# Overview of the protocol

**IMPORTANT**

**This is an Early Access product**

For more information about our Early Access programmes, please seethis article on product release phases

Please ensure you always use the most recent version of the protocol.

### Native Barcoding Kit 24 V14 features

This kit is recommended for users who:

- want to obtain duplex data
- want to achieve median raw read accuracy of Q20+ (99%) and above
- want to optimise their sequencing experiment for accuracy and output
- wish to multiplex up to 24 samples to reduce price per sample
- need a PCR-free method of multiplexing to preserve additional information such as base modifications
- require control over read length
- would like to utilise upstream processes such as size selection or whole genome amplification

**IMPORTANT**

**Kit 14 sequencing and duplex basecalling info sheet**

The Kit 14 chemistry is a new development from Oxford Nanopore Technologies with improved duplex basecalling, which requires a different set of tools. For more information, please see the Kit 14 sequencing and duplex basecalling info sheet. We strongly recommend that you read it before proceeding with Kit 14 chemistry sequencing experiments and basecalling duplex data.

### Introduction to the Native Barcoding Kit 24 V14 protocol

This protocol describes how to carry out native barcoding of genomic DNA (gDNA) using the Native Barcoding Kit 24 V14 (SQK-NBD114.24). There are 24 unique barcodes available, 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:

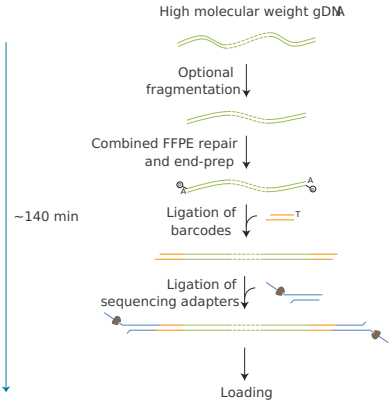
- Extract your DNA, and check its length, quantity and purity. **The quality checks performed during the protocol are essential in ensuring experimental success.**
- Ensure you have your sequencing kit, the correct equipment and third-party reagents
- Download the software for acquiring and analysing 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
- Demultiplex barcoded reads in MinKNOW or the Guppy basecalling, choosing the SQK-NBD114.24 kit option
- Start the EPI2ME software and select a workflow for further analysis (this step is optional)

**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:

- Native Barcoding Kit 24 V14 (SQK-NBD114.24)
- R10.4.1 flow cells (FLO-PRO114M)
- Flow Cell Wash Kit (EXP-WSH004)
- Sequencing Auxiliary Vials V14 (EXP-AUX003)
- Native Barcoding Expansion V14 (EXP-NBA114)

# Equipment and consumables

**Materials**

- Native Barcoding Kit 24 V14 (SQK-NBD114.24)
- 400 ng gDNA per sample if using >4 barcodes
- OR 1000 ng gDNA per sample if using ≤4 barcodes

**Consumables**

- NEB Blunt/TA Ligase Master Mix (NEB, cat # M0367)
- NEBNext FFPE Repair Mix (NEB, M6630)
- NEBNext Ultra II End repair/dA-tailing Module (NEB, cat # E7546)
- NEBNext Quick Ligation Module (NEB, cat # E6056)
- Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, cat # 0030129504) with heat seals
- 1.5 ml Eppendorf DNA LoBind tubes
- 2 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 80% ethanol in nuclease-free water
- Qubit™ Assay Tubes (Invitrogen, cat # Q32856)
- Qubit dsDNA HS Assay Kit (ThermoFisher, cat # Q32851)

**Equipment**

- Hula mixer (gentle rotator mixer)
- Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, cat # 11766427)
- Microfuge
- Magnetic rack suitable for 0.2 ml thin-walled PCR tubes or 96-well plates
- Vortex mixer
- Thermal cycler
- Multichannel pipette
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips

| | |
|---|---|
| | • P10 pipette and tips<br>• P2 pipette and tips<br>• Ice bucket with ice<br>• Timer<br>• Eppendorf 5424 centrifuge (or equivalent)<br>• Qubit fluorometer (or equivalent for QC check) |
| **Optional Equipment** | • Nanodrop spectrophotometer |

**For this protocol, we recommend the following inputs:**

- 400 ng per sample for >4 barcodes
- 1000 ng per sample for ≤4 barcodes

#### Input DNA

### How to QC your input DNA

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

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

### Chemical contaminants

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

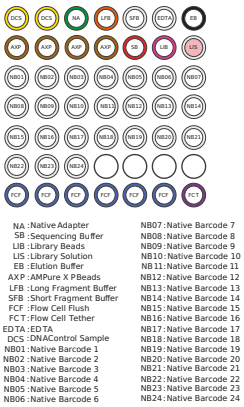
#### Third-party reagents

We have validated and recommend the use of all the third-party reagents used in this protocol. Alternatives have not been tested by Oxford Nanopore Technologies.

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

> **IMPORTANT**
>
> **The Native Adapter (NA) used in this kit and protocol is not interchangeable with other sequencing adapters.**

#### Native Barcoding Kit 24 V14 (SQK-NBD114.24) contents




| Name | Acronym | Cap colour | No. of vials | Fill volume per vial (µl) |
|---|---|---|---|---|
| Native Barcodes | NB01-24 | Clear | 24 (one per barcode) | 20 |
| DNA Control Sample | DCS | Yellow | 2 | 35 |
| Native Adapter | NA | Green | 1 | 40 |
| Sequencing Buffer | SB | Red | 1 | 700 |
| Library Beads | LIB | Pink | 1 | 600 |
| Library Solution | LIS | White cap, pink label | 1 | 600 |
| Elution Buffer | EB | Black | 1 | 500 |
| AMPure XP Beads | AXP | Amber | 4 | 1,200 |
| Long Fragment Buffer | LFB | Orange | 1 | 1,800 |
| Short Fragment Buffer | SFB | Clear | 1 | 1,800 |
| EDTA | EDTA | Clear | 1 | 700 |
| Flow Cell Flush | FCF | Blue | 6 | 1,170 |
| Flow Cell Tether | FCT | Purple | 1 | 200 |

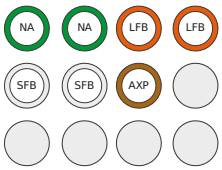
**Note:** This Product Contains AMPure XP Reagent Manufactured by Beckman Coulter, Inc. and can be stored at -20°C with the kit without detriment to reagent stability.

**To maximise the use of the Native Barcoding Kits, the Native Barcode Auxiliary V14 (EXP-NBA114) and the Sequencing Auxiliary Vials V14 (EXP-AUX003) expansion packs are available.**

These expansions provide extra library preparation and flow cell priming reagents to allow users to utilise any unused barcodes for those running in smaller subsets.

Both expansion packs used together will provide enough reagents for 12 reactions.

**Native Barcode Auxiliary V14 (EXP-NBA114) contents:**

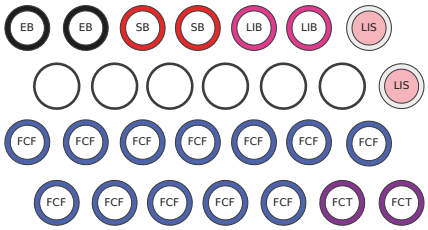



NA : NativeAdapter
LFB : Long Fragment Buffer
SFB : Short Fragment Buffer
AXP : AMPure XP Beads

| Name | Acronym | Cap colour | No. of vials | Fill volume per vial (µl) |
|---|---|---|---|---|
| Native Adapter | NA | Green | 2 | 40 |
| AMPure XP Beads | AXP | Amber | 1 | 400 |
| Long Fragment Buffer | LFB | Orange | 2 | 1,800 |
| Short Fragment Buffer | SFB | Clear | 2 | 1,800 |

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

**Sequencing Auxiliary Vials V14 (EXP-AUX003) contents:**




EB : Elution Buffer
SB : Sequencing Buffer
LIB : Library Beads
LIS : Library Solution
FCF : Flow Cell Flush
FCT : Flow Cell Tether

| Name | Acronym | Cap colour | No. of vials | Fill volume per vial (µl) |
|---|---|---|---|---|
| Elution Buffer | EB | Black | 2 | 500 |
| Sequencing Buffer | SB | Red | 2 | 700 |
| Library Solution | LIS | White cap, pink label | 2 | 600 |
| Library Beads | LIB | Pink | 2 | 600 |
| Flow Cell Flush | FCF | Blue | 12 | 1,170 |
| Flow Cell Tether | FCT | Purple | 2 | 200 |

**Native barcode sequences**

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

# Computer requirements and software

#### PromethION 24/48 IT requirements

The PromethION device contains all the hardware required to control up to 24 (for the P24 model) or 48 (for the P48 model) sequencing experiments and acquire the data. The device is further enhanced with high performance GPU technology for real-time basecalling. Read more in the PromethION IT Requirements document.

#### PromethION 2 Solo IT requirements

The PromethION 2 (P2) Solo is a device which directly connects into a GridION Mk1 or a stand-alone computer that meets the miminum specifications for real-time data streaming and analysis. Up to two PromethION flow cells can be can be run and each is independently addressable, meaning experiments can be run concurrently or individually. For information on the computer IT requirements, please see the PromethION 2 Solo IT requirements document.

**Check your PromethION 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 PromethION flow cells. Oxford Nanopore Technologies will replace any flow cell with fewer than **5000 pores** 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.

**Software for nanopore sequencing**

### MinKNOW

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

**MinKNOW use**

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

### Guppy

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

**Guppy installation and use**

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

### EPI2ME (optional)

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

**EPI2ME installation and use**

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

# DNA repair and end-prep

~20 minutes

**Materials**

- 400 ng gDNA per barcode
- OR 1000 ng gDNA per sample if using ≤4 barcodes

- AMPure XP Beads (AXP)
- DNA Control Sample (DCS)

**Consumables**

- NEBNext FFPE DNA Repair Mix (NEB, cat # M6630)
- NEBNext Ultra II End Repair / dA-tailing Module (NEB, cat # E7546)
- Freshly prepared 80% ethanol in nuclease-free water
- 1.5 ml Eppendorf DNA LoBind tubes
- Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, cat # 0030129504) with heat seals
- OR 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Qubit™ Assay Tubes (Invitrogen, cat # Q32856)
- Qubit dsDNA HS Assay Kit (Invitrogen, cat # Q32851)

**Equipment**

- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips
- Multichannel pipette
- Thermal cycler at 20°C and 65°C
- Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, cat # 11766427)
- Microfuge
- Ice bucket with ice
- Magnetic rack suitable for 0.2 ml thin-walled PCR tubes or 96-well plates
- Vortex mixer
- Hula mixer
- Qubit fluorometer (or equivalent)

**TIP**

**For samples containing long gDNA fragments, we recommend using wide-bore pipette tips for the mixing steps to preserve the DNA length.**

**1 Thaw the AMPure XP Beads (AXP) and DNA Control Sample (DCS) at room temperature and mix by vortexing. Keep the beads at room temperature and store the DNA Control Sample (DCS) on ice.**

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

**IMPORTANT**

**Do not vortex the NEBNext FFPE DNA Repair Mix or NEBNext Ultra II End Prep Enzyme Mix.**

**IMPORTANT**

**It is important that the NEBNext FFPE DNA Repair Buffer and NEBNext Ultra II End Prep Reaction Buffer are mixed well by vortexing.**

Check for any visible precipitate; vortexing for at least 30 seconds may be required to solubilise any precipitate.

3 **Dilute your DNA Control Sample (DCS) by adding 105 µl Elution Buffer (EB) directly to one DCS tube. Mix gently by pipetting and spin down.**

One tube of diluted DNA Control Sample (DCS) is enough for 140 samples. Excess can be stored at -20°C in the freezer.

**TIP**

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

4 **In clean 0.2 ml thin-walled PCR tubes (or a clean 96-well plate), prepare your DNA samples:**

- For >4 barcodes, aliquot 400 ng per sample
- For ≤4 barcodes, aliquot 1000 ng per sample

5 **Make up each sample to 11 µl using nuclease-free water. Mix gently by pipetting and spin down.**

**TIP**

**We recommend making up a mastermix of the end-prep and DNA repair reagents for the total number of samples and adding 3 µl to each well.**

6 **Combine the following components per tube/well:**

Between each addition, pipette mix 10 - 20 times.

| Reagent | Volume |
|---|---|
| DNA sample | 11 µl |
| Diluted DNA Control Sample (DCS) | 1 µl |
| NEBNext FFPE DNA Repair Buffer | 0.875 µl |
| Ultra II End-prep Reaction Buffer | 0.875 µl |
| Ultra II End-prep Enzyme Mix | 0.75 µl |
| NEBNext FFPE DNA Repair Mix | 0.5 µl |
| **Total** | **15 µl** |

7 **Ensure the components are thoroughly mixed by pipetting and spin down in a centrifuge.**

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

9 **Transfer each sample into a clean 1.5 ml Eppendorf DNA LoBind tube.**

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

11 **Add 15 µl of resuspended AMPure XP Beads (AXP) to each end-prep reaction and mix by flicking the tube.**

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

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

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

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

If the pellet was disturbed, wait for beads to pellet again before removing the ethanol.

16 **Repeat the previous step.**

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

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

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

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

- Dispose of the pelleted beads

Quantify 1 µl of each eluted sample using a Qubit fluorometer.

**END OF STEP**

**Take forward an equimolar mass of samples to be barcoded and pooled forward 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

~60 minutes

**Materials**

- Native Barcodes (NB01-24)
- AMPure XP Beads (AXP)
- EDTA (EDTA)

**Consumables**

- NEB Blunt/TA Ligase Master Mix (NEB, cat # M0367)
- Freshly prepared 80% ethanol in nuclease-free water
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- 1.5 ml Eppendorf DNA LoBind tubes
- Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, cat # 0030129504) with heat seals
- OR 0.2 ml thin-walled PCR tubes
- Qubit™ Assay Tubes (Invitrogen, cat # Q32856)
- Qubit dsDNA HS Assay Kit (ThermoFisher, cat # Q32851)

**Equipment**

- Magnetic rack
- Vortex mixer
- Hula mixer (gentle rotator mixer)
- Microfuge
- Thermal cycler
- Ice bucket with ice
- Multichannel pipette
- P1000 pipette and tips

- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips
- Qubit fluorometer (or equivalent for QC check)

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

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

**2** **Thaw the EDTA at room temperature, mix by vortexing, spin down and place on ice.**

**3** **Thaw the Native Barcodes (NB01-24) required for your number of samples at room temperature. Individually mix the barcodes by pipetting, spin down, and place them on ice.**

**4** **Select a unique barcode for each sample to be run together on the same flow cell. Up to 24 samples can be barcoded and combined in one experiment.**

**Please note:** Only use one barcode per sample.

**5** **In clean 0.2 ml PCR-tubes or a 96-well plate, add the reagents in the following order per well:**

Between each addition, pipette mix 10 - 20 times.

| Reagent | Volume |
|---|---|
| End-prepped DNA | 7.5 µl |
| Native Barcode (NB01-24) | 2.5 µl |
| Blunt/TA Ligase Master Mix | 10 µl |
| **Total** | **20 µl** |

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

**7** **Incubate for 20 minutes at room temperature.**

**8** **Add 2 µl of EDTA to each well and mix thoroughly by pipetting and spin down briefly.**

> **TIP**
>
> **EDTA is added at this step to stop the reaction.**

**9 Pool all the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.**

| | Volume per sample | For 6 samples | For 12 samples | For 24 samples |
|---|---|---|---|---|
| Total volume | 22 µl | 132 µl | 264 µl | 528 µl |

**TIP**

**We recommend checking the base of your tubes/plate are all the same volume before pooling and after to ensure all the liquid has been taken forward.**

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

**11 Add AMPure XP Beads (AXP) to the pooled reaction, and mix by pipetting for a 0.4X clean.**

| | Volume per sample | For 6 samples | For 12 samples | For 24 samples |
|---|---|---|---|---|
| Volume of AXP | 9 µl | 53 µl | 106 µl | 211 µl |

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

**13 Prepare 2 ml of fresh 80% ethanol in nuclease-free water.**

**14 Spin down the sample and pellet on a magnet for 5 minutes. Keep the tube on the magnetic rack until the eluate is clear and colourless, and pipette off the supernatant.**

**15 Keep the tube on the magnetic rack and wash the beads with 700 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

If the pellet was disturbed, wait for beads to pellet again before removing the ethanol.

**16 Repeat the previous step.**

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

**18 Remove the tube from the magnetic rack and resuspend the pellet in 35 µl nuclease-free water by gently flicking.**

**19 Incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.**

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

**21 Remove and retain 35 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Quantify 1 µl of eluted sample using a Qubit fluorometer.

**END OF STEP**

**Take forward the barcoded DNA library to the adapter ligation and clean-up 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 (EB)
- Native Adapter (NA)
- AMPure XP Beads (AXP)

**Consumables**

- Quick T4 DNA Ligase in NEBNext® Quick Ligation Module (NEB, cat # E6056)
- NEBNext® Quick Ligation Reaction Buffer (NEB, cat # B6058)
- 1.5 ml Eppendorf DNA LoBind tubes
- Qubit™ Assay Tubes (Invitrogen, cat # Q32856)
- Qubit dsDNA HS Assay Kit (ThermoFisher, cat # Q32851)

**Equipment**

- Microfuge
- Magnetic rack
- Vortex mixer
- Hula mixer (gentle rotator mixer)
- Thermal cycler
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- Ice bucket with ice
- Qubit fluorometer (or equivalent for QC check)

**IMPORTANT**

**The Native Adapter (NA) used in this kit and protocol is not interchangeable with other sequencing adapters.**

1 **Prepare the NEBNext Quick Ligation Reaction Module according to the manufacturer's instructions, and place on ice:**

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

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

**IMPORTANT**

**Do not vortex the Quick T4 DNA Ligase.**

2 **Spin down the Native Adapter (NA) and Quick T4 DNA Ligase, pipette mix and place on ice.**

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

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

Between each addition, pipette mix 10 - 20 times.

| Reagent | Volume |
|---|---|
| Pooled barcoded sample | 30 µl |
| Native Adapter (NA) | 5 µl |
| NEBNext Quick Ligation Reaction Buffer (5X) | 10 µl |
| Quick T4 DNA Ligase | 5 µl |
| **Total** | **50 µl** |

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

7 **Incubate the reaction for 20 minutes at room temperature.**

> **IMPORTANT**
>
> **The next clean-up step uses Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) rather than 80% ethanol to wash the beads. The use of ethanol will be detrimental to the sequencing reaction.**

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

9 **Add 20 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting.**

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

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

12 **Wash the beads by adding either 125 µl Long Fragment Buffer (LFB) or 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 25 µl Elution Buffer (EB).**

16 **Spin down and incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.**

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

18 **Remove and retain 25 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Dispose of the pelleted beads

Quantify 1 µl of eluted sample using a Qubit fluorometer.

19 **Make up the library to 32 µl at 10-20 fmol, using Elution Buffer (EB).**

**IMPORTANT**

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

Loading more than 20 fmol of DNA can reduce the rate of duplex read capture.

**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.

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

Depending on how many flow cells the library will be split across, more Elution Buffer (EB) than what is supplied in the kit will be required.

# Priming and loading the PromethION flow cell

**Materials**

- Sequencing Buffer (SB)
- Library Beads (LIB)
- Library Solution (LIS)
- Flow Cell Tether (FCT)
- Flow Cell Flush (FCF)

**Consumables**

- PromethION Flow Cell
- 1.5 ml Eppendorf DNA LoBind tubes

**Equipment**

- PromethION 2 Solo device
- PromethION 24/48 device
- P1000 pipette and tips
- P200 pipette and tips
- P20 pipette and tips

**IMPORTANT**

**This kit is only compatible with R10.4.1 flow cells (FLO-PRO114M).**

### Using the Library Solution

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

**Note:** Some customers have noticed that viscous libraries can be loaded more easily when not using Library Beads (LIB).

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

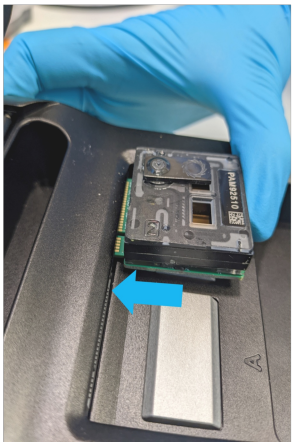
2. **To prepare the flow cell priming mix, add 30 µl of thawed and mixed Flow Cell Tether (FCT) directly to the tube of thawed and mixed Flow Cell Flush (FCF), and mix by vortexing at room temperature.**

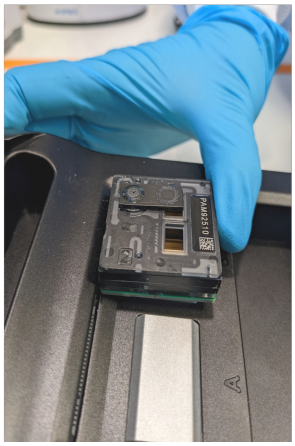
> **IMPORTANT**
>
> **After taking flow cells out of the fridge, wait 20 minutes before inserting the flow cell into the PromethION for the flow cell to come to room temperature. Condensation can form on the flow cell in humid environments. Inspect the gold connector pins on the top and underside of the flow cell for condensation and wipe off with a Kimwipe if any is observed. Ensure the heat pad (black pad) is present on the underside of the flow cell.**

**3 For PromethION P2 Solo, load the flow cell(s) as follows:**

1. Place the flow cell flat on the thermoelectrical control plate

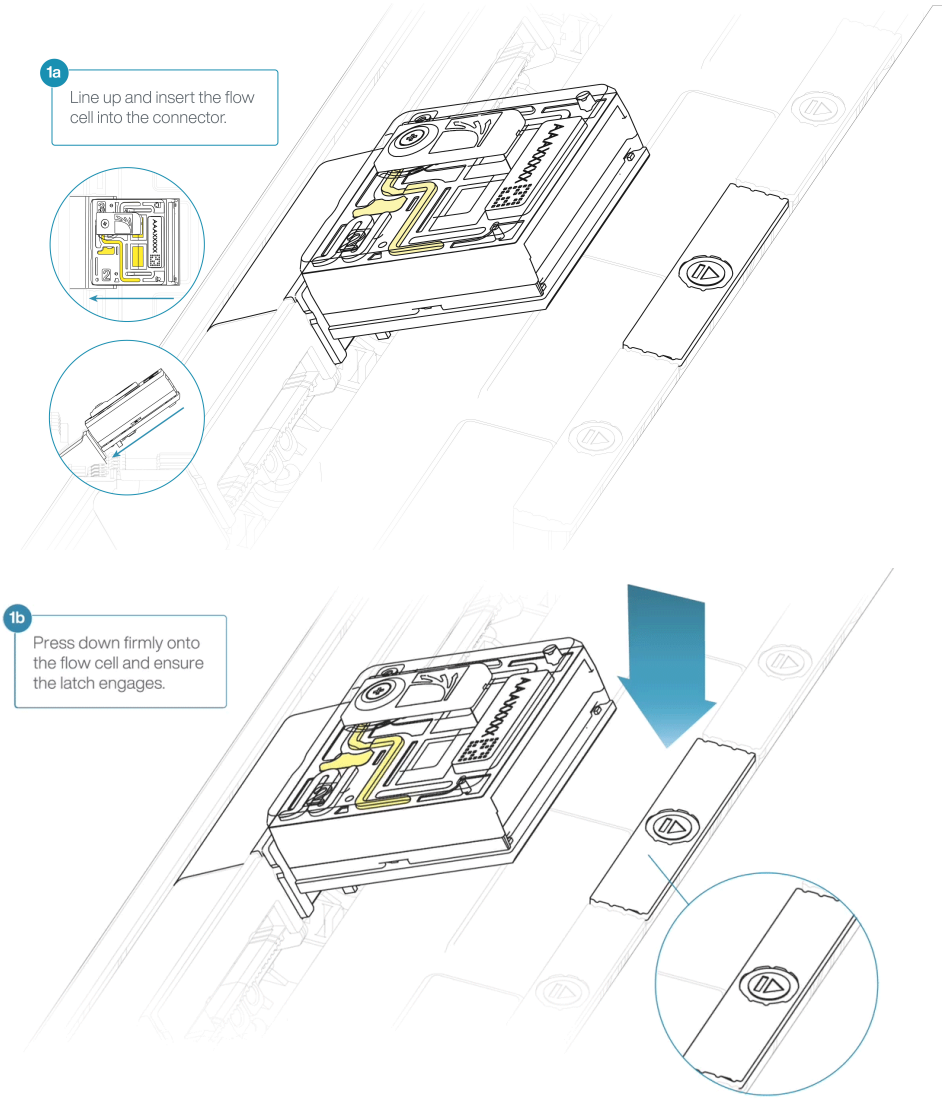
2. Slide the flow cell into the docking port until the gold pins or green board cannot be seen





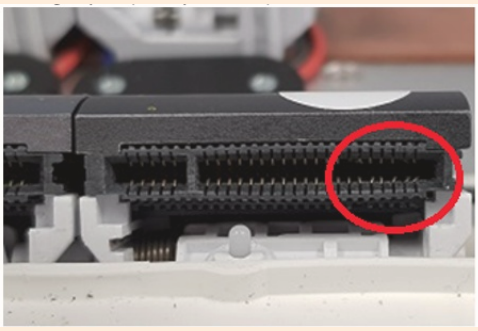
**4 For the PromethION 24/48, load the flow cell(s) into the docking ports:**

1. Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position.
2. Press down firmly onto the flow cell and ensure the latch engages and clicks into place.

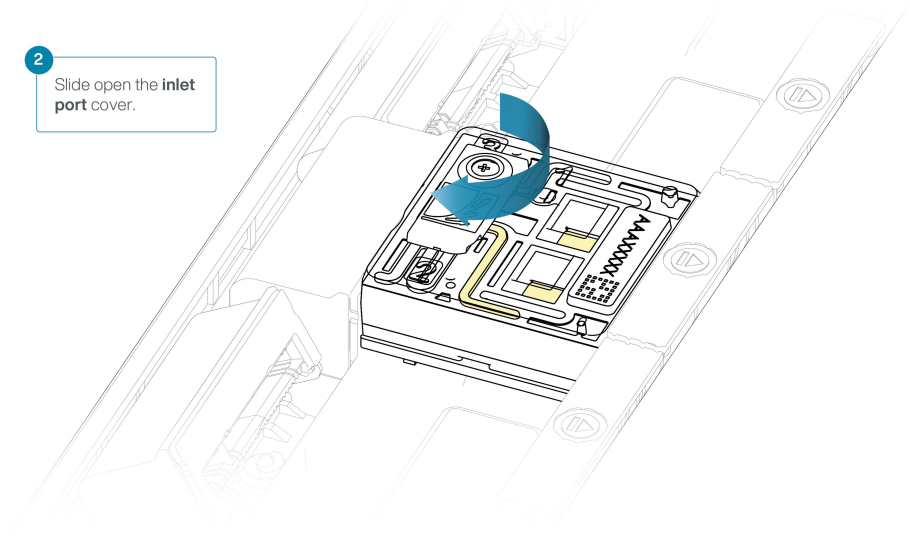


**IMPORTANT**

**Insertion of the flow cells at the wrong angle can cause damage to the pins on the PromethION and affect your sequencing results. If you find the pins on a PromethION position are damaged, please contact support@nanoporetech.com for assistance.**



5 **Turn the valve clockwise to expose the inlet port.**

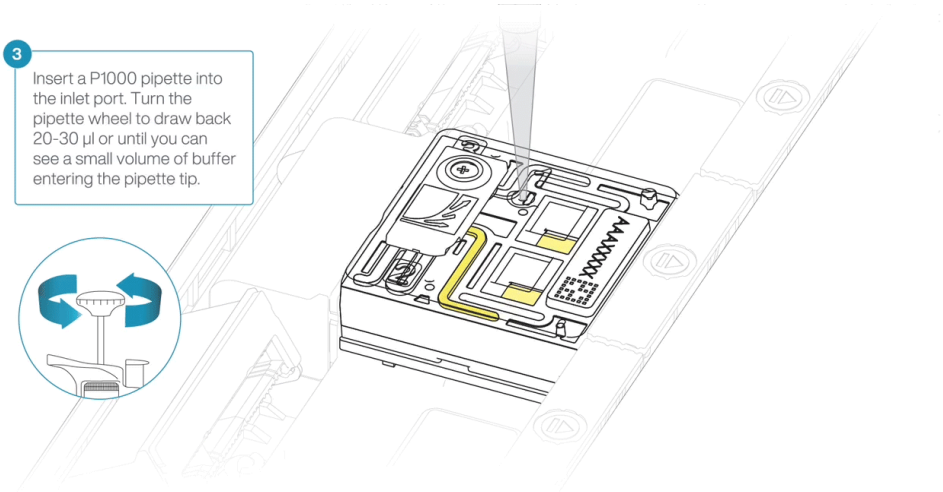



**IMPORTANT**

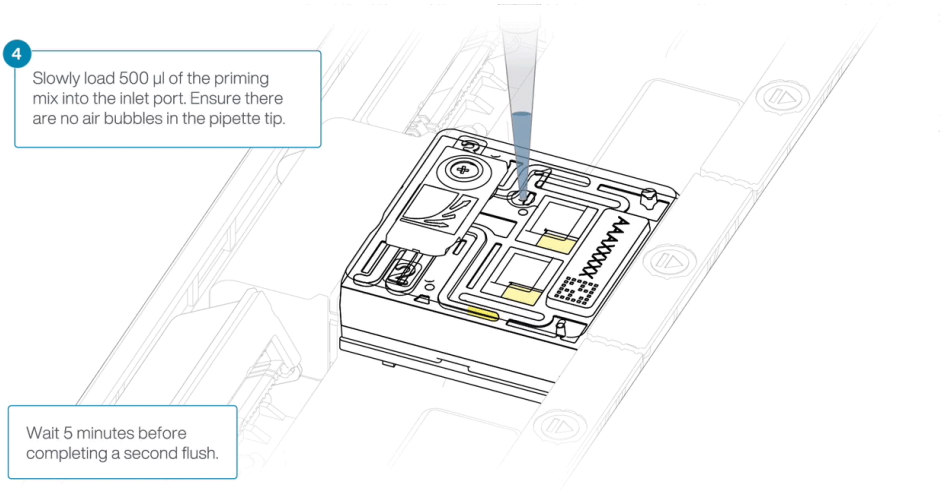
**Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.**

6 **After opening the inlet port, draw back a small volume to remove any air bubbles:**

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




7 **Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes. During this time, prepare the library for loading using the next steps in the protocol.**




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

**IMPORTANT**

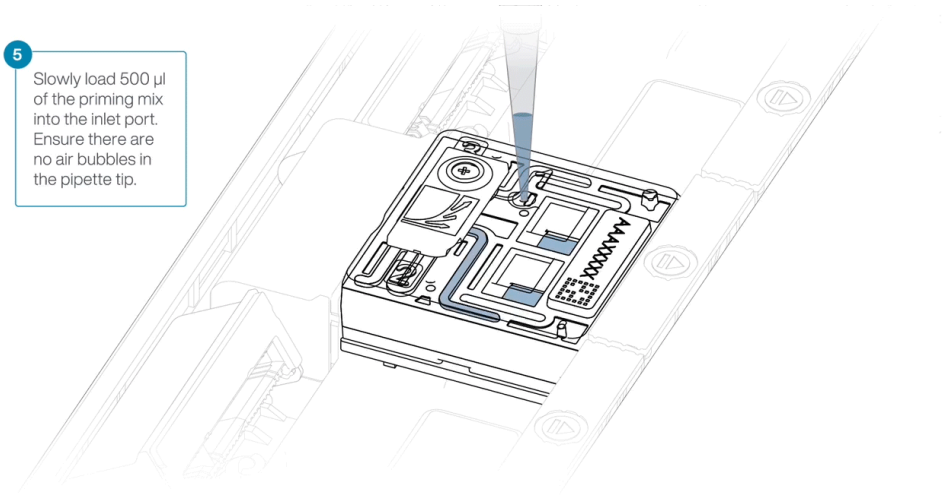
**The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.**

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

| Reagent | Volume per flow cell |
|---|---|
| Sequencing Buffer (SB) | 100 µl |
| Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS), if using | 68 µl |
| DNA library | 32 µl |
| **Total** | **200 µl** |

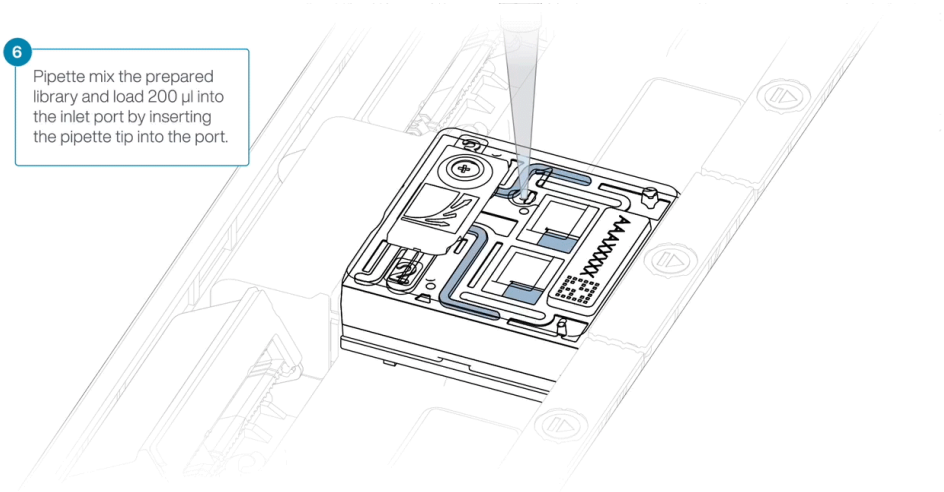
**Note:** Library loading volume has been increased to improve array coverage.

**10 Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port.**



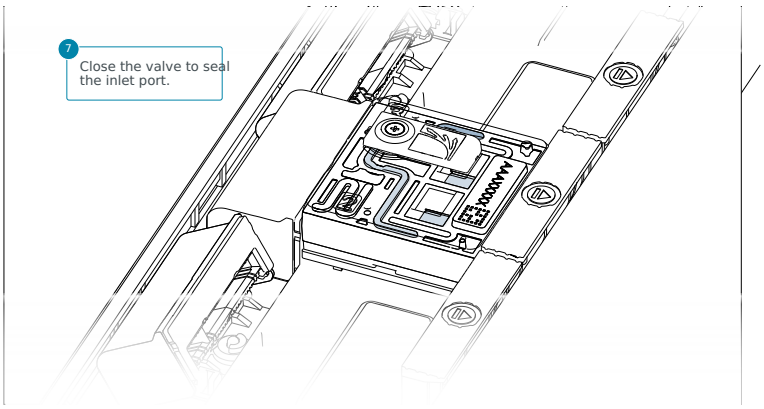

**11 Mix the prepared library gently by pipetting up and down just prior to loading.**

**12 Using a P1000, insert the pipette tip into the inlet port and load 200 µl of library.**




**13 Close the valve to seal the inlet port and close the PromethION lid when ready.**

Wait a minimum of 10 minutes after loading the flow cells onto the PromethION before initiating any experiments. This will help to increase the sequencing output.




# Data acquisition and basecalling

### Overview of nanopore data analysis

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

### How to start sequencing

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

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

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

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

Follow the instructions in the GridION user manual.

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

Follow the instructions in the MinION Mk1C user manual.

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

Follow the instructions in the PromethION user manual or the PromethION 2 Solo user manual.

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

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

# Downstream analysis

**Post-basecalling analysis**

There are several options for further analysing your basecalled data:

### 1. EPI2ME platform

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

### 2. EPI2ME Labs tutorials and workflows

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

### 3. Research analysis tools

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

### 4. Community-developed analysis tools

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

# Ending the experiment

**Materials**

- Flow Cell Wash Kit (EXP-WSH004)

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

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

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

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

Instructions for returning flow cells can be foundhere.

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

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

# Issues during DNA/RNA extraction and library preparation for Kit 14

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

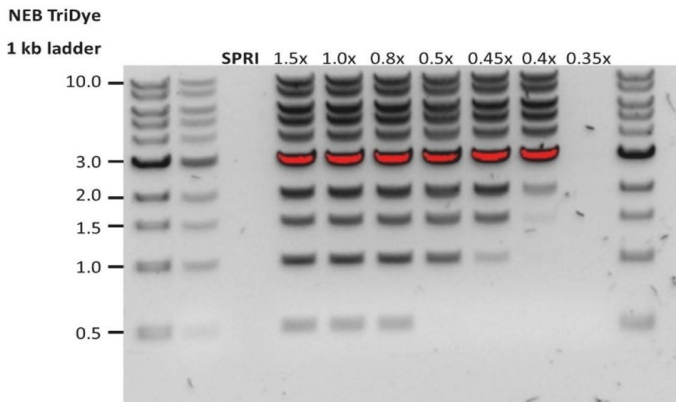
We also have an FAQ section available on the Nanopore Community Support section.

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.<br><br>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.<br><br>We recommend working in an RNase-free environment, and to keep your lab equipment RNase-free when working with RNA. |

### Low DNA recovery after AMPure bead clean-up

| Observation | Possible cause | Comments and actions |
|---|---|---|
| **Low recovery** | DNA loss due to a lower than intended AMPure beads-to-sample ratio | 1. AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample.<br><br>2. When the AMPure beads-to-sample ratio is lower than 0.4:1, DNA fragments of any size will be lost during the clean-up. |
| **Low recovery** | DNA fragments are shorter than expected | The lower the AMPure beads-to-sample ratio, the more stringent the selection against short fragments. Please always determine the input DNA length on an agarose gel (or other gel electrophoresis methods) and then calculate the appropriate amount of AMPure beads to use.<br>  |

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

# Issues during the sequencing run for Kit 14

**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

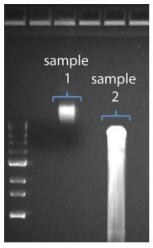
| Observation | Possible cause | Comments and actions |
|---|---|---|
| **MinKNOW shows "Script failed"** | | Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support. |

### Pore occupancy below 40%

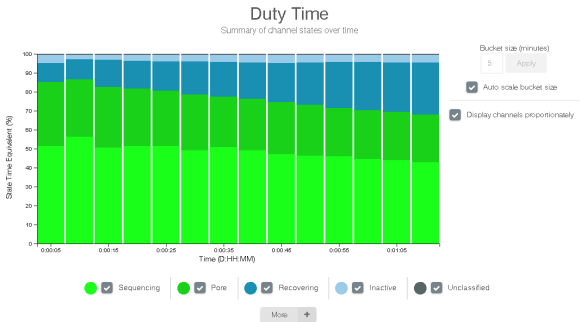
| Observation | Possible cause | Comments and actions |
|---|---|---|
| **Pore occupancy <40%** | Not enough library was loaded on the flow cell | 10–20 fmol of good quality library can be loaded on to a MinION/GridION flow cell. Please quantify the library before loading and calculate mols using tools like the Promega Biomath Calculator, choosing "dsDNA: µg to pmol" |
| **Pore occupancy close to 0** | The Native Barcoding 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 (FCT tube). Make sure FCT was added to FCF before priming. |

### Shorter than expected read length

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

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

**Large proportion of recovering pores**

| Observation | Possible cause | Comments and actions |
|---|---|---|
| **Large proportion of recovering pores (shown as dark blue in the channels panel and duty time plot)** | Contaminants are present in the sample | Some contaminants can be cleared from the pores by the unblocking function built into MinKNOW. If this is successful, the pore status will change to "single pores". If the portion of recovering pores (unavailable pores in the extended view) stays large or increases:<br><br>1. A nuclease flush using the Flow Cell Wash Kit (EXP-WSH004) can be performed, or<br>2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.<br><br>  <br>The duty time plot above shows an increasing proportion of "recovering" pores over the course of a sequencing experiment |

**Large proportion of inactive pores**

| Observation | Possible cause | Comments and actions |
|---|---|---|
| **Large proportion of inactive pores (shown as light blue in the channels panel and duty time plot. Pores or membranes are irreversibly damaged)** | Air bubbles have been introduced into the flow cell | Air bubbles introduced through flow cell priming and library loading can irreversibly damage the pores. Watch the Priming and loading your flow cell video for best practice |
| **Large proportion of inactive pores** | Certain compounds co-purified with DNA | Known compounds, include polysaccharides, typically associate with plant genomic DNA.<br><br>1. Please refer to the Plant leaf DNA extraction method.<br>2. Clean-up using the QIAGEN PowerClean Pro kit.<br>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 (~10–20 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"** | The instrument was placed in a location that is colder than normal room temperature, or a location with poor ventilation (which leads to the flow cells overheating) | MinKNOW has a default timeframe for the flow cell to reach the target temperature. Once the timeframe is exceeded, an error message will appear and the sequencing experiment will continue. However, sequencing at an incorrect temperature may lead to a decrease in throughput and lower q-scores. Please adjust the location of the sequencing device to ensure that it is placed at room temperature with good ventilation, then re-start the process in MinKNOW. Please refer to this FAQ for more information on MinION Mk 1B temperature control. |

**Guppy – no input .fast5 was found or basecalled**

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

**Guppy – no Pass or Fail folders were generated after basecalling**

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

**Guppy – unusually slow processing on a GPU computer**

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