.

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, data acquisition and real-time basecalling are carried out by the MinKNOW software. It is assumed you have already installed MinKNOW on your computer. Further instructions for setting up a sequencing run can be found in the MinKNOW protocol.

In the current MinKNOW software version, the PCR Barcoding Expansions are not available in "Kit selection" when setting up a sequencing run. These will be included in the next software update.

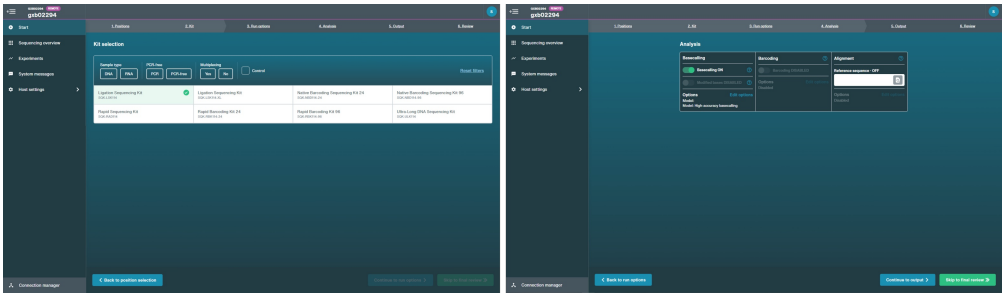
For the meantime, we recommend starting a sequencing run in the MinKNOW software using Ligation Sequencing Kit V14 (SQK-LSK114) to perform real-time basecalling. Once basecalling is complete, the data can be demultiplexed using post-run barcoding on MinKNOW, as outlined below.

MinKNOW settings for real-time basecalling and post-run barcoding:

Real-time basecalling

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

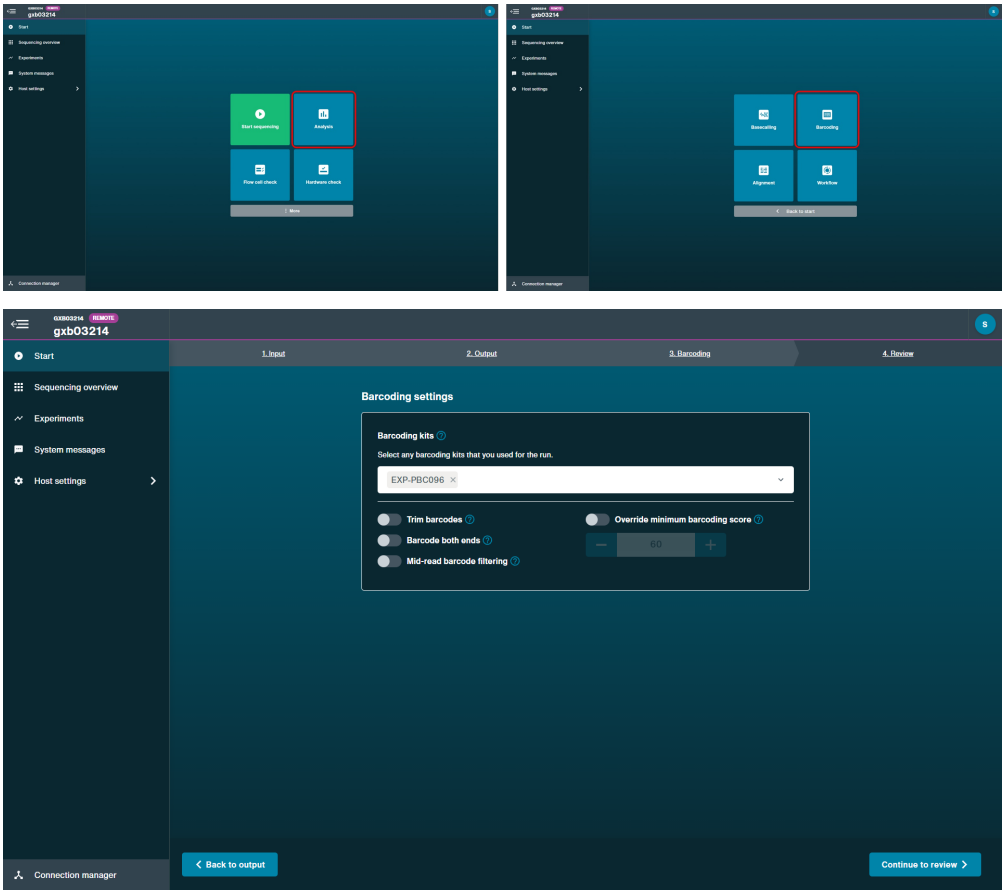
Select Ligation Sequencing Kit (SQK-LSK114) in "Kit selection". The barcoding option will be unavailable as default. Other parameters can be kept at their default settings.



Post-run barcoding

Follow the instructions in "Post-run barcoding" of the MinKNOW protocol.

Click Analysis to open post-run options. Choose Barcoding and input your fastq data files. Select the PCR Barcoding Expansion used during library preparation in "Barcode settings" and use the default settings for other parameters.



Downstream analysis

Post-basecalling analysis

There are several options for further analysing your basecalled data:

1. EPI2ME platform

The EPI2ME platform is a cloud-based data analysis service developed by Metrichor Ltd., a subsidiary of Oxford Nanopore Technologies. The EPI2ME platform offers a range of analysis workflows, e.g. for metagenomic identification, barcoding, alignment, and structural variant calling. The analysis requires no additional equipment or compute power, and provides an easy-to-interpret report with the results. For instructions on how to run an analysis workflow in EPI2ME, please follow the instructions in the EPI2ME protocol, beginning at the "Starting data analysis" step.

2. EPI2ME Labs tutorials and workflows

For more in-depth data analysis, Oxford Nanopore Technologies offers a range of bioinformatics tutorials and workflows available in EPI2ME Labs, which are available in the EPI2ME Labs section of the Community. The platform provides a vehicle where workflows deposited in GitHub by our Research and Applications teams can be showcased with descriptive texts, functional bioinformatics code

and example data.

3. Research analysis tools

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

4. Community-developed analysis tools

If a data analysis method for your research question is not provided in any of the resources above, please refer to the Bioinformatics section of the Resource centre. Numerous members of the Nanopore Community have developed their own tools and pipelines for analysing nanopore sequencing data, most of which are available on GitHub. Please be aware that these tools are not supported by Oxford Nanopore Technologies, and are not guaranteed to be compatible with the latest chemistry/software configuration.

Ending the experiment

Materials

- Flow Cell Wash Kit (EXP-WSH004)

1 **After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the 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.

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

IMPORTANT

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

Issues during 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.

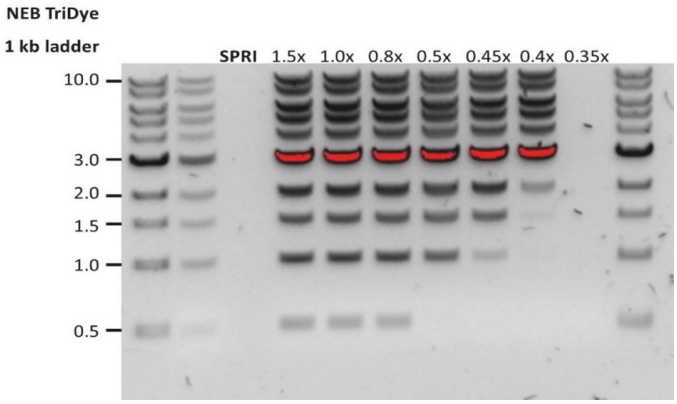
If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via LiveChat in the Nanopore Community.

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.

Observation	Possible cause	Comments and actions
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.

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

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

Observation	Possible cause	Comments and actions
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	An air bubble was introduced into the nanopore array	After the Flow Cell Check it is essential to remove any air bubbles near the priming port before priming the flow cell. If not removed, the air bubble can travel to the nanopore array and irreversibly damage the nanopores that have been exposed to air. The best practice to prevent this from happening is demonstrated in this video.
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	The flow cell is not correctly inserted into the device	Stop the sequencing run, remove the flow cell from the sequencing device and insert it again, checking that the flow cell is firmly seated in the device and that it has reached the target temperature. If applicable, try a different position on the device (GridION/PromethION).
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	Contaminations in the library damaged or blocked the pores	The pore count during the Flow Cell Check is performed using the QC DNA molecules present in the flow cell storage buffer. At the start of sequencing, the library itself is used to estimate the number of active pores. Because of this, variability of about 10% in the number of pores is expected. A significantly lower pore count reported at the start of sequencing can be due to contaminants in the library that have damaged the membranes or blocked the pores. Alternative 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

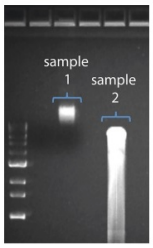
Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support.

Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	Ensure the correct volume and concentration as stated on the appropriate protocol for your sequencing library is loaded onto the flow cell. Please quantify the library before loading and calculate fmols using tools like the Promega Biomath Calculator, choosing "dsDNA: µg to fmol"

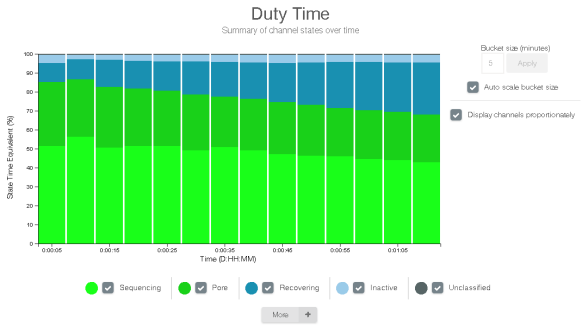
Observation	Possible cause	Comments and actions
Pore occupancy close to 0	The Ligation Sequencing Kit was used, and sequencing adapters did not ligate to the DNA	Make sure to use the NEBNext Quick Ligation Module (E6056) and Oxford Nanopore Technologies Ligation Buffer (LNB, provided in the SQK-LSK110 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 for Kit 9, 10, 11, and FCT for Kit 14). Make sure FLT/FCT was added to the buffer (FB for Kit 9, 10, 11, and FCF for Kit 14) 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

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.