

# Overview of the protocol

## PCR Barcoding Expansion Pack 1-96 features

This kit is recommended for users who:

- want to optimise their sequencing experiment for throughput
- require control over read length
- would like to utilise upstream processes such as size selection or whole genome amplification
- want to multiplex up to 96 different samples

### 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 (100 ng–500 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.

## Introduction to the PCR Barcoding Expansion Pack 1-96

This protocol describes how to carry out PCR barcoding using the Ligation Sequencing Kit 1D (SQK-LSK109) and the PCR Barcoding Expansion Pack 1-96 (EXP-PBC096). It is highly recommended that a Lambda control experiment is completed first to become familiar with the technology.

### 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.**

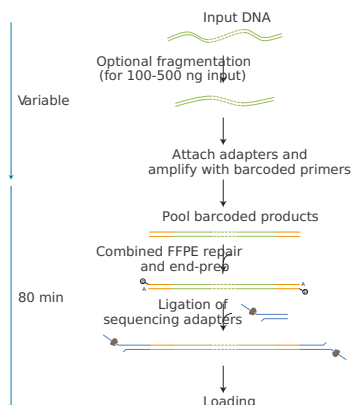
- Ensure you have your sequencing kit, the correct equipment and third-party reagents
- Download the software for acquiring and analysing your data
- Check your flow cell to ensure it has enough pores for a good sequencing run

#### Library preparation

You will need to:

- Prepare the DNA ends for adapter attachment
- Attach barcoding adapters supplied in the kit to the DNA ends
- Amplify each barcoded sample by PCR, then pool the samples together
- 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
- Start the EPI2ME software and select the barcoding workflow

### 96 barcode sequences

Component	Sequence
BC01 / RB01	AAGAAAGTTGTCGGTGTCTTTGTG
BC02 / RB02	TCGATTCCGTTTGTAGTCGTCTGT
BC03 / RB03	GAGTCTTGTGTCCCAGTTACCAGG
BC04 / RB04	TTCGGATTCTATCGTGTTCCCTA
BC05 / RB05	CTTGTCAGGGTTTGTGTAACCTT
BC06 / RB06	TTCTCGCAAAGGCAGAAAGTAGTC
BC07 / RB07	GTGTTACCGTGGGAATGAATCCTT
BC08 / RB08	TTCAGGGAACAAACCAAGTTACGT
BC09 / RB09	AACTAGGCACAGCGAGTCTTGTT
BC10 / RB10	AAGCGTTGAAACCTTTGTCCTCTC
BC11 / RB11	GTTTCATCTATCGGAGGGAATGGA
BC12 / RB12	CAGGTAGAAAGAAGCAGAATCGGA
BC13 / 16S13 / RB13	AGAACGACTTCCATACTCGTGTGA
BC14 / 16S14 / RB14	AACGAGTCTCTTGGGACCCATAGA
BC15 / 16S15 / RB15	AGGTCTACCTCGCTAACACCACTG
BC16 / 16S16 / RB16	CGTCAACTGACAGTGGTTCGTA

Component	Sequence
BC17 / 16S17 / RB17	ACCCTCCAGGAAAGTACCTCTGAT
BC18 / 16S18 / RB18	CCAAACCCAACAACCTAGATAGGC
BC19 / 16S19 / RB19	GTTCTCTGTCAGTGTCAAGAGAT
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	CGACGGTTAGATTACCTCTTACA
BC28 / RB28	TGAAACCTAAGAAGGCACCGTATC
BC29 / RB29	CTAGACACCTTGGGTTGACAGACC
BC30 / RB30	TCAGTGAGGATCTACTTCGACCCA
BC31 / RB31	TGCGTACAGCAATCAGTTACATTG
BC32 / RB32	CCAGTAGAAGTCCGACAACGTCAT
BC33 / RB33	CAGACTTGGTACGGTTGGGTA ACT
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

Component	Sequence
BC46 / RB46	GCTGTGTTCCACTTCATTCTCCTG
BC47 / RB47	GTGCAACTTTCCACAGGTAGTTC
BC48 / RB48	CATCTGGAACGTGGTACACCTGTA
BC49 / RB49	ACTGGTGCAGCTTTGAACATCTAG
BC50 / RB50	ATGGACTTTGGTAACTTCCTGCGT
BC51 / RB51	GTTGAATGAGCCTACTGGGTCCTC
BC52 / RB52	TGAGAGACAAGATTGTTCTGGGAC
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
BC64 / RB64	GACAGACACCGTTCATCGACTTTC
BC65 / RB65	TTCTCAGTCTTCTCCAGACAAGG
BC66 / RB66	CCGATCCTTGTGGCTTCTAACTTC
BC67 / RB67	GTTTGTCTACTCGTGTGCTCACC
BC68 / RB68	GAATCTAAGCAAACACGAAGGTGG
BC69 / RB69	TACAGTCCGAGCCTCATGTGATCT
BC70 / RB70	ACCGAGATCCTACGAATGGAGTGT
BC71 / RB71	CCTGGGAGCATCAGGTAGTAACAG
BC72 / RB72	TAGCTGACTGTCTTCCATACCGAC
BC73 / RB73	AAGAAACAGGATGACAGAACCCTC
BC74 / RB74	TACAAGCATCCCAACTTCCACT
BC75 / RB75	GACCATTGTGATGAACCCTGTTGT
BC76 / RB76	ATGCTTGTTACATCAACCCTGGAC
BC77 / RB77	CGACCTGTTTCTCAGGGATACAAC
BC78 / RB78	AACAACCGAACCTTTGAATCAGAA
BC79 / RB79	TCTCGGAGATAGTTCTCACTGCTG

Component	Sequence
BC80 / RB80	CGGATGAACATAGGATAGCGATTC
BC81 / RB81	CCTCATCTTGTGAAGTTGTTTCGG
BC82 / RB82	ACGGTATGTCGAGTTCAGGACTA
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	GATTGTCCTCAAACCTGCCACCTAC
BC95 / RB95	CCTGTCTGGAAGAAGAATGGACTT
BC96 / RB96	CTGAACGGTCATAGAGTCCACCAT

#### 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 1-96 (EXP-PBC096)
- Ligation Sequencing Kit (SQK-LSK109)
- R9.4.1 (FLO-MIN106) flow cells
- Flow Cell Wash Kit (EXP-WSH004)

## Equipment and consumables

### Materials

- <1 µg of each DNA sample to be barcoded in 45 µl

- PCR Barcoding Expansion Pack 1-96 (EXP-PBC096)
- Ligation Sequencing Kit (SQK-LSK109)
- Flow Cell Priming Kit (EXP-FLP002)

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### Consumables

- Agencourt AMPure XP beads (Beckman Coulter™, A63881)
- NEB Blunt/TA Ligase Master Mix (M0367)
- NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (cat # E7180S or E7180L). Alternatively, you can use the three NEBNext® products below:
- NEBNext FFPE Repair Mix (M6630)
- NEBNext Ultra II End repair/dA-tailing Module (E7546)
- NEBNext Quick Ligation Module (E6056)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 70% ethanol in nuclease-free water
- LongAmp Taq 2X Master Mix (e.g. NEB M0287)

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### 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

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### Optional Equipment

- Agilent Bioanalyzer (or equivalent)
- Qubit fluorometer (or equivalent for QC check)
- Eppendorf 5424 centrifuge (or equivalent)

**For this protocol, you will need <1 µg of each DNA sample to be barcoded in 45 µl.**

### 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.

**The reagents provided in the NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing are sufficient for 8 reactions when following this protocol. If performing more than 8 reactions, an additional 168 µl of NEBNext® Ultra™ II End Prep Reaction Buffer is required.**

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

The kit allows up to 96 different libraries to be combined and loaded into a flow cell at the same time. Each of these libraries will be barcoded with one of the 96 PCR barcodes (BC1-BC96). The kit allows for up to 960 libraries to be made and sequenced, based on 96 libraries being combined for each of ten separate flow cell loadings. It is possible to work with fewer than 96 libraries and PCR barcodes if required.

The reagents are divided between two 96 tube plates. One plate (blue caps) contains barcode adapters that are ligated to the DNA. All tubes in this plate have identical content. The other plate (white caps) contains the barcodes, one barcode per tube.



Name	No. of plates	Fill volume per well (µl)
PCR Primer mix	1	24
Barcode adapter plate	1	240

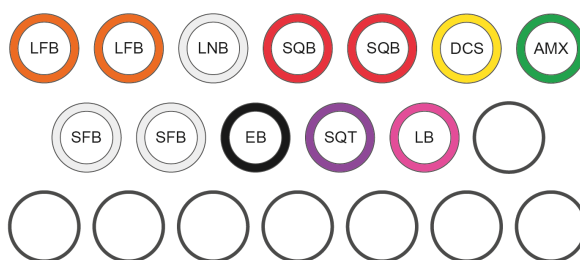
**IMPORTANT**

The blue-capped Barcode adapter tubes are used in the PCR 96 Barcoding genomic DNA protocol only. They are not required for preparing amplicons or cDNA.

**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.

A1 BC01	A2 BC02	A3 BC03	A4 BC04	A5 BC05	A6 BC06	A7 BC07	A8 BC08	A9 BC09	A10 BC10	A11 BC11	A12 BC12
B1 BC13	B2 BC14	B3 BC15	B4 BC16	B5 BC17	B6 BC18	B7 BC19	B8 BC20	B9 BC21	B10 BC22	B11 BC23	B12 BC24
C1 BC25	C2 BC26	C3 BC27	C4 BC28	C5 BC29	C6 BC30	C7 BC31	C8 BC32	C9 BC33	C10 BC34	C11 BC35	C12 BC36
D1 BC37	D2 BC38	D3 BC39	D4 BC40	D5 BC41	D6 BC42	D7 BC43	D8 BC44	D9 BC45	D10 BC46	D11 BC47	D12 BC48
E1 BC49	E2 BC50	E3 BC51	E4 BC52	E5 BC53	E6 BC54	E7 BC55	E8 BC56	E9 BC57	E10 BC58	E11 BC59	E12 BC60
F1 BC61	F2 BC62	F3 BC63	F4 BC64	F5 BC65	F6 BC66	F7 BC67	F8 BC68	F9 BC69	F10 BC70	F11 BC71	F12 BC72
G1 BC73	G2 BC74	G3 BC75	G4 BC76	G5 BC77	G6 BC78	G7 BC79	G8 BC80	G9 BC81	G10 BC82	G11 BC83	G12 BC84
H1 BC85	H2 BC86	H3 BC87	H4 BC88	H5 BC89	H6 BC90	H7 BC91	H8 BC92	H9 BC93	H10 BC94	H11 BC95	H12 BC96

**Ligation Sequencing Kit contents (SQK-LSK109)**

LFB : L fragment buffer  
 LNB : Ligation buffer  
 SQB : Sequencing buffer  
 DCS : DNA control strand  
 AMX : Adapter mix

SFB : S fragment buffer  
 EB : Elution buffer  
 SQT : Sequencing tether  
 LB : Loading beads

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
DNA CS	DCS	Yellow	1	50



Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Adapter Mix	AMX	Green	1	40
Ligation Buffer	LNB	Clear	1	200
Long Fragment Buffer	LFB	White cap, orange stripe on label	2	1,800
Short Fragment Buffer	SFB	Grey	2	1,800
Sequencing Buffer	SQB	Red	2	300
Elution Buffer	EB	Black	1	200
Loading Beads	LB	Pink	1	360
Sequencing Tether	SQT	Purple	1	10

#### Flow Cell Priming Kit contents (EXP-FLP002)



FLB : Flush buffer

FLT : Flush tether

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Flush Buffer	FB	Blue	6	1,170
Flush Tether	FLT	Purple	1	200

## Computer requirements and software

### 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](#).

### 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](#).

### 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

## **End-prep**

~50 minutes

#### **Materials**

- <1 µg of each DNA sample to be barcoded in 45 µl

#### **Consumables**

- NEBNext Ultra II End repair/dA-tailing Module (E7546)
- Freshly prepared 70% ethanol in nuclease-free water
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

- Agencourt AMPure XP beads (Beckman Coulter™, A63881)
- 0.2 ml 96-well PCR plate

#### Equipment

- Thermal cycler
- Ice bucket with ice
- Centrifuge capable of taking 96-well plates
- Magnetic separator, suitable for 1.5 ml Eppendorf tubes

#### Optional Equipment

- Qubit fluorometer (or equivalent for QC check)

#### 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 (100 ng–500 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.

#### 1 Prepare the DNA in nuclease-free water.

- Transfer <1 µg DNA into a 1.5 ml Eppendorf DNA LoBind tube
- Adjust the volume to 45 µl with nuclease-free water
- Mix thoroughly by flicking the tube to avoid unwanted shearing
- Spin down briefly in a microfuge

#### 2 In a 0.2 ml 96 well PCR plate, set up the end-repair / dA-tailing reactions as follows:

Between each addition, pipette mix 10-20 times.

Reagent	Volume
<1 µg DNA	45 µl
Ultra II End-prep reaction buffer	7 µl
Ultra II End-prep enzyme mix	3 µl
Nuclease-free water	5 µl
<b>Total</b>	<b>60 µl</b>

#### 3 Ensure the components are thoroughly mixed by pipetting, and spin down.

- 4 Seal the plate with adhesive film or PCR strip caps, spin down in a centrifuge and incubate for 5 minutes at 20 °C and 5 minutes at 65 °C using the thermal cycler.**

If condensation is observed in the plate after the thermocycling, briefly spin down the plate contents in a centrifuge.

- 5 Resuspend the AMPure XP beads by vortexing.**

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

End-prep cleanup demo

- 7 Allow DNA to bind to beads for 5 minutes at room temperature.**

- 8 Prepare sufficient fresh 70% ethanol in nuclease-free water.**

- 9 Place on magnetic rack, allow beads to pellet and pipette off supernatant.**

- 10 Keep the tube on the magnet and wash the beads with 180 µl of freshly-prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

- 11 Repeat the previous step.**

- 12 Cover the plate with adhesive film and leave plate on magnet for 2 minutes to allow residual liquid to collect at the bottom. Remove the adhesive film, return the plate to the magnet and aspirate residual wash solution.**

- 13 Briefly incubate the plate on a thermal cycler at 37° C with the lid open and the plate wells unsealed.**

- 14 Remove the plate from the magnet and resuspend pellet in 31 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 96-well PCR plate.**

- 17 Quantify 1 µl of end-prepped DNA using a Qubit fluorometer - recovery aim > 700 ng.**

#### END OF STEP

Take forward approximately 700 ng of end-prepped DNA in 30 µl nuclease-free water into adapter ligation. 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 (M0367)
- Agencourt AMPure XP beads (Beckman Coulter™, A63881)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 70% ethanol in nuclease-free water
- 10 mM Tris-HCl pH 8.5
- 0.2 ml 96-well PCR plate

## Equipment

- Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, #11766427)
- Hula mixer (gentle rotator mixer)
- Vortex mixer
- Magnetic separator suitable for 96-well PCR plates, e.g. DynaMag™-96 Side Skirted Magnet (Thermo Fisher, # 12027)
- Ice bucket with ice
- Multichannel pipette

## Optional Equipment

- Agilent Bioanalyzer (or equivalent)

### 1 Add the reagents to a fresh 96-well plate, in the order given below:

Between each addition, pipette mix 10-20 times.

Reagent	Volume
End-prepped DNA	30 µl
Barcode Adapter	20 µl
Blunt/TA Ligase Master Mix	50 µl
<b>Total</b>	<b>100 µl</b>

### 2 Ensure the components are thoroughly mixed by pipetting.

### 3 Seal the plate with adhesive film or PCR strip caps and briefly spin down in a plate spinner.

### 4 Incubate the reaction for 10 minutes at room temperature.

- 5 Resuspend the AMPure XP beads by vortexing.
- 6 Add 40  $\mu\text{l}$  of resuspended AMPure XP beads to each sample and mix by pipetting up and down ten times.
- 7 Allow DNA to bind to beads for 5 minutes at room temperature.
- 8 Prepare sufficient fresh 70% ethanol in nuclease-free water.
- 9 Place on magnetic rack, allow beads to pellet and pipette off supernatant.
- 10 Keep the tube on the magnet and wash the beads with 180  $\mu\text{l}$  of freshly-prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
- 11 Repeat the previous step.
- 12 Cover the plate with adhesive film and leave plate on magnet for 2 minutes to allow residual liquid to collect at the bottom. Remove the adhesive film, return the plate to the magnet and aspirate residual wash solution.
- 13 Briefly incubate the plate on a thermal cycler at 37° C with the lid open and the plate wells unsealed.
- 14 Remove the plate from the magnet and resuspend pellet in 25  $\mu\text{l}$  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 96-well PCR plate.
- 17 Quantify 1  $\mu\text{l}$  of end-prepped DNA using a Qubit fluorometer.
- 18 Dilute the library to a concentration of 10 ng/ $\mu\text{l}$  with nuclease-free water or 10 mM Tris-HCl pH 8.5.

**END OF STEP**

Take forward the samples to the next step. However, at this point it is also possible to store the sample at 4°C overnight.

## Barcoding PCR

~15 minutes

**Materials**

- PCR Barcodes (BC01-96, at 10  $\mu$ M)

**Consumables**

- LongAmp Taq 2X Master Mix (e.g. NEB M0287)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- 1.5 ml Eppendorf DNA LoBind tubes

**Equipment**

- Thermal cycler

**Optional  
Equipment**

- Qubit fluorometer (or equivalent for QC check)

**Capping and decapping the 96 well format****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  $\mu$ M concentration and to be used at a final concentration of 0.2  $\mu$ M.

A1 BC01	A2 BC02	A3 BC03	A4 BC04	A5 BC05	A6 BC06	A7 BC07	A8 BC08	A9 BC09	A10 BC10	A11 BC11	A12 BC12
B1 BC13	B2 BC14	B3 BC15	B4 BC16	B5 BC17	B6 BC18	B7 BC19	B8 BC20	B9 BC21	B10 BC22	B11 BC23	B12 BC24
C1 BC25	C2 BC26	C3 BC27	C4 BC28	C5 BC29	C6 BC30	C7 BC31	C8 BC32	C9 BC33	C10 BC34	C11 BC35	C12 BC36
D1 BC37	D2 BC38	D3 BC39	D4 BC40	D5 BC41	D6 BC42	D7 BC43	D8 BC44	D9 BC45	D10 BC46	D11 BC47	D12 BC48

E1 BC49	E2 BC50	E3 BC51	E4 BC52	E5 BC53	E6 BC54	E7 BC55	E8 BC56	E9 BC57	E10 BC58	E11 BC59	E12 BC60
F1 BC61	F2 BC62	F3 BC63	F4 BC64	F5 BC65	F6 BC66	F7 BC67	F8 BC68	F9 BC69	F10 BC70	F11 BC71	F12 BC72
G1 BC73	G2 BC74	G3 BC75	G4 BC76	G5 BC77	G6 BC78	G7 BC79	G8 BC80	G9 BC81	G10 BC82	G11 BC83	G12 BC84
H1 BC85	H2 BC86	H3 BC87	H4 BC88	H5 BC89	H6 BC90	H7 BC91	H8 BC92	H9 BC93	H10 BC94	H11 BC95	H12 BC96

### 1 Set up a barcoding PCR reaction as follows for each library:

The following is written for LongAmp Taq, but can be adapted for use with other polymerases.

Between each addition, pipette mix 10-20 times.

Reagent	Volume
PCR Barcode (one of BC1-BC96, at 10 $\mu$ M)	1 $\mu$ l
Adapter-ligated DNA	2 $\mu$ l
LongAmp Taq 2x master mix	25 $\mu$ l
Nuclease-free water	22 $\mu$ l
<b>Total volume</b>	<b>50 <math>\mu</math>l</b>

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

### 2 Mix by pipetting.

### 3 Seal the plate with adhesive film or PCR strip caps and briefly spin down in a plate spinner.



#### 4 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	15-18 (b)
Annealing	62 °C (a)	15 secs (a)	15-18 (b)
Extension	65 °C (c)	dependent on length of target fragment (d)	15-18 (b)
Final extension	65 °C	dependent on length of target fragment (d)	1
Hold	4 °C	∞	

- This is specific to the Oxford Nanopore primer and should be maintained
- Adjust accordingly if input quantities are altered
- This temperature is determined by the type of polymerase that is being used (given here for LongAmp Taq polymerase)
- Adjust accordingly for different lengths of amplicons and the type of polymerase that is being used (LongAmp Taq amplifies at a rate of 50 seconds per kb)

#### 5 Purify the barcoded DNA using standard methods which are suitable for the fragment size.

#### 6 Quantify the barcoded library using standard techniques, and pool all barcoded libraries in the desired ratios in a 1.5 ml DNA LoBind Eppendorf tube.

#### 7 Prepare 1 µg of pooled barcoded libraries in 47 µl nuclease-free water.

##### Optional Action

If the volume of your pool exceeds the 47 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 nanopore sequencing. However, at this point it is also possible to store the sample at 4°C overnight.**

## DNA repair and end-prep

~35 minutes

### Materials

- Pooled barcoded DNA in 47 µl nuclease-free water
- DNA CS

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**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 (M6630)
- NEBNext Ultra II End repair / dA-tailing Module (E7546)
- Agencourt AMPure XP beads
- Freshly prepared 70% ethanol in nuclease-free water

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**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

**1 Thaw DNA CS (DCS) at room temperature, spin down, mix by pipetting, and place on ice.**

**2 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 reagent tube to ensure they are well mixed.
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 several seconds to ensure the reagent is thoroughly mixed.
5. The FFPE DNA repair buffer may have a yellow tinge and is fine to use if yellow.

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

Between each addition, pipette mix 10-20 times.

Reagent	Volume
DNA CS	1 $\mu$ l
DNA	47 $\mu$ l
NEBNext FFPE DNA Repair Buffer	3.5 $\mu$ l
NEBNext FFPE DNA Repair Mix	2 $\mu$ l
Ultra II End-prep reaction buffer	3.5 $\mu$ l
Ultra II End-prep enzyme mix	3 $\mu$ l
<b>Total</b>	<b>60 <math>\mu</math>l</b>

**4 Ensure the components are thoroughly mixed by pipetting.**

**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 by vortexing.**

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

**8 Add 60  $\mu$ l of resuspended AMPure XP beads 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  $\mu$ l of fresh 70% 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 70% 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.**

Optional Action

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

- Adapter Mix (AMX)
- Ligation Buffer (LNB)
- Long Fragment Buffer (LFB)
- Short Fragment Buffer (SFB)
- Elution Buffer from the Oxford Nanopore kit (EB)

### Consumables

- NEBNext Quick Ligation Module (E6056)
- 1.5 ml Eppendorf DNA LoBind tubes
- Agencourt AMPure XP beads

### Equipment

- Magnetic rack
- Microfuge
- Vortex mixer
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

**IMPORTANT**

Although the recommended 3rd party ligase is supplied with its own buffer, the ligation efficiency of Adapter Mix (AMX) is higher when using Ligation Buffer supplied within the Ligation Sequencing Kit.

- 1 Spin down the Adapter Mix (AMX) 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.

**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)**

- 4 To enrich for DNA fragments of 3 kb or longer, thaw one tube of Long Fragment Buffer (LFB) at room temperature, mix by vortexing, spin down and place on ice.
- 5 To retain DNA fragments of all sizes, thaw one tube of 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
Adapter Mix (AMX)	5 µl
<b>Total</b>	<b>100 µl</b>

- 7 Ensure the components are thoroughly mixed by pipetting, and spin down.
- 8 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.

- 9 Resuspend the AMPure XP beads by vortexing.
- 10 Add 40  $\mu$ l of resuspended AMPure XP beads 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  $\mu$ l Long Fragment Buffer (LFB) or 250  $\mu$ l 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  $\mu$ l 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  $\mu$ l of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

Dispose of the pelleted beads

Quantify 1  $\mu$ l of eluted sample using a Qubit fluorometer.

#### IMPORTANT

**We recommend loading 5-50 fmol of the final prepared library onto a flow cell.**

Loading more than the maximal recommended amount of DNA can have a detrimental effect on output as higher quantities of DNA results in a larger number of ligated DNA ends with loaded motor protein. This depletes fuel in the Sequencing Buffer, regardless of whether or not the DNA fragments are being sequenced. This leads to fuel depletion and speed drop-off early in the sequencing run. Dilute the libraries in Elution Buffer if required.

If you are using the Flongle for sample prep development, we recommend loading 3-20 fmol instead.

#### 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.

For further information, please refer to the [DNA library stability Know-How document](#).

#### 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-AUX001), available to purchase separately. This expansion also contains additional vials of Sequencing Buffer (SQB) and Loading Beads (LB), required for loading the libraries onto flow cells.

## Priming and loading the SpotON flow cell

~10 minutes

### Materials

- Flow Cell Priming Kit (EXP-FLP002)
- Loading Beads (LB)
- Sequencing Buffer (SQB)

### Consumables

- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

### Equipment

- MinION Mk1B or Mk1C
- SpotON Flow Cell
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

#### IMPORTANT

Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol.

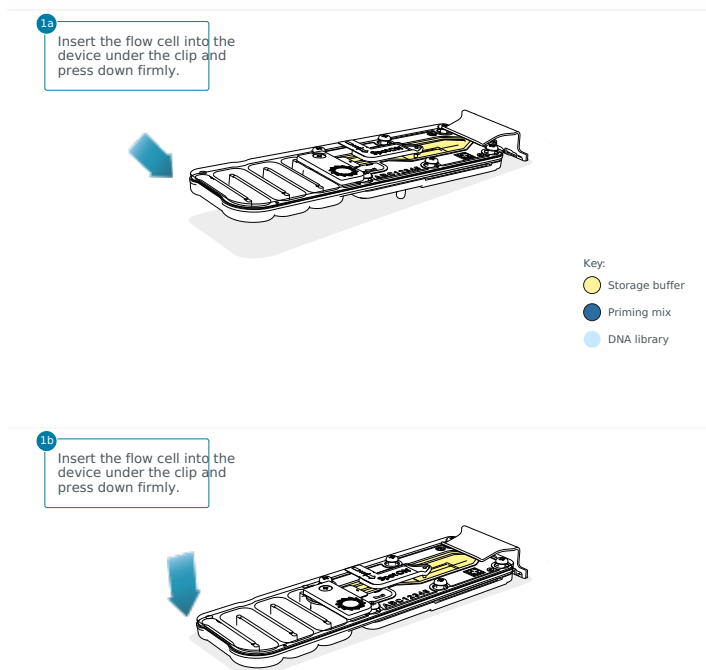
## TIP

### Priming and loading a flow cell

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

- 1 Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature before mixing the reagents by vortexing, and spin down at room temperature.**
- 2 To prepare the flow cell priming mix, add 30  $\mu$ l of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at room temperature.**
- 3 Open the MinION 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.

Please see the MinKNOW [Flow cell check protocol](#) for more information



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

**IMPORTANT**

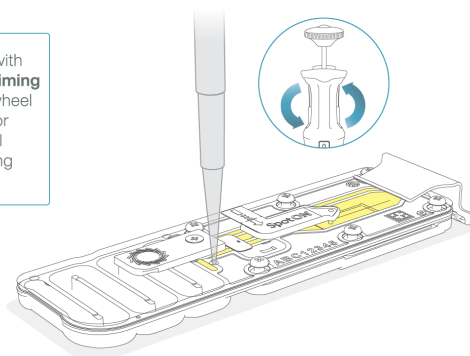
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30  $\mu\text{l}$ , and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

5 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few  $\mu\text{l}$ ):

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

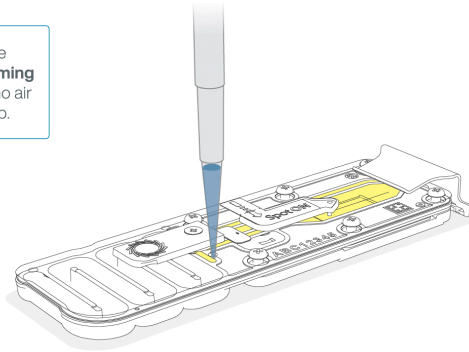
**Note:** Visually check that there is continuous buffer from the priming port across the sensor array.

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



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

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



Wait 5 minutes before proceeding to the next step.

- 7 Thoroughly mix the contents of the Loading Beads (LB) by pipetting.

**IMPORTANT**

The Loading Beads (LB) 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
Sequencing Buffer (SQB)	37.5 µl
Loading Beads (LB), mixed immediately before use	25.5 µl
DNA library	12 µl
<b>Total</b>	<b>75 µl</b>

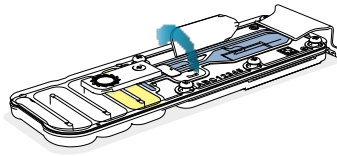
**Note:** Load the library onto the flow cell immediately after adding the Sequencing Buffer (SQB) and Loading Beads (LB) because the fuel in the buffer will start to be consumed by the adapter.

## 9 Complete the flow cell priming:

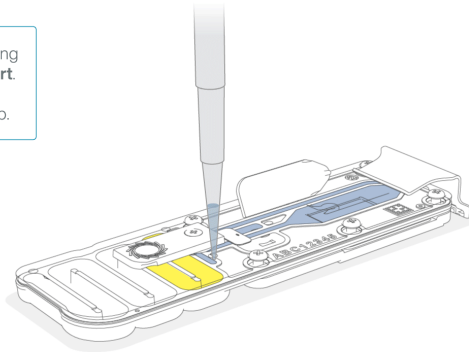
1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load **200 µl** of the priming mix into the flow cell via the priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.

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

5 Gently flip open SpotON sample port cover.



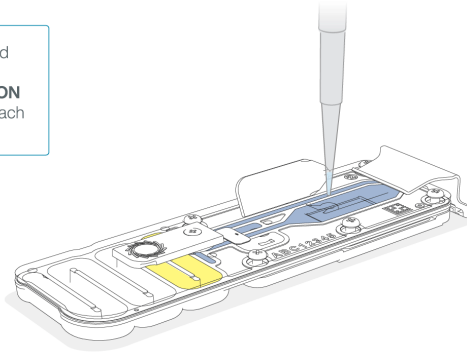
6 Load 200 µl of the priming mix into the **Priming Port**. Ensure there are no air bubbles in the pipette tip.



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

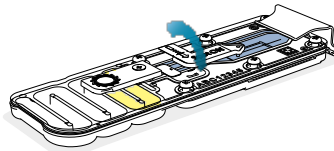
**11 Add 75  $\mu$ l of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.**

**7** Pipette mix the prepared library and load 75  $\mu$ l dropwise into the **SpotON** sample port, ensuring each drop flows into the port.

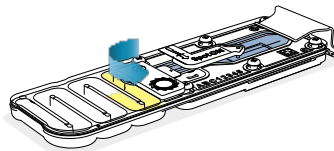


**12 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION device lid.**

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



**9** Gently close the **Priming port**.



# Data acquisition and basecalling

## Overview of nanopore data analysis

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

## How to start sequencing

The sequencing device control, data acquisition and real-time basecalling are carried out by the MinKNOW software. It is assumed you have already installed MinKNOW on your computer, or that you are using the MinIT device for data acquisition and basecalling. There are three options for how to carry out sequencing:

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

Follow the instructions in the [MinKNOW protocol](#) beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section.

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

Follow the instructions in the [GridION user manual](#).

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

Follow the instructions in the [MinION Mk1C user manual](#).

### 4. Data acquisition and basecalling in real-time using the MinIT device

Follow the instructions in the [MinIT protocol](#).

### 5. Data acquisition using MinKNOW on a computer and basecalling at a later time using MinKNOW or Guppy

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

# Downstream analysis

## 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 by washing out the flow cell ready to send back to Oxford Nanopore.**

Instructions for returning flow cells can be found [here](#).

### 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.

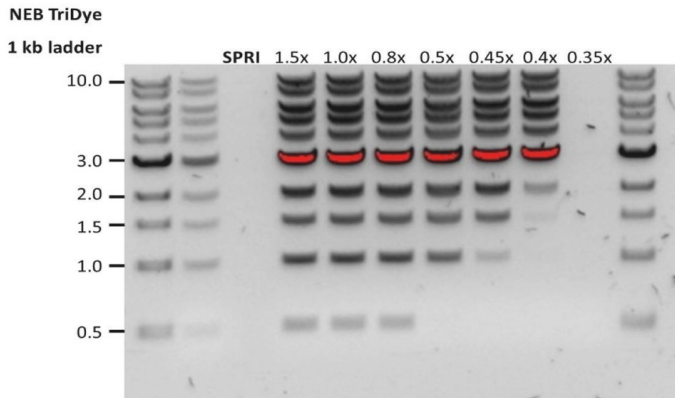
If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email ([support@nanoporetech.com](mailto:support@nanoporetech.com)) or via [LiveChat](#) in the Nanopore Community.

## Low sample quality

Observation	Possible cause	Comments and actions
<b>Low DNA purity (Nanodrop reading for DNA OD 260/280 is &lt;1.8 and OD 260/230 is &lt;2.0-2.2)</b>	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the <a href="#">Contaminants Know-how</a> piece. Please try an alternative <a href="#">extraction method</a> that does not result in contaminant carryover.  Consider performing an additional SPRI clean-up step.
<b>Low RNA integrity (RNA integrity number &lt;9.5 RIN, or the rRNA band is shown as a smear on the gel)</b>	The RNA degraded during extraction	Try a different <a href="#">RNA extraction method</a> ). For more info on RIN, please see the <a href="#">RNA Integrity Number Know-how piece</a> .
<b>RNA has a shorter than expected fragment length</b>	The RNA degraded during extraction	Try a different <a href="#">RNA extraction method</a> ). For more info on RIN, please see the <a href="#">RNA Integrity Number Know-how piece</a> .  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
<b>Low recovery</b>	DNA loss due to a lower than intended AMPure beads-to-sample ratio	<ol style="list-style-type: none"><li>1. AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample.</li><li>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.</li></ol>

Observation	Possible cause	Comments and actions
<b>Low recovery</b>	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.  
<b>Low recovery after end-prep</b>	The wash step used ethanol <70%	DNA will be eluted from the beads when using ethanol <70%. Make sure to use the correct percentage.

#### The VoITRAX run terminated in the middle of the library prep

Observation	Possible cause	Comments and actions
<b>The green light was switched off</b>  <b>or</b> <b>An adapter was used to connect the VoITRAX USB-C cable to the computer</b>	Insufficient power supply to the VoITRAX	The green LED signals that 3 A are being supplied to the device. This is the requirement for the full capabilities of the VoITRAX V2 device. Please use computers that meet the requirements listed on the <a href="#">VoITRAX V2 protocol</a> .

#### The VoITRAX software shows an inaccurate amount of reagents loaded

Observation	Possible cause	Comments and actions
<b>The VoITRAX software shows an inaccurate amount of reagents loaded</b>	Pipette tips do not fit the VoITRAX cartridge ports	TRainin 20 µl or 30 µl and Gilson 10 µl, 20 µl or 30 µl pipette tips are compatible with loading reagents into the VoITRAX cartridge. Rainin 20 µl is the most suitable.
<b>The VoITRAX software shows an inaccurate amount of reagents loaded</b>	The angle at which reagents are pipetted into the cartridge is incorrect	The pipetting angle should be slightly greater than the cartridge inlet angle. Please watch the demo video included in the VoITRAX software before loading.

## 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](mailto: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
<b>MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check</b>	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 <a href="#">this video</a> .
<b>MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check</b>	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).
<b>MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check</b>	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 <a href="#">Contaminants Know-how piece</a> . Please try an alternative <a href="#">extraction method</a> that does not result in contaminant carryover.

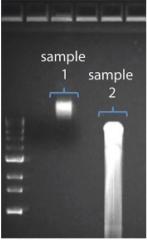
### MinKNOW script failed

Observation	Possible cause	Comments and actions
<b>MinKNOW shows "Script failed"</b>		Restart the computer and then restart MinKNOW. If the issue persists, please collect the <a href="#">MinKNOW log files</a> and contact Technical Support.

### Pore occupancy below 40%

Observation	Possible cause	Comments and actions
<b>Pore occupancy &lt;40%</b>	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 <a href="#">Promega Biomath Calculator</a> , choosing "dsDNA: µg to pmol"
<b>Pore occupancy close to 0</b>	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.
<b>Pore occupancy close to 0</b>	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.
<b>Pore occupancy close to 0</b>	No tether on the flow cell	Tethers are added 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
<b>Shorter than expected read length</b>	Unwanted fragmentation of DNA sample	<p>Read length reflects input DNA fragment length. Input DNA can be fragmented during extraction and library prep.</p> <ol style="list-style-type: none"> <li>1. Please review the <a href="#">Extraction Methods</a> in the Nanopore Community for best practice for extraction.</li> <li>2. Visualise the input DNA fragment length distribution on an agarose gel before proceeding to the library prep.</li> </ol>  <p>In the image above, Sample 1 is of high molecular weight, whereas Sample 2 has been fragmented.</p> <ol style="list-style-type: none"> <li>3. During library prep, avoid pipetting and vortexing when mixing reagents. Flicking or inverting the tube is sufficient.</li> </ol>

### Large proportion of recovering pores

Observation	Possible cause	Comments and actions
<p><b>Large proportion of recovering pores (shown as dark blue in the channels panel and duty time plot)</b></p>	<p>Contaminants are present in the sample</p>	<p>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:</p> <ol style="list-style-type: none"> <li>1. A <a href="#">nuclease flush</a> can be performed, or</li> <li>2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.</li> </ol> <div style="text-align: center;"> </div> <p>The duty time plot above shows an increasing proportion of "recovering" pores over the course of a sequencing experiment</p>

### Large proportion of inactive pores

Observation	Possible cause	Comments and actions
<p><b>Large proportion of inactive pores (shown as light blue in the channels panel and duty time plot. Pores or membranes are irreversibly damaged)</b></p>	<p>Air bubbles have been introduced into the flow cell</p>	<p>Air bubbles introduced through flow cell priming and library loading can irreversibly damage the pores. Watch the <a href="#">Priming and loading your flow cell</a> video for best practice</p>
<p><b>Large proportion of inactive pores</b></p>	<p>Certain compounds co-purified with DNA</p>	<p>Known compounds, include polysaccharides, typically associate with plant genomic DNA.</p> <ol style="list-style-type: none"> <li>1. Please refer to the <a href="#">Plant leaf DNA extraction method</a>.</li> <li>2. Clean-up using the QIAGEN PowerClean Pro kit.</li> <li>3. Perform a whole genome amplification with the original gDNA sample using the QIAGEN REPLI-g kit.</li> </ol>
<p><b>Large proportion of inactive pores</b></p>	<p>Contaminants are present in the sample</p>	<p>The effects of contaminants are shown in the <a href="#">Contaminants</a> Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover.</p>

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

Observation	Possible cause	Comments and actions
<b>Reduction in sequencing speed and q-score later into the run</b>	Fast fuel consumption is typically seen when the flow cell is overloaded with library (~5-50 fmol of library is recommended).	Add more fuel to the flow cell by following the instructions in the <a href="#">MinKNOW protocol</a> . In future experiments, load lower amounts of library to the flow cell.

### Temperature fluctuation

Observation	Possible cause	Comments and actions
<b>Temperature fluctuation</b>	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
<b>MinKNOW shows "Failed to reach target temperature"</b> (37°C for Flow Cell Check, 34°C for sequencing on MinION Mk 1B/PromethION flow cells, and 35°C for sequencing on Flongle)	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 <a href="#">this FAQ</a> for more information on MinION Mk 1B temperature control.

### Guppy - no input .fast5 was found or basecalled

Observation	Possible cause	Comments and actions
<b>No input .fast5 was found or basecalled</b>	<i>input_path</i> did not point to the .fast5 file location	The <i>--input_path</i> 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.
<b>No input .fast5 was found or basecalled</b>	The .fast5 files were in a subfolder at the <i>input_path</i> location	To allow Guppy to look into subfolders, add the <i>--recursive</i> flag to the command

### Guppy - no Pass or Fail folders were generated after basecalling


Observation	Possible cause	Comments and actions
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Observation	Possible cause	Comments and actions
<b>No Pass or Fail folders were generated after basecalling</b>	The <code>--qscore_filtering</code> flag was not included in the command	The <code>--qscore_filtering</code> 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
<b>Unusually slow processing on a GPU computer</b>	The <code>--device</code> flag wasn't included in the command	The <code>--device</code> 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 <code>--device cuda:0 cuda:1</code> , 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
<b>The MinKNOW interface is not shown in the web browser</b>	Browser compatibility issue	Always use Google Chrome as the browser to view MinKNOW. Alternatively, instead of typing <code>//mt-xxxxxx</code> (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.
<b>The MinKNOW interface is not shown in the web browser</b>	The MinIT Wi-Fi was not used for connecting to the computer or mobile device	<p>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:</p>  <p>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.</p>
<b>The MinKNOW interface is not shown in the web browser</b>	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
<b>The MinIT software cannot be updated</b>	The firewall is blocking IPs for update	Please consult your IT department, as the MinIT software requires access to the <a href="#">following AWS IP ranges</a> . Access to the following IP addresses is also needed: 178.79.175.200 96.126.99.215

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