

Overview of the protocol

Native Barcoding Expansion 1-12 and 13-24 features

These kits are recommended for users who:

- wish to multiplex samples to reduce price per sample
- need a PCR-free method of multiplexing to preserve additional information such as base modifications
- want to optimise their sequencing experiment for throughput
- require control over read length
- are interested in utilising upstream processes such as size selection or whole genome amplification

Introduction to the Native Barcoding protocol

This protocol describes how to carry out native barcoding of genomic DNA using the Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114), in conjunction with the Ligation Sequencing Kit (SQK-LSK109). There are 24 unique barcodes if using both expansion kits, allowing the user to pool up to 24 different samples in one sequencing experiment. 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.

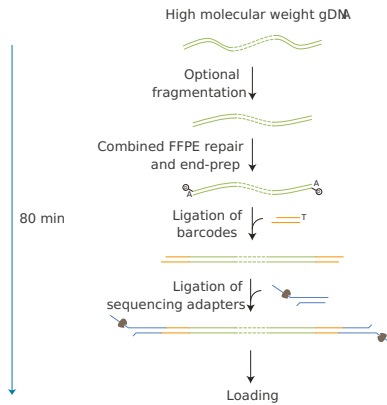
The quality checks performed during the protocol are essential in ensuring experimental success.

- 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

Prepare your library

You will need to:

- Repair the DNA, and prepare the DNA ends for adapter attachment
- Ligate Native barcodes supplied in the kit to the DNA ends
- Ligate sequencing adapters supplied in the kit to the DNA ends
- Prime the flow cell, and load your DNA library into the flow cell



Sequencing

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

IMPORTANT

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

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.

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Ligation Sequencing Kit (SQK-LSK109)
- Native Barcoding Expansions 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114)
- FLO-MIN106 (R9.4.1) flow cells
- Flow Cell Wash Kit (EXP-WSH004)

Equipment and consumables

Materials

- 1 µg (or 100-200 fmol) high molecular weight genomic DNA for every sample to be barcoded
- 1.5-3 µg (or 150-300 fmol) high molecular weight genomic DNA for every sample to be barcoded, if using R10.3 flow cells
- OR 100+ ng high molecular weight genomic DNA if performing DNA fragmentation
- Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) if multiplexing more than 12 samples
- Ligation Sequencing Kit (SQK-LSK109)
- Flow Cell Priming Kit (EXP-FLP002)
- Adapter Mix II Expansion (EXP-AMII001)

Consumables

- NEB Blunt/TA Ligase Master Mix (M0367)
- NEBNext FFPE Repair Mix (M6630)
- NEBNext Ultra II End repair/dA-tailing Module (E7546)
- NEBNext Quick Ligation Module (E6056)
- Agencourt AMPure XP beads (Beckman Coulter™, A63881)
- 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

Equipment

- Hula mixer (gentle rotator mixer)
- Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- Microfuge
- Vortex mixer
- Thermal cycler
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips
- Ice bucket with ice
- Timer

Optional Equipment

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

For this protocol, you will need the following amounts of high molecular DNA for every sample to be barcoded

depending on the flow cell type used:

- 1 µg (or 100-200 fmol) gDNA is required for R9.4.1 flow cells
- 1.5-3 µg (or 150-300 fmol) gDNA is required for R10.3 flow cells

Users can start with lower input quantities (down to 100 ng) if performing [DNA fragmentation](#) to increase the number of DNA molecules in the sample, or if amplifying the sample by PCR.

Input DNA

How to QC your input DNA

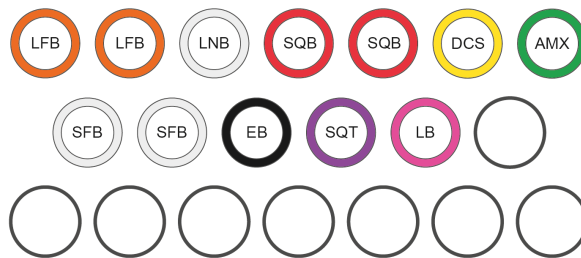
It is important that the input DNA meets the quantity and quality requirements. Using too little or too much DNA, or DNA of poor quality (e.g. highly fragmented or containing RNA or chemical contaminants) can affect your library preparation.

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.

Ligation Sequencing Kit contents (SQK-LSK109)



LFB : L fragment buffer SFB : S fragment buffer
LNB : Ligation buffer EB : Elution buffer
SQB : Sequencing buffer SQT : Sequencing tether
DCS : DNA control strand LB : Loading beads
AMX : Adapter mix

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

| Name | Acronym | Cap colour | No. of vials | Fill volume per vial (µl) |
|-------------------|---------|------------|--------------|---------------------------|
| 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)

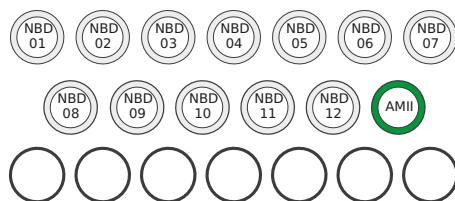


FLB : Flush buffer
FLT : Flush tether

| 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 |

Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) contents

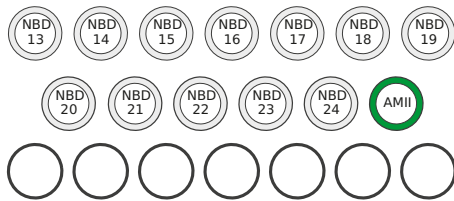
EXP-NBD104 kit contents



NBD01: Native barcode 1
NBD02: Native barcode 2
NBD03: Native barcode 3
NBD04: Native barcode 4
NBD05: Native barcode 5
NBD06: Native barcode 6
NBD07: Native barcode 7
NBD08: Native barcode 8
NBD09: Native barcode 9
NBD10: Native barcode 10
NBD11: Native barcode 11
NBD12: Native barcode 12
AMII: Adapter mix II

| Name | Acronym | Cap colour | No. of vials | Fill volume per vial (µl) |
|----------------------|---------|------------|--------------|---------------------------|
| Native Barcode 01-12 | NB01-12 | White | 12 | 20 |
| Adapter Mix II | AMII | Green | 1 | 40 |

EXP-NBD114 kit contents



NBD13: Native barcode 13
 NBD14: Native barcode 14
 NBD15: Native barcode 15
 NBD16: Native barcode 16
 NBD17: Native barcode 17
 NBD18: Native barcode 18
 NBD19: Native barcode 19

NBD20: Native barcode 20
 NBD21: Native barcode 21
 NBD22: Native barcode 22
 NBD23: Native barcode 23
 NBD24: Native barcode 24
 AMII: Adapter mix II

| Name | Acronym | Cap colour | No. of vials | Fill volume per vial (µl) |
|----------------------|---------|------------|--------------|---------------------------|
| Native Barcode 13-24 | NB13-24 | White | 12 | 20 |
| Adapter Mix II | AMII | Green | 1 | 40 |

Adapter Mix II Expansion contents (EXP-AMII001)



AMII : Adapter Mix II

| Name | Acronym | Cap colour | No. of tubes | Fill volume per vial (µl) |
|----------------|---------|------------|--------------|---------------------------|
| Adapter Mix II | AMII | Green | 2 | 40 |

Adapter Mix II Expansion use

Protocols that use the Native Barcoding Expansions require 5 µl of AMII per reaction. Native Barcoding Expansions EXP-NBD104/NBD114 and EXP-NBD196 contain sufficient AMII for 6 and 12 reactions, respectively (or 12 and 24 reactions when sequencing on Flongle). This assumes that all barcodes are used in one sequencing run.

The Adapter Mix II expansion provides additional AMII for customers who are running subsets of barcodes, and allows a further 12 reactions (24 on Flongle).

Native barcode sequences

The native barcode sequences are the reverse complement of the corresponding barcode sequence in other kits. The first 24 unique barcodes are available in the Native Barcoding Kit 24 (SQK-NBD112.24) and the Native Barcoding Expansion 1-12 and 13-24 (EXP-NBD104 and EXP-NBD114). The Native Barcoding Kit 96 (SQK-NBD112.96) and Native Barcoding Expansion 96 (EXP-NBD196) include the first 24 native barcodes, with an additional 48 unique barcodes.

Native Barcoding Kit 24 (SQK-NBD112.24) and Native Barcoding Expansion 1-12 and 13-24 (EXP-NBD104 and EXP-NBD114)

| Component | Forward sequence | Reverse sequence |
|-----------|---------------------------|--------------------------|
| NB01 | CACAAAGACACCGACAACCTTTCTT | AAGAAAGTTGTCGGTGTCTTTGTG |
| NB02 | ACAGACGACTACAAACGGAATCGA | TCGATTCCGTTTGTAGTCGTCTGT |
| NB03 | CCTGGTAACTGGGACACAAGACTC | GAGTCTTGTGTCCCAGTTACCAGG |
| NB04 | TAGGGAAACACGATAGAATCCGAA | TTCGGATTCTATCGTGTTCCTA |
| NB05 | AAGGTTACACAAACCCTGGACAAG | CTTGTCCAGGGTTTGTGTAACCTT |
| NB06 | GACTACTTTCTGCCTTTGCGAGAA | TTCTCGCAAAGGCAGAAAGTAGTC |
| NB07 | AAGGATTCATTCCCACGGTAACAC | GTGTTACCGTGGGAATGAATCCTT |
| NB08 | ACGTAACCTGGTTTGTTCCTGAA | TTCAGGGAACAAACCAAGTTACGT |
| NB09 | AACCAAGACTCGCTGTGCCTAGTT | AACTAGGCACAGCGAGTCTTGGTT |
| NB10 | GAGAGGACAAAGGTTTCAACGCTT | AAGCGTTGAAACCTTTGTCCTCTC |
| NB11 | TCCATTCCCTCCGATAGATGAAAC | GTTTCATCTATCGGAGGGAATGGA |
| NB12 | TCCGATTCTGCTTCTTTCTACCTG | CAGGTAGAAAGAAGCAGAATCGGA |
| NB13 | AGAACGACTTCCATACTCGTGTGA | TCACACGAGTATGGAAGTCGTTCT |
| NB14 | AACGAGTCTCTTGGGACCCATAGA | TCTATGGGTCCCAAGAGACTCGTT |
| NB15 | AGGTCTACCTCGCTAACACCACTG | CAGTGGTGTAGCGAGGTAGACCT |
| NB16 | CGTCAACTGACAGTGGTTCGTACT | AGTACGAACCACTGTCAGTTGACG |
| NB17 | ACCCTCCAGGAAAGTACCTCTGAT | ATCAGAGGTACTTTCCTGGAGGGT |
| NB18 | CCAAACCAACAACCTAGATAGGC | GCCTATCTAGGTTGTTGGGTTTGG |
| NB19 | GTTCCCTCGTGCAGTGTCAAGAGAT | ATCTCTTGACACTGCACGAGGAAC |
| NB20 | TTGCGTCTGTTACGAGAACTCAT | ATGAGTTCTCGTAACAGGACGCAA |
| NB21 | GAGCCTCTCATTGTCCGTTCTCTA | TAGAGAACGGACAATGAGAGGCTC |
| NB22 | ACCACTGCCATGTATCAAAGTACG | CGTACTTTGATACATGGCAGTGGT |
| NB23 | CTTACTACCCAGTGAACCTCCTCG | CGAGGAGGTTCACTGGGTAGTAAG |
| NB24 | GCATAGTTCTGCATGATGGGTTAG | CTAACCCATCATGCAGAACTATGC |

Native Barcoding Kit 96 (SQK-NBD112.96) and Native Barcoding Expansion 96 (EXP-NBD196)

| Component | Forward sequence | Reverse sequence |
|-----------|---------------------------|---------------------------|
| NB01 | CACAAAGACACCGACAACCTTTCTT | AAGAAAGTTGTCGGTGTCTTTGTG |
| NB02 | ACAGACGACTACAAACGGAATCGA | TCGATTCCGTTTGTAGTCGTCTGT |
| NB03 | CCTGGTAACTGGGACACAAGACTC | GAGTCTTGTGTCCCAGTTACCAGG |
| NB04 | TAGGGAAACACGATAGAATCCGAA | TTCGATTCTATCGTGTTCCTA |
| NB05 | AAGGTTACACAAACCCTGGACAAG | CTTGTCCAGGGTTTGTGTAACCTT |
| NB06 | GACTACTTTCTGCCTTTGCGAGAA | TTCTCGCAAAGGCAGAAAGTAGTC |
| NB07 | AAGGATTCATTCCCACGGTAACAC | GTGTTACCGTGGGAATGAATCCTT |
| NB08 | ACGTAACCTGGTTTGTCCCTGAA | TTCAGGGAACAAACCAAGTTACGT |
| NB09 | AACCAAGACTCGCTGTGCCTAGTT | AACTAGGCACAGCGAGTCTTGGTT |
| NB10 | GAGAGGACAAAGGTTTCAACGCTT | AAGCGTTGAAACCTTTGCCTCTC |
| NB11 | TCCATTCCCTCCGATAGATGAAAC | GTTTCATCTATCGGAGGGAATGGA |
| NB12 | TCCGATTCTGCTTCTTTCTACCTG | CAGGTAGAAAGAAGCAGAATCGGA |
| NB13 | AGAACGACTTCCATACTCGTGTGA | TCACACGAGTATGGAAGTCGTTCT |
| NB14 | AACGAGTCTCTTGGGACCCATAGA | TCTATGGGTCCCAAGAGACTCGTT |
| NB15 | AGGTCTACCTCGCTAACACCACTG | CAGTGGTGTAGCGAGGTAGACCT |
| NB16 | CGTCAACTGACAGTGGTTCGTA | AGTACGAACCACTGTCAGTTGACG |
| NB17 | ACCTCCAGGAAAGTACCTCTGAT | ATCAGAGGTACTTTCCTGGAGGGT |
| NB18 | CCAAACCAACAACCTAGATAGGC | GCCTATCTAGGTTGTTGGGTTTGG |
| NB19 | GTTCCCTCGTGCAGTGTCAAGAGAT | ATCTCTTGACACTGCACGAGGAAC |
| NB20 | TTGCGTCTGTTACGAGAACTCAT | ATGAGTTCTCGTAACAGGACGCAA |
| NB21 | GAGCCTCTCATTGTCCGTTCTCTA | TAGAGAACGGACAATGAGAGGCTC |
| NB22 | ACCACTGCCATGTATCAAAGTACG | CGTACTTTGATACATGGCAGTGGT |
| NB23 | CTTACTACCCAGTGAACCTCCTCG | CGAGGAGGTTCACTGGGTAGTAAG |
| NB24 | GCATAGTTCTGCATGATGGGTTAG | CTAACCCATCATGCAGAACTATGC |
| NB25 | GTAAGTTGGGTATGCAACGCAATG | CATTGCGTTGCATACCCAACCTTAC |
| NB26 | CATACAGCGACTACGATTCTCAT | ATGAGAATGCGTAGTCGCTGTATG |
| NB27 | CGACGGTTAGATTACCTCTTACA | TGTAAGAGGTGAATCTAACCGTCG |
| NB28 | TGAAACCTAAGAAGGCACCGTATC | GATACGGTGCCTTCTTAGGTTTCA |
| NB29 | CTAGACACCTTGGGTTGACAGACC | GGTCTGTCAACCCAAGGTGTCTAG |
| NB30 | TCAGTGAGGATCTACTTCGACCCA | TGGGTGCAAGTAGATCCTCACTGA |
| NB31 | TGCGTACAGCAATCAGTTACATTG | CAATGTAACCTGATTGCTGTACGCA |
| NB32 | CCAGTAGAAGTCCGACAACGTCAT | ATGACGTTGTCGGACTTCTACTGG |

| Component | Forward sequence | Reverse sequence |
|-----------|---------------------------|---------------------------|
| NB33 | CAGACTTGGTACGGTTGGGTA ACT | AGTTACCCAACCGTACCAAGTCTG |
| NB34 | GGACGAAGA ACTCAAGTCAAAGGC | GCCTTTGACTTGAGTTCTTCGTCC |
| NB35 | CTACTTACGAAGCTGAGGGACTGC | GCAGTCCCTCAGCTTCGTAAGTAG |
| NB36 | ATGTCCCAGTTAGAGGAGGAAACA | TGTTTCCTCCTCTAACTGGGACAT |
| NB37 | GCTTGCGATTGATGCTTAGTATCA | TGATACTAAGCATCAATCGCAAGC |
| NB38 | ACCACAGGAGGACGATACAGAGAA | TTCTCTGTATCGTCCCTCTGTGGT |
| NB39 | CCACAGTGTCAACTAGAGCCTCTC | GAGAGGCTCTAGTTGACACTGTGG |
| NB40 | TAGTTTGGATGACCAAGGATAGCC | GGCTATCCTTGGTCATCCAAACTA |
| NB41 | GGAGTTCGTCCAGAGAAGTACACG | CGTGTACTTCTCTGGACGAACTCC |
| NB42 | CTACGTGTAAGGCATACCTGCCAG | CTGGCAGGTATGCCTTACACGTAG |
| NB43 | CTTTGTTGTTGACTCGACGGTAG | CTACCGTCGAGTCAACAACGAAAG |
| NB44 | AGTAGAAAGGGTTCCTTCCCCTC | GAGTGGGAAGGAACCTTTCTACT |
| NB45 | GATCCAACAGAGATGCCTTCAGTG | CACTGAAGGCATCTCTGTTGGATC |
| NB46 | GCTGTGTTCCACTTCATTCTCCTG | CAGGAGAATGAAGTGGAACACAGC |
| NB47 | GTGCAACTTTCCACAGGTAGTTC | GAACTACCTGTGGGAAAGTTGCAC |
| NB48 | CATCTGGAACGTGGTACACCTGTA | TACAGGTGTACCACGTTCCAGATG |
| NB49 | ACTGGTGCAGCTTTGAACATCTAG | CTAGATGTTCAAAGCTGCACCAGT |
| NB50 | ATGGACTTTGGTAACTTCCTGCGT | ACGCAGGAAGTTACCAAAGTCCAT |
| NB51 | GTTGAATGAGCCTACTGGGTCTC | GAGGACCCAGTAGGCTCATTCAAC |
| NB52 | TGAGAGACAAGATTGTTTCGTGGAC | GTCCACGAACAATCTTGTCTCTCA |
| NB53 | AGATTCAGACCGTCTCATGCAAAG | CTTTGCATGAGACGGTCTGAATCT |
| NB54 | CAAGAGCTTTGACTAAGGAGCATG | CATGCTCCTTAGTCAAAGCTCTTG |
| NB55 | TGGAAGATGAGACCCTGATCTACG | CGTAGATCAGGGTCTCATCTTCCA |
| NB56 | TCACTACTCAACAGGTGGCATGAA | TTCATGCCACCTGTTGAGTAGTGA |
| NB57 | GCTAGGTCAATCTCCTTCGGAAGT | ACTTCCGAAGGAGATTGACCTAGC |
| NB58 | CAGGTTACTCCTCCGTGAGTCTGA | TCAGACTCACGGAGGAGTAACTG |
| NB59 | TCAATCAAGAAGGGAAAGCAAGGT | ACCTTGCTTTCCCTTCTTGATTGA |
| NB60 | CATGTTCAACCAAGGCTTCTATGG | CCATAGAAGCCTTGGTTGAACATG |
| NB61 | AGAGGGTACTATGTGCCTCAGCAC | GTGCTGAGGCACATAGTACCCTCT |
| NB62 | CACCCACACTTACTTCAGGACGTA | TACGTCCTGAAGTAAAGTGTGGGTG |
| NB63 | TTCTGAAGTTCCTGGGTCTTGAAC | GTTCAAGACCCAGGAACTTCAGAA |
| NB64 | GACAGACACCGTTCATCGACTTTC | GAAAGTCGATGAACGGTGTCTGTC |

| Component | Forward sequence | Reverse sequence |
|-----------|---------------------------|---------------------------|
| NB65 | TTCTCAGTCTTCCTCCAGACAAGG | CCTTGTCTGGAGGAAGACTGAGAA |
| NB66 | CCGATCCTTGTGGCTTCTAACTTC | GAAGTTAGAAGCCACAAGGATCGG |
| NB67 | GTTTGTCACTACTCGTGTGCTCACC | GGTGAGCACACGAGTATGACAAAC |
| NB68 | GAATCTAAGCAAACACGAAGGTGG | CCACCTTCGTGTTTGCTTAGATTC |
| NB69 | TACAGTCCGAGCCTCATGTGATCT | AGATCACATGAGGCTCGGACTGTA |
| NB70 | ACCGAGATCCTACGAATGGAGTGT | ACACTCCATTTCGTAGGATCTCGGT |
| NB71 | CCTGGGAGCATCAGGTAGTAACAG | CTGTTACTACCTGATGCTCCCAGG |
| NB72 | TAGCTGACTGTCTTCCATACCGAC | GTCGGTATGGAAGACAGTCAGCTA |
| NB73 | AAGAAACAGGATGACAGAACCCTC | GAGGGTTCTGTCATCCTGTTTCTT |
| NB74 | TACAAGCATCCCAACACTTCCACT | AGTGGAAGTGTTGGGATGCTTGTA |
| NB75 | GACCATTGTGATGAACCCTGTTGT | ACAACAGGGTTCATCACAATGGTC |
| NB76 | ATGCTTGTTACATCAACCCTGGAC | GTCCAGGGTTGATGTAACAAGCAT |
| NB77 | CGACCTGTTTCTCAGGGATACAAC | GTTGTATCCCTGAGAAACAGGTCCG |
| NB78 | AACAACCGAACCTTTGAATCAGAA | TTCTGATTCAAAGGTTTCGGTTGTT |
| NB79 | TCTCGGAGATAGTTCTCACTGCTG | CAGCAGTGAGAACTATCTCCGAGA |
| NB80 | CGGATGAACATAGGATAGCGATTC | GAATCGCTATCCTATGTTTCATCCG |
| NB81 | CCTCATCTTGTGAAGTTGTTTCGG | CCGAAACAACCTTCACAAGATGAGG |
| NB82 | ACGGTATGTGCGAGTTCCAGGACTA | TAGTCCTGGAACCTCGACATACCGT |
| NB83 | TGGCTTGATCTAGGTAAGGTCGAA | TTCGACCTTACCTAGATCAAGCCA |
| NB84 | GTAGTGGACCTAGAACCTGTGCCA | TGGCACAGGTTCTAGGTCCACTAC |
| NB85 | AACGGAGGAGTTAGTTGGATGATC | GATCATCCAACCTAACCTCCGTT |
| NB86 | AGGTGATCCCAACAAGCGTAAGTA | TACTTACGCTTGTTGGGATCACCT |
| NB87 | TACATGCTCCTGTTGTTAGGGAGG | CCTCCCTAACAACAGGAGCATGTA |
| NB88 | TCTTCTACTACCGATCCGAAGCAG | CTGCTTCGGATCGGTAGTAGAAGA |
| NB89 | ACAGCATCAATGTTTGGCTAGTTG | CAACTAGCCAAACATTGATGCTGT |
| NB90 | GATGTAGAGGGTACGGTTTGAGGC | GCCTCAAACCGTACCCTCTACATC |
| NB91 | GGCTCCATAGGAACTCACGCTACT | AGTAGCGTGAGTTCCTATGGAGCC |
| NB92 | TTGTGAGTGGAAAGATACAGGACC | GGTCCTGTATCTTCCACTCACAA |
| NB93 | AGTTTCCATCACTTCAGACTTGGG | CCCAAGTCTGAAGTGATGGAAACT |
| NB94 | GATTGTCCTCAAACCTGCCACCTAC | GTAGGTGGCAGTTTGAGGACAATC |
| NB95 | CCTGTCTGGAAGAAGAATGGACTT | AAGTCCATTCTTCTCCAGACAGG |
| NB96 | CTGAACGGTCATAGAGTCCACCAT | ATGGTGGACTCTATGACCGTTCAG |

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 |

DNA repair and end-prep

~35 minutes

Materials

- gDNA in 48 µl nuclease-free water

Consumables

- 0.2 ml thin-walled PCR 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
- 1.5 ml Eppendorf DNA LoBind tubes

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

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

For optimal performance, NEB recommend the following:

1. Thaw all reagents on ice.
2. Flick and/or invert the reagent tubes to ensure they are well mixed.
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.

2 Prepare the DNA in nuclease-free water.

- For R9.4.1 flow cells, transfer 1 μg (or 100–200 fmol) genomic DNA into a 1.5 ml Eppendorf DNA LoBind tube, or 1.5–3 μg (or 150–300 fmol) genomic DNA if using R10.3 flow cells.
- Adjust the volume to 48 μl with nuclease-free water
- Mix thoroughly by flicking the tube
- Spin down briefly in a microfuge

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

Between each addition, pipette mix 10-20 times

| Reagent | Volume |
|-----------------------------------|-----------------------------|
| DNA | 48 μ l |
| NEBNext FFPE DNA Repair Buffer | 3.5 μ l |
| Ultra II End-prep reaction buffer | 3.5 μ l |
| Ultra II End-prep enzyme mix | 3 μ l |
| NEBNext FFPE DNA Repair Mix | 2 μ l |
| Total | 60 μl |

4 Mix well by pipetting using wide-bore pipette tips. Alternatively, if you are concerned about preserving the integrity of very long DNA fragments, mix gently by flicking the tube, and spin down.

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

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

7 Resuspend the AMPure XP beads by vortexing.

8 Add 60 μ 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 μ 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 μ l 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 25 µl nuclease-free water. Spin down and 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 25 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

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



Figure NSRL: Typical DNA recovery levels at the end of two steps during the library preparation.

END OF STEP

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

Native barcode ligation

~50 minutes

Materials

- Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) if multiplexing more than 12 samples

Consumables

- Freshly prepared 70% ethanol in nuclease-free water
- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Agencourt AMPure XP beads (Beckman Coulter™, A63881)
- NEB Blunt/TA Ligase Master Mix (M0367)

Equipment

- Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- Hula mixer (gentle rotator mixer)
- Vortex mixer
- Ice bucket with ice
- Microfuge
- P1000 pipette and tips
- P100 pipette and tips
- P10 pipette and tips

Optional Equipment

- Qubit fluorometer (or equivalent for QC check)

- 1 Thaw the native barcodes at room temperature. Use one barcode per sample. Individually mix the barcodes by pipetting, spin down, and place them on ice.
- 2 Select a unique barcode for every sample to be run together on the same flow cell, from the provided 24 barcodes. Up to 24 samples can be barcoded and combined in one experiment.
- 3 Dilute 500 ng (750 ng if sequencing on R10.3 flow cells) of each end-prepped sample to be barcoded to 22.5 μ l in nuclease-free water.
- 4 Add the reagents in the order given below, mixing by flicking the tube between each sequential addition:

| Reagent | Volume |
|---|-----------------------------|
| 500 ng end-prepped DNA (750 ng if using R10.3 flow cells) | 22.5 μ l |
| Native Barcode | 2.5 μ l |
| Blunt/TA Ligase Master Mix | 25 μ l |
| Total | 50 μl |

- 5 Mix well by pipetting using wide-bore pipette tips. Alternatively, if you are concerned about preserving the integrity of very long DNA fragments, mix gently by flicking the tube, and spin down.
- 6 Incubate the reaction for 10 minutes at room temperature.
- 7 Resuspend the AMPure XP beads by vortexing.
- 8 Add 50 μ l of resuspended AMPure XP beads to the reaction and mix by pipetting.
- 9 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
- 10 Prepare 500 μ l of fresh 70% ethanol in nuclease-free water.
- 11 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
- 12 Keep the tube on the magnet and wash the beads with 200 μ l 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 26 μ l nuclease-free water. Incubate for 2 minutes at room temperature.

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

17 Remove and retain 26 μ l of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

Dispose of the pelleted beads

Quantify 1 μ l of eluted sample using a Qubit fluorometer.

IMPORTANT

Please first refer to the ligation step below to ensure that the library is diluted to the correct volume.

18 Pool equimolar amounts of each barcoded sample into a 1.5 ml Eppendorf DNA LoBind tube, ensuring that sufficient sample is combined to produce a pooled sample of 700 ng total (1050 ng if sequencing on R10.3 flow cells).

19 Quantify 1 μ l of pooled and barcoded DNA using a Qubit fluorometer.

20 Dilute 700 ng (1050 ng for R10.3 flow cells) pooled sample to 65 μ l in nuclease-free water.

Optional Action

If 700 ng (1050 ng for R10.3 flow cells) of pooled sample exceeds 65 μ l in volume, perform an AMPure clean-up with 2.5x Agencourt AMPure XP beads to pooled sample volume, eluting in 65 μ l of nuclease-free water.

Fragment size and adapter ligation

The amount of adapter has been optimised for fragment sizes greater or equal to 8 kb. If the fragments are generally smaller than 3 kb, adjustments should be made to use 0.1–0.2 pmoles of DNA in the adapter ligation step.

END OF STEP

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

Adapter ligation and clean-up

~50 minutes

Materials

- Long Fragment Buffer (LFB)
- Short Fragment Buffer (SFB)
- Elution Buffer from the Oxford Nanopore kit (EB)
- Adapter Mix II (AMII)

Consumables

- Quick T4 DNA Ligase in NEBNext® Quick Ligation Module (NEB E6056)
- NEBNext® Quick Ligation Reaction Buffer (NEB B6058)
- Agencourt AMPure XP beads
- 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- Microfuge
- Magnetic rack
- Vortex mixer
- Hula mixer (gentle rotator mixer)

Optional Equipment

- Qubit fluorometer (or equivalent for QC check)

Adapter Mix II Expansion use

Protocols that use the Native Barcoding Expansions require 5 µl of AMII per reaction. Native Barcoding Expansions EXP-NBD104/NBD114 and EXP-NBD196 contain sufficient AMII for 6 and 12 reactions, respectively (or 12 and 24 reactions when sequencing on Flongle). This assumes that all barcodes are used in one sequencing run.

The Adapter Mix II expansion provides additional AMII for customers who are running subsets of barcodes, and allows a further 12 reactions (24 on Flongle).

- 1 Thaw the Elution Buffer (EB) and NEBNext Quick Ligation Reaction Buffer (5x) at room temperature, mix by vortexing, spin down and place on ice. Check the contents of each tube are clear of any precipitate.**
- 2 Spin down the T4 Ligase and the Adapter Mix II (AMII), 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)**

- 3 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.**

4 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.

5 Taking the pooled and barcoded DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition.

| Reagent | Volume |
|---|------------------------------|
| 700 ng (1050 ng if using R10.3 flow cells) pooled barcoded sample | 65 μ l |
| Adapter Mix II (AMII) | 5 μ l |
| NEBNext Quick Ligation Reaction Buffer (5X) | 20 μ l |
| Quick T4 DNA Ligase | 10 μ l |
| Total | 100 μl |

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

7 Incubate the reaction for 10 minutes at room temperature.

8 Resuspend the AMPure XP beads by vortexing.

9 Add 50 μ l of resuspended AMPure XP beads to the reaction and mix by pipetting.

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

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

12 Wash the beads by adding either 250 μ l Long Fragment Buffer (LFB) or 250 μ 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.

13 Repeat the previous step.

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

15 Remove the tube from the magnetic rack and resuspend the pellet in 15 μ 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.

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

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

Dispose of the pelleted beads

18 Quantify 1 µl of adapter ligated and barcoded DNA using a Qubit fluorometer - recovery aim ~430 ng.

IMPORTANT

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

Loading more than the recommended input can have a detrimental effect on output. Dilute the library in Elution Buffer (EB) 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 onto 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.

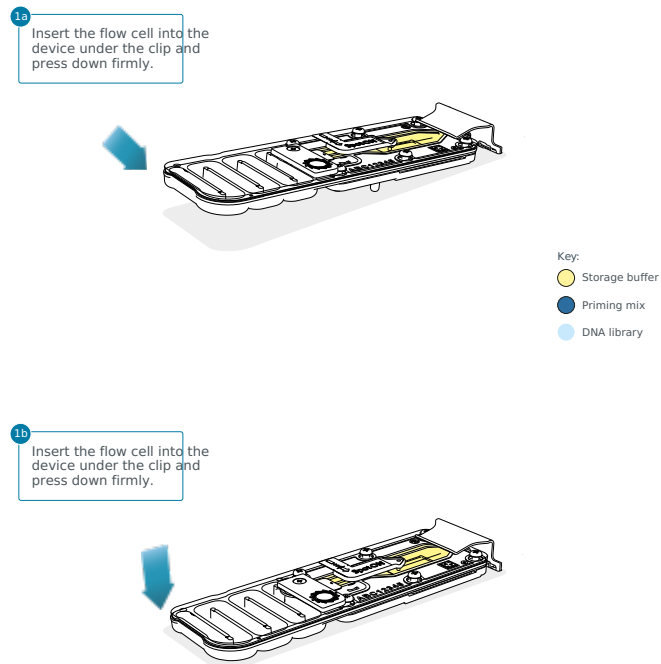
TIP**Priming and loading a flow cell**

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 μ 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

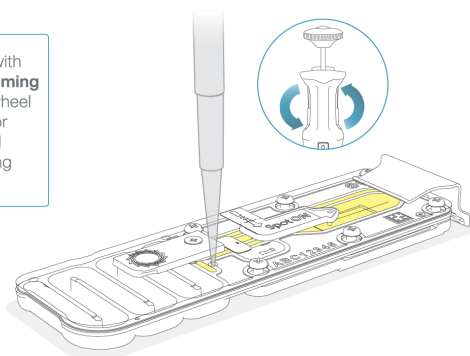
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 μl , and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

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

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

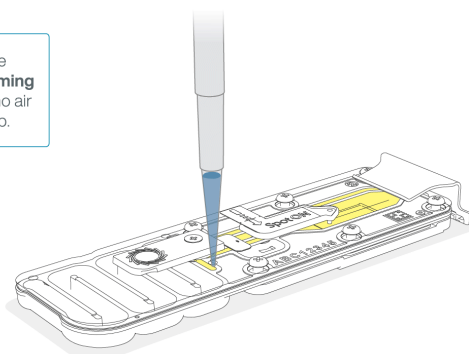
Note: Visually check that there is continuous buffer from the priming port across the sensor array.

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



- 6 Load 800 μl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 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 per flow cell |
|--|------------------------------------|
| Sequencing Buffer (SQB) | 37.5 μl |
| Loading Beads (LB), mixed immediately before use | 25.5 μl |
| DNA library | 12 μl |
| Total | 75 μl |

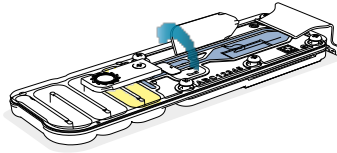
Note: Load the library onto the flow cell immediately after adding the Sequencing Buffer (SQB) and Loading Beads (LB) because the fuel in the buffer will start to be consumed by the adapter.

9 Complete the flow cell priming:

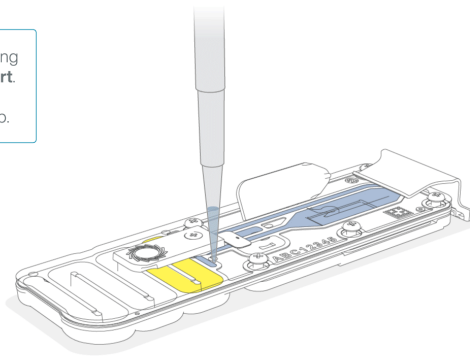
1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load **200 µl** of the priming mix into the flow cell via the priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.

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

5 Gently flip open SpotON sample port cover.



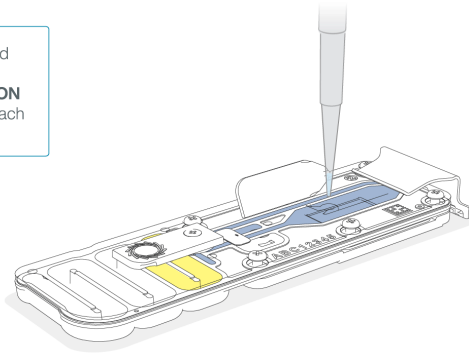
6 Load 200 µl of the priming mix into the **Priming Port**. Ensure there are no air bubbles in the pipette tip.



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

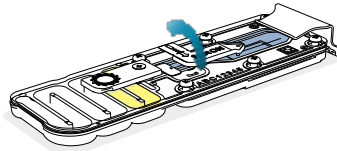
11 Add 75 μ l of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

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

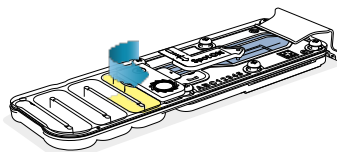


12 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION device lid.

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.

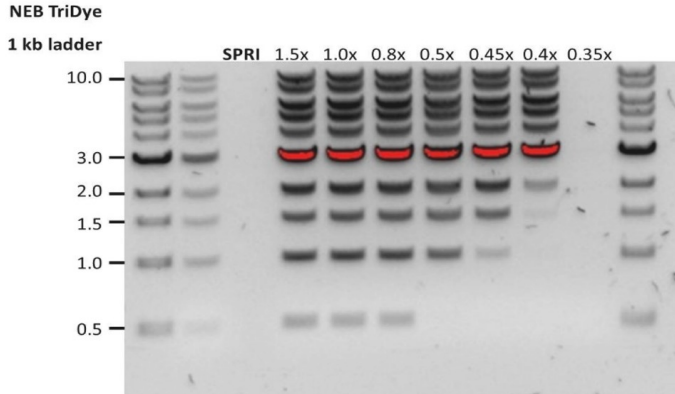
If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via [LiveChat](#) in the Nanopore Community.

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 |
|-------------|----------------|----------------------|
|-------------|----------------|----------------------|

| Observation | Possible cause | Comments and actions |
|------------------------------------|--|--|
| Low recovery | DNA loss due to a lower than intended AMPure beads-to-sample ratio | <p>1. AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample.</p> <p>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.</p> |
| Low recovery | DNA fragments are shorter than expected | <p>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.</p>  |
| 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. |

The VoITRAX run terminated in the middle of the library prep

| Observation | Possible cause | Comments and actions |
|---|--|--|
| <p>The green light was switched off</p> <p>or</p> <p>An adapter was used to connect the VoITRAX USB-C cable to the computer</p> | 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 VoITRAX V2 protocol . |

The VoITRAX software shows an inaccurate amount of reagents loaded

| Observation | Possible cause | Comments and actions |
|---|---|---|
| The VoITRAX software shows an inaccurate amount of reagents loaded | Pipette tips do not fit the VoITRAX cartridge ports | Rainin 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. |

| Observation | Possible cause | Comments and actions |
|---|--|---|
| The VolTRAX software shows an inaccurate amount of reagents loaded | 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 VolTRAX 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) or via [LiveChat](#) in the Nanopore Community.

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

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

| Observation | Possible cause | Comments and actions |
|--|--|--|
| MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check | Contaminations in the library damaged or blocked the pores | The pore count during the Flow Cell Check is performed using the QC DNA molecules present in the flow cell storage buffer. At the start of sequencing, the library itself is used to estimate the number of active pores. Because of this, variability of about 10% in the number of pores is expected. A significantly lower pore count reported at the start of sequencing can be due to contaminants in the library that have damaged the membranes or blocked the pores. Alternative DNA/RNA extraction or purification methods may be needed to improve the purity of the input material. The effects of contaminants are shown in the Contaminants Know-how piece . Please try an alternative extraction method that does not result in contaminant carryover. |

MinKNOW script failed

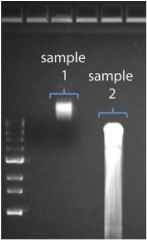
| Observation | Possible cause | Comments and actions |
|--------------------------------------|----------------|---|
| MinKNOW shows "Script failed" | | Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support. |

Pore occupancy below 40%

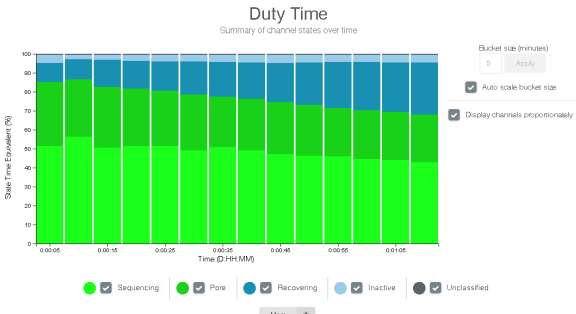
| Observation | Possible cause | Comments and actions |
|----------------------------------|---|--|
| Pore occupancy <40% | Not enough library was loaded on the flow cell | 5–50 fmol of good quality library can be loaded on to a MinION Mk1B/GridION flow cell. Please quantify the library before loading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

Shorter than expected read length

| Observation | Possible cause | Comments and actions |
|-------------|----------------|----------------------|
|-------------|----------------|----------------------|

| Observation | Possible cause | Comments and actions |
|--|--------------------------------------|---|
| Shorter than expected read length | Unwanted fragmentation of DNA sample | <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"> 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.  <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"> 3. During library prep, avoid pipetting and vortexing when mixing reagents. Flicking or inverting the tube is sufficient. |

Large proportion of recovering pores

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

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

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

Temperature fluctuation

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

Failed to reach target temperature

| Observation | Possible cause | Comments and actions |
|--|--|---|
| MinKNOW shows "Failed to reach target temperature" (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 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 | <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. |
| No input .fast5 was found or basecalled | 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 |
|---|--|--|
| No Pass or Fail folders were generated after basecalling | The <i>--qscore_filtering</i> flag was not included in the command | The <i>--qscore_filtering</i> 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 <i>--device</i> flag wasn't included in the command | The <i>--device</i> 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 <i>--device cuda:0 cuda:1</i> , when 2 GPUs are specified to use by the Guppy command. |

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

| Observation | Possible cause | Comments and actions |
|--|--|--|
| The MinKNOW interface is not shown in the web browser | Browser compatibility issue | Always use Google Chrome as the browser to view MinKNOW. Alternatively, instead of typing //mt-xxxxxx (x is a number) in the address bar, type in the generic IP address, 10.42.0.1, which identifies the MinIT Wi-Fi router. |
| The MinKNOW interface is not shown in the web browser | The MinIT Wi-Fi was not used for connecting to the computer or mobile device | <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> |
| The MinKNOW interface is not shown in the web browser | The MinIT was not on the same network that the computer was connected to. | Make sure that the wall sockets used by the Ethernet cables from the MinIT and computer belong to the same local network. |

MinIT - the MinIT software cannot be updated

| Observation | Possible cause | Comments and actions |
|---|---|---|
| The MinIT software cannot be updated | The firewall is blocking IPs for update | Please consult your IT department, as the MinIT software requires access to the following AWS IP ranges . Access to the following IP addresses is also needed: 178.79.175.200 96.126.99.215 |
| The MinIT software cannot be updated | The device already has the latest version of the software | Occasionally, the MinIT software admin page displays "updates available" even when the software is already up-to-date. Please compare the version listed on the admin page with the one on the Software Downloads page . Alternatively, SSH into the MinIT through a SSH Client (e.g. Bitwise or Putty, as described in the MinIT protocol) 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. |