Overview of the protocol

IMPORTANT

This is a Legacy product

This kit has now been discontinued and we recommend all customers upgrade to the latest chemistry for their relevant kit which is available on the Store. For further information on please see the product update page.

Native Barcoding Kit 96 features

This kit is recommended for users who:

- wish to multiplex up to 96 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 accuracy
- require control over read length
- would like to utilise upstream processes such as size selection or whole genome amplification

IMPORTANT

Kit 12 chemistry device and informatics info sheet

The Kit 12 chemistry is a new development from Oxford Nanopore Technologies, the data acquisition, basecalling and analysis will require a different set of tools to the typical sequencing run. This is decribed in more detail in the Kit 12 chemistry device and informatics info sheet. We strongly recommend that you read it before proceeding with Kit 12 chemistry sequencing experiments.

Introduction to the Native Barcoding Kit 96 protocol

This protocol describes how to carry out native barcoding of amplicons using the Native Barcoding Kit 96 (SQK-NBD112.96). There are 96 unique barcodes available, allowing the user to pool up to 96 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

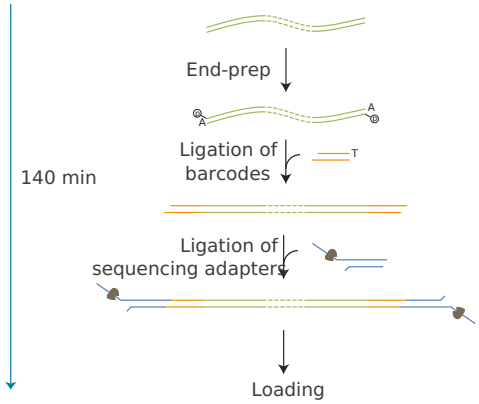
You will need to:

- 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-NBD112.96 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

Compatibility of this protocol

This protocol should only be used in combination with:

- Native Barcoding Kit 96 (SQK-NBD112.96)
- R10.4 flow cells (FLO-MIN112)
- R9.4.1 flow cells (FLO-MIN106)
- Flow Cell Wash Kit (EXP-WSH004)
- Native Barcoding Expansion Kit (EXP-NBD112)

Equipment and consumables

Materials	• Native Barcoding Kit 96 (SQK-NBD112.96) • 200 fmol (130 ng for 1 kb amplicons) DNA per sample to be barcoded
Consumables	• NEB Blunt/TA Ligase Master Mix (NEB, M0367) • NEBNext® Ultra™ II End Repair/dA-Tailing Module (E7546) • NEBNext Quick Ligation Module (NEB, 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 70% ethanol in nuclease-free water • Qubit™ Assay Tubes (Invitrogen, 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) • Magnetic rack • 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 • 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)

IMPORTANT

The Kit 12 chemistry runs at 30°C on nanopore sequencing devices. This is several degrees cooler than other chemistries. While the protocol was initially developed on GridION and PromethION, we also support its use on MinION Mk1C, as the MinION Mk1C device's temperature control allows the flow cell to be maintained at 30°C for the duration of the run. However, we cannot guarantee the same level of temperature control on the MinION Mk1B. Therefore, if you are running Kit 12 chemistry on the MinION Mk1B, ensure that the ambient temperature does not exceed 23°C.

For this protocol, we recommend using 200 fmol (130 ng for 1 kb amplicons) DNA per sample to be barcoded.

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

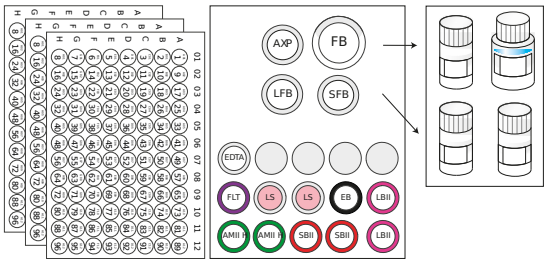
Depending on how the DNA is extracted from the raw sample, certain chemical contaminants may remain in the purified DNA, which can affect library preparation efficiency and sequencing quality. Read more about contaminants on the Contaminants page of the Community.

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.

Native Barcoding Kit 96 (SQK-NBD112.96) contents



AXP: AMPure XP Beads
FB: Flush Buffer
LFB: Long Fragment Buffer
SFB: Short Fragment Buffer
EDTA: EDTA
LBII: Loading Beads II
EB: Elution Buffer
LS: Loading Solution
FLT: Flush Tether
SBII: Sequencing Buffer II
AMII H: Adapter Mix II H

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Rapid Barcode plate	RB96	-	3 plates	8 µl per well
Adapter Mix II H	AMII H	Green	2	40
Sequencing Buffer II	SBII	Red	2	500
Loading Beads II	LBII	Pink	2	360
Loading Solution	LS	White cap, pink label	2	400
Elution Buffer	EB	Black	1	500
AMPure XP Beads	AXP	White	1	6,000
Long Fragment Buffer	LFB	White	1	7,500
Short Fragment Buffer	SFB	White	1	7,500

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
EDTA	EDTA	Clear	1	700
Flush Buffer	FB	White	1	15,500
Flush Tether	FLT	Purple	1	400

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.

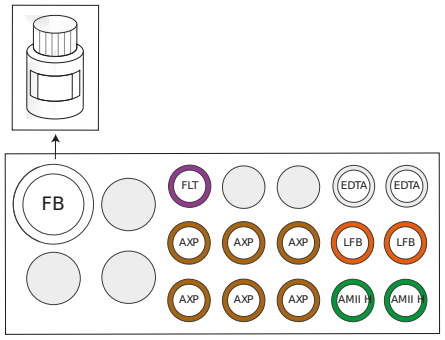
The barcodes are orientated in columns in the barcode plates:

	01	02	03	04	05	06	07	08	09	10	11	12
A	NB01	NB09	NB17	NB25	NB33	NB41	NB49	NB57	NB65	NB73	NB81	NB89
B	NB02	NB10	NB18	NB26	NB34	NB42	NB50	NB58	NB66	NB74	NB82	NB90
C	NB03	NB11	NB19	NB27	NB35	NB43	NB51	NB59	NB67	NB75	NB83	NB91
D	NB04	NB12	NB20	NB28	NB36	NB44	NB52	NB60	NB68	NB76	NB84	NB92
E	NB05	NB13	NB21	NB29	NB37	NB45	NB53	NB61	NB69	NB77	NB85	NB93
F	NB06	NB14	NB22	NB30	NB38	NB46	NB54	NB62	NB70	NB78	NB86	NB94
G	NB07	NB15	NB23	NB31	NB39	NB47	NB55	NB63	NB71	NB79	NB87	NB95
H	NB08	NB16	NB24	NB32	NB40	NB48	NB56	NB64	NB72	NB80	NB88	NB96

The Native Barcoding Expansion kit (EXP-NBD112) is available to provide enough reagents for 12 reactions depending on how barcodes are used.

This kit contains reagents used in the Native Barcoding Kit 24 (SQK-NBD112.24) and the Native Barcoding Kit 96 (SQK-NBD112.96).

Native Barcoding Expansion Kit (EXP-NBD112) contents:



FB: Flush Buffer
FLT: Flush Tether
AXP: AMPure XP Beads
LFB: Long Fragment Buffer
EDTA: EDTA
AMII H: Adapter Mix II H

Reagent	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Adapter II H	AMII H	Green	2	40
AMPure XP Beads	AXP	Amber	6	1,200
EDTA	EDTA	Clear	2	700
Long Fragment Buffer	LFB	Orange	2	1,800

Reagent	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Flush Buffer	FB	Bottle	1	15,500
Flush Tether	FLT	Purple	1	400

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.

IMPORTANT

Adapter Mix II H (AMII H) used in this kit and protocol is not interchangeable with other sequencing adapters.

This kit and protocol is only compatible with Adapter Mix II H (AMII H). If more sequencing adapter is required, the Native Barcoding Expansion (EXP-NBD112) is available for additional reagents.

Adapter Mix II H (AMII H) is a new sequencing adapter for Kit 12 chemistry and is loaded with an updated sequencing enzyme with improved accuracies and has a higher capture rate to lower flow cell loading amounts. It also contains the fuel fix technology to enable longer runs without the need for fuel addition during a run. Therefore, sequencing adapters from other kits are not compatible with this kit or protocol.

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

Component	Forward sequence	Reverse sequence
NB19	GTTCCTCGTGCAGTGTCAAGAGAT	ATCTCTTGACACTGCACGAGGAAC
NB20	TTGCGTCCTGTTACGAGAACTCAT	ATGAGTTCTCGTAACAGGACGCAA
NB21	GAGCCTCTCATTGTCCGTTCTCTA	TAGAGAACGGACAATGAGAGGCTC
NB22	ACCACTGCCATGTATCAAAGTACG	CGTACTTTGATACATGGCAGTGGT
NB23	CTTACTACCCAGTGAACCTCCTCG	CGAGGAGGTTCACTGGGTAGTAAG
NB24	GCATAGTTCTGCATGATGGGTTAG	CTAACCCATCATGCAGAACTATGC
NB25	GTAAGTTGGGTATGCAACGCAATG	CATTGCGTTGCATACCCAACTTAC
NB26	CATACAGCGACTACGCATTCTCAT	ATGAGAATGCGTAGTCGCTGTATG
NB27	CGACGGTTAGATTCACCTCTTACA	TGTAAGAGGTGAATCTAACCGTCG
NB28	TGAAACCTAAGAAGGCACCGTATC	GATACGGTGCCTTCTTAGGTTTCA
NB29	CTAGACACCTTGGGTTGACAGACC	GGTCTGTCAACCCAAGGTGTCTAG
NB30	TCAGTGAGGATCTACTTCGACCCA	TGGGTCGAAGTAGATCCTCACTGA
NB31	TGCGTACAGCAATCAGTTACATTG	CAATGTAACTGATTGCTGTACGCA
NB32	CCAGTAGAAGTCCGACAACGTCAT	ATGACGTTGTCGGACTTCTACTGG
NB33	CAGACTTGGTACGGTTGGGTAACT	AGTTACCCAACCGTACCAAGTCTG
NB34	GGACGAAGAACTCAAGTCAAAGGC	GCCTTTGACTTGAGTTCTTCGTCC
NB35	CTACTTACGAAGCTGAGGGACTGC	GCAGTCCCTCAGCTTCGTAAGTAG
NB36	ATGTCCCAGTTAGAGGAGGAAACA	TGTTTCCTCCTCTAACTGGGACAT
NB37	GCTTGCGATTGATGCTTAGTATCA	TGATACTAAGCATCAATCGCAAGC
NB38	ACCACAGGAGGACGATACAGAGAA	TTCTCTGTATCGTCCTCCTGTGGT
NB39	CCACAGTGTCAACTAGAGCCTCTC	GAGAGGCTCTAGTTGACACTGTGG
NB40	TAGTTTGGATGACCAAGGATAGCC	GGCTATCCTTGGTCATCCAAACTA
NB41	GGAGTTCGTCCAGAGAAGTACACG	CGTGTACTTCTCTGGACGAACTCC
NB42	CTACGTGTAAGGCATACCTGCCAG	CTGGCAGGTATGCCTTACACGTAG
NB43	CTTTCGTTGTTGACTCGACGGTAG	CTACCGTCGAGTCAACAACGAAAG
NB44	AGTAGAAAGGGTTCCTTCCCACTC	GAGTGGGAAGGAACCCTTTCTACT
NB45	GATCCAACAGAGATGCCTTCAGTG	CACTGAAGGCATCTCTGTTGGATC
NB46	GCTGTGTTCCACTTCATTCTCCTG	CAGGAGAATGAAGTGGAACACAGC
NB47	GTGCAACTTTCCCACAGGTAGTTC	GAACTACCTGTGGGAAAGTTGCAC
NB48	CATCTGGAACGTGGTACACCTGTA	TACAGGTGTACCACGTTCCAGATG
NB49	ACTGGTGCAGCTTTGAACATCTAG	CTAGATGTTCAAAGCTGCACCAGT
NB50	ATGGACTTTGGTAACTTCCTGCGT	ACGCAGGAAGTTACCAAAGTCCAT
NB51	GTTGAATGAGCCTACTGGGTCCTC	GAGGACCCAGTAGGCTCATTCAAC
NB52	TGAGAGACAAGATTGTTCGTGGAC	GTCCACGAACAATCTTGTCTCTCA

Component	Forward sequence	Reverse sequence
NB53	AGATTCAGACCGTCTCATGCAAAG	CTTTGCATGAGACGGTCTGAATCT
NB54	CAAGAGCTTTGACTAAGGAGCATG	CATGCTCCTTAGTCAAAGCTCTTG
NB55	TGGAAGATGAGACCCTGATCTACG	CGTAGATCAGGGTCTCATCTTCCA
NB56	TCACTACTCAACAGGTGGCATGAA	TTCATGCCACCTGTTGAGTAGTGA
NB57	GCTAGGTCAATCTCCTTCGGAAGT	ACTTCCGAAGGAGATTGACCTAGC
NB58	CAGGTTACTCCTCCGTGAGTCTGA	TCAGACTCACGGAGGAGTAACCTG
NB59	TCAATCAAGAAGGGAAAGCAAGGT	ACCTTGCTTTCCCTTCTTGATTGA
NB60	CATGTTCAACCAAGGCTTCTATGG	CCATAGAAGCCTTGGTTGAACATG
NB61	AGAGGGTACTATGTGCCTCAGCAC	GTGCTGAGGCACATAGTACCCTCT
NB62	CACCCACACTTACTTCAGGACGTA	TACGTCCTGAAGTAAGTGTGGGTG
NB63	TTCTGAAGTTCCTGGGTCTTGAAC	GTTCAAGACCCAGGAACTTCAGAA
NB64	GACAGACACCGTTCATCGACTTTC	GAAAGTCGATGAACGGTGTCTGTC
NB65	TTCTCAGTCTTCCTCCAGACAAGG	CCTTGTCTGGAGGAAGACTGAGAA
NB66	CCGATCCTTGTGGCTTCTAACTTC	GAAGTTAGAAGCCACAAGGATCGG
NB67	GTTTGTCATACTCGTGTGCTCACC	GGTGAGCACACGAGTATGACAAAC
NB68	GAATCTAAGCAAACACGAAGGTGG	CCACCTTCGTGTTTGCTTAGATTC
NB69	TACAGTCCGAGCCTCATGTGATCT	AGATCACATGAGGCTCGGACTGTA
NB70	ACCGAGATCCTACGAATGGAGTGT	ACACTCCATTCGTAGGATCTCGGT
NB71	CCTGGGAGCATCAGGTAGTAACAG	CTGTTACTACCTGATGCTCCCAGG
NB72	TAGCTGACTGTCTTCCATACCGAC	GTCGGTATGGAAGACAGTCAGCTA
NB73	AAGAAACAGGATGACAGAACCCTC	GAGGGTTCTGTCATCCTGTTTCTT
NB74	TACAAGCATCCCAACACTTCCACT	AGTGGAAGTGTTGGGATGCTTGTA
NB75	GACCATTGTGATGAACCCTGTTGT	ACAACAGGGTTCATCACAATGGTC
NB76	ATGCTTGTTACATCAACCCTGGAC	GTCCAGGGTTGATGTAACAAGCAT
NB77	CGACCTGTTTCTCAGGGATACAAC	GTTGTATCCCTGAGAAACAGGTCG
NB78	AACAACCGAACCTTTGAATCAGAA	TTCTGATTCAAAGGTTCGGTTGTT
NB79	TCTCGGAGATAGTTCTCACTGCTG	CAGCAGTGAGAACTATCTCCGAGA
NB80	CGGATGAACATAGGATAGCGATTC	GAATCGCTATCCTATGTTCATCCG
NB81	CCTCATCTTGTGAAGTTGTTTCGG	CCGAAACAACTTCACAAGATGAGG
NB82	ACGGTATGTCGAGTTCCAGGACTA	TAGTCCTGGAACTCGACATACCGT
NB83	TGGCTTGATCTAGGTAAGGTCGAA	TTCGACCTTACCTAGATCAAGCCA
NB84	GTAGTGGACCTAGAACCTGTGCCA	TGGCACAGGTTCTAGGTCCACTAC
NB85	AACGGAGGAGTTAGTTGGATGATC	GATCATCCAACTAACTCCTCCGTT
NB86	AGGTGATCCCAACAAGCGTAAGTA	TACTTACGCTTGTTGGGATCACCT

Component	Forward sequence	Reverse sequence
NB87	TACATGCTCCTGTTGTTAGGGAGG	CCTCCCTAACAACAGGAGCATGTA
NB88	TCTTCTACTACCGATCCGAAGCAG	CTGCTTCGGATCGGTAGTAGAAGA
NB89	ACAGCATCAATGTTTGGCTAGTTG	CAACTAGCCAAACATTGATGCTGT
NB90	GATGTAGAGGGTACGGTTTGAGGC	GCCTCAAACCGTACCCTCTACATC
NB91	GGCTCCATAGGAACTCACGCTACT	AGTAGCGTGAGTTCCTATGGAGCC
NB92	TTGTGAGTGGAAAGATACAGGACC	GGTCCTGTATCTTTCCACTCACAA
NB93	AGTTTCCATCACTTCAGACTTGGG	CCCAAGTCTGAAGTGATGGAAACT
NB94	GATTGTCCTCAAACTGCCACCTAC	GTAGGTGGCAGTTTGAGGACAATC
NB95	CCTGTCTGGAAGAAGAATGGACTT	AAGTCCATTCTTCTTCCAGACAGG
NB96	CTGAACGGTCATAGAGTCCACCAT	ATGGTGGACTCTATGACCGTTCAG

Computer requirements and software

MinION Mk1C IT requirements

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

MinION Mk1B IT requirements

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

Check your flow cell

We highly recommend that you check the number of pores in your flow cell prior to starting a sequencing experiment. This should be done within three months of purchasing for MinION/GridION/PromethION flow cells, or within four weeks of purchasing for Flongle flow cells. Oxford Nanopore Technologies will replace any flow cell with fewer than the number of pores in the table below, when the result is reported within two days of performing the flow cell check, and when the storage recommendations have been followed. To do the flow cell check, please follow the instructions in the Flow Cell Check document.

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

End-prep

~20 minutes

Materials	• 200 fmol (130 ng for 1 kb amplicons) DNA per sample to be barcoded
Consumables	• Nuclease-free water (e.g. ThermoFisher, AM9937) • NEBNext Ultra II End Repair / dA-tailing Module (NEB, E7546) • 1.5 ml Eppendorf DNA LoBind tubes • Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, cat # 0030129504) with heat seals
Equipment	• Multichannel pipette • P1000 pipette and tips • P100 pipette and tips • P10 pipette and tips • Thermal cycler at 20°C and 65°C • Microfuge • Ice bucket with ice

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

For optimal perfomance, NEB recommend the following:

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

IMPORTANT

Do not vortex the NEBNext Ultra II End Prep Enzyme Mix.

IMPORTANT

It is important that the NEBNext Ultra II End Prep Reaction Buffer is mixed well by vortexing.

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

2 **In a clean 96-well plate, aliquot 200 fmol (130 ng for 1 kb amplicons) of DNA per sample.**

3 **Make up each sample to 12.5 µl using nuclease-free water. Mix gently by pipetting and spin down.**

4 **Combine the following components per sample:**

Between each addition, pipette mix 10 - 20 times.

Reagent	Volume
Ultra II End-prep reaction buffer	1.75 µl
Ultra II End-prep enzyme mix	0.75 µl
Total	**2.5 µl**

TIP

We recommend making up a mastermix for the total number of samples and adding 2.5 µl to each sample.

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

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

END OF STEP

Take forward the end-prepped DNA into the native barcode ligation step.

If users want to pause the library preparation here, we recommend cleaning up your sample with 1X AMPure XP Beads (AXP) and eluting in nuclease-free water before storing at 4°C.

Please note, extra AMPure XP Beads (AXP) will be required for this optional step.

Native barcode ligation

~60 minutes

Materials

- Native Barcodes (NB01-NB96)
- AMPure XP Beads (AXP)
- EDTA (EDTA)

Consumables

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

	• 2 ml Eppendorf DNA LoBind tubes • Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Eppendorf™, cat # 0030129504) with heat seals • Qubit™ Assay Tubes (Invitrogen, Q32856) • Qubit dsDNA HS Assay Kit (ThermoFisher, cat # Q32851)
Equipment	• Thermal cycler • Hula mixer (gentle rotator mixer) • Magnetic rack • 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 **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 AMPure XP Beads (AXP) at room temperature and mix by vortexing. Keep the beads at room temperature.**

4 **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.**

5 **Thaw the tube of Short Fragment Buffer (SFB) at room temperature, mix by vortexing, spin down and place on ice.**

6 **Select a unique barcode for every sample to be run.**

7 In a new 96-well plate, add the reagents in the order given below per well:

Mix well by pipetting and spin down in a centrifuge.

Reagent	Volume
Nuclease-free water	3 µl
End-prepped DNA	0.75 µl
Native Barcode	1.25 µl
Blunt/TA Ligase Master Mix	5 µl
Total	**10 µl**

8 Mix contents thoroughly by pipetting and spin down briefly.

9 Incubate for 20 minutes at room temperature.

10 Add 1 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.**

11 Pool the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.

We expect to have about ~10 µl per sample.

	x24 samples	x48 samples	x96 samples
Total volume	~240 µl	~480 µl	~960 µl

12 Resuspend the AMPure XP Beads (AXP) by vortexing.

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

	Volume per sample	For 24 samples	For 48 samples	For 96 samples
Volume of AXP	4 µl	96 µl	192 µl	384 µl

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

15 Prepare 2 ml of fresh 70% ethanol in nuclease-free water.

16 **Spin down the sample and pellet the beads on a magnet for 5 mins. Keep the tube on the magnet until the eluate is clear and colourless, and pipette off the supernatant.**

17 **Keep the tube on the magnetic rack and wash the beads with 700 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

18 **Repeat the previous step.**

19 **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.**

20 **Remove the tube from the magnetic rack and resuspend the pellet in 35 µl nuclease-free water.**

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

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

23 **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 - recovery aim 2 ng/µl.

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)
- Adapter Mix II H (AMII H)
- 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, 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
Optional Equipment	• Qubit fluorometer (or equivalent for QC check)

IMPORTANT

Adapter Mix II H (AMII H) used in this kit and protocol is not interchangeable with other sequencing adapters.

This kit and protocol is only compatible with Adapter Mix II H (AMII H). If more sequencing adapter is required, the Native Barcoding Expansion (EXP-NBD112) is available for additional reagents.

Adapter Mix II H (AMII H) is a new sequencing adapter for Kit 12 chemistry and is loaded with an updated sequencing enzyme with improved accuracies and has a higher capture rate to lower flow cell loading amounts. It also contains the fuel fix technology to enable longer runs without the need for fuel addition during a run. Therefore, sequencing adapters from other kits are not compatible with this kit or protocol.

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 Adapter Mix II (AMII H), 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
Adapter Mix II H (AMII H)	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 70% 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 15 µl Elution Buffer (EB).**

16 **Spin down and incubate for 10 mins at 37°C. Periodically agitate the sample by gently flicking 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 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Dispose of the pelleted beads

Quantify 1 µl of eluted sample using a Qubit fluorometer.

IMPORTANT

We recommend loading 2 - 5 ng of this final prepared library onto the flow cell.

If required, dilute the library in Elution Buffer (EB) to make up the final volume of 12 µl. Loading more than 20 fmol of DNA can reduce the rate of duplex read capture. The loading concentration is the same across both R9.4.1 and R10.4 flow cells.

The new sequencing adapter used in Kit 12 chemistry has a higher capture rate, enabling lower flow cell loading amounts to give optimal pore occupancy.

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.

Optional Action
If quantities allow, the libraries 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-AUX002), available to purchase separately. This expansion also contains additional vials of Sequencing Buffer (SBII) and Loading Beads (LBII), required for loading the libraries onto flow cells.

Priming and loading the SpotON flow cell

~10 minutes

Materials	• Flush Buffer (FB) • Flush Tether (FLT) • Loading Beads II (LBII) • Sequencing Buffer II (SBII) • Loading Solution (LS)
Consumables	• 1.5 ml Eppendorf DNA LoBind tubes • Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
Equipment	• SpotON Flow Cell • P1000 pipette and tips • P100 pipette and tips • P20 pipette and tips • P10 pipette and tips

IMPORTANT

The Kit 12 chemistry runs at 30°C on nanopore sequencing devices. This is several degrees cooler than other chemistries. While the protocol was initially developed on GridION and PromethION, we also support its use on MinION Mk1C, as the MinION Mk1C device's temperature control allows the flow cell to be maintained at 30°C for the duration of the run. However, we cannot guarantee the same level of temperature control on the MinION Mk1B. Therefore, if you are running Kit 12 chemistry on the MinION Mk1B, ensure that the ambient temperature does not exceed 23°C.

TIP

Priming and loading a MinION flow cell

We recommend all new users watch the Priming and loading your flow cell video before your first run.

Using the Loading Solution

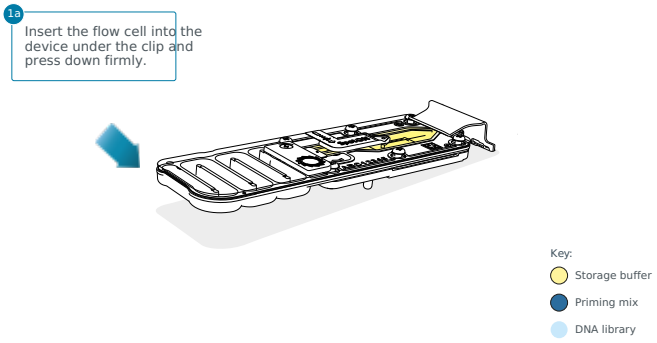
We recommend using the Loading Beads II (LBII) for loading your library onto the flow cell for most sequencing experiments. However, if you have previously used water to load your library, you must use Loading Solution (LS) instead of water.
Note: some customers have noticed that viscous libraries can be loaded more easily when not using Loading Beads II.

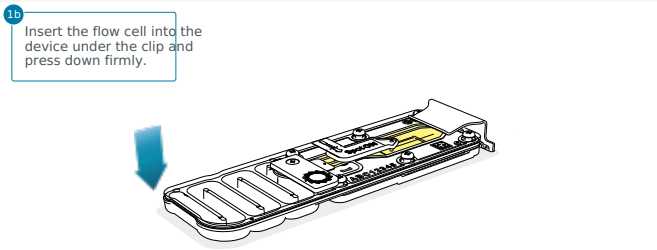
1 **Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) or Loading Solution (LS, if using), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature before mixing the reagents by vortexing and spin down at room temperature.**

2 **Prepare the flow cell priming mix in a suitable vial for the number of flow cells to flush. Once combined, mix well by briefly vortexing.**

Reagent	Volume per flow cell
Flush Tether (FLT)	30 µl
Flush Buffer (FB)	1,170 µl

3 **Open the MinION lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact.**





Optional Action
Complete a flow cell check to assess the number of pores available before loading the library.

This step can be omitted if the flow cell has been checked previously.

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

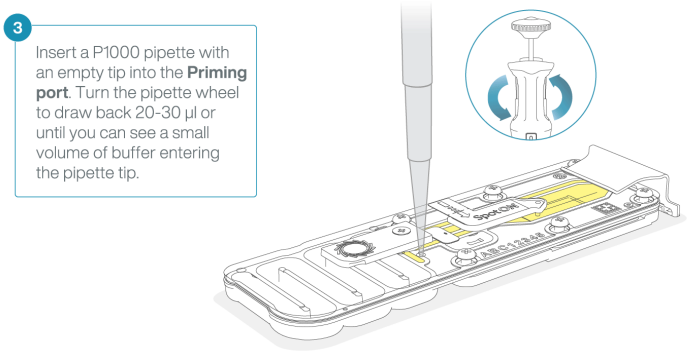
4 **Slide the flow cell priming port cover clockwise to open the priming port.**

IMPORTANT

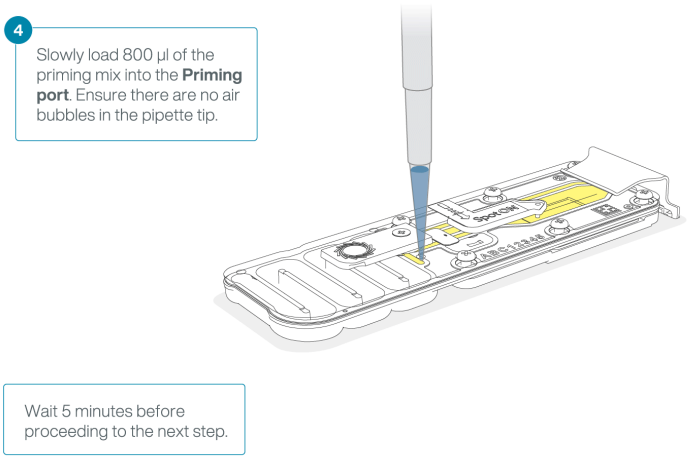
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

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

1. Set a P1000 pipette to 200 µl
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 ul, to draw back 20-30 ul, or until you can see a small volume of buffer entering the pipette tip
   Note: Visually check that there is continuous buffer from the priming port across the sensor array.



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



7 **Thoroughly mix the contents of the Loading Beads II (LBII) by pipetting.**

IMPORTANT

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

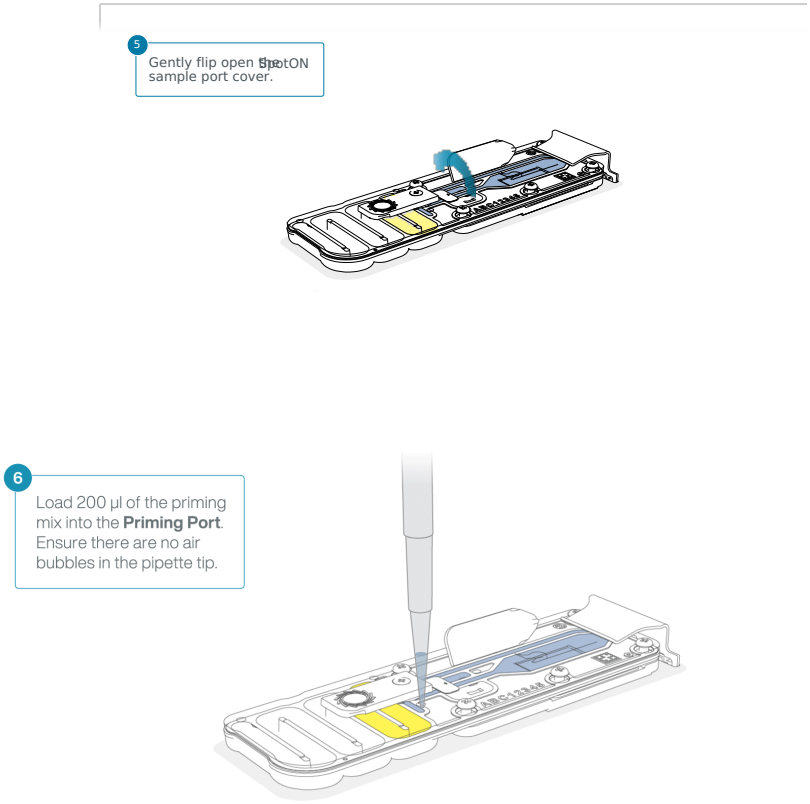
8 **In a new tube, prepare the library for loading as follows:**

Reagent	Volume per flow cell
Sequencing Buffer II (SBII)	37.5 µl
Loading Beads II (LBII) mixed immediately before use, or Loading Solution (LS), if using	25.5 µl
DNA library	12 µl
Total	**75 µl**

Note: Load the library onto the flow cell immediately after adding the Sequencing Buffer II (SBII) and Loading Beads II (LBII).

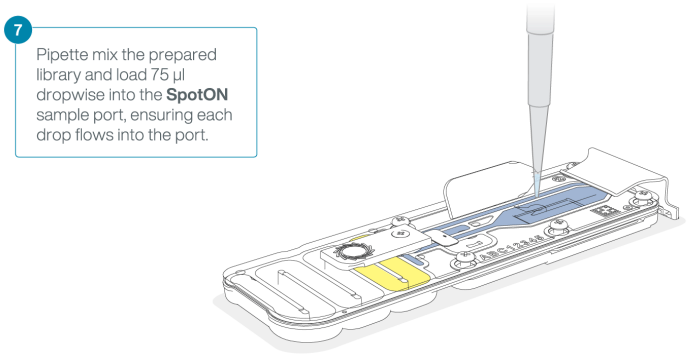
9 Complete the flow cell priming:

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

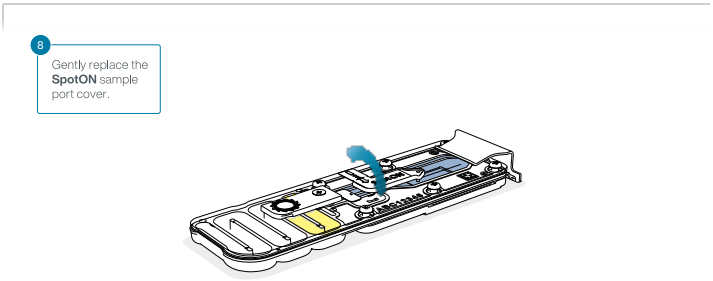


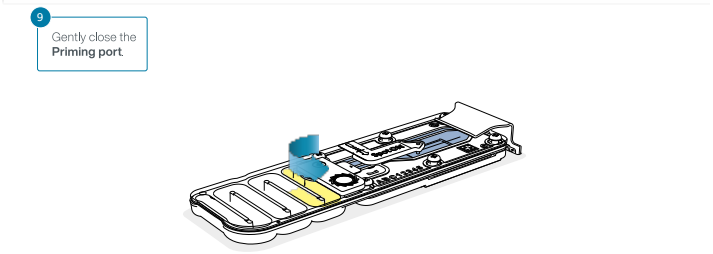
10 Mix the prepared library gently by pipetting up and down just prior to loading.

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



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





Data acquisition and basecalling

Overview of nanopore data analysis

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

How to start sequencing

The sequencing device control, data acquisition and real-time basecalling are carried out by the MinKNOW software. It is assumed you have already installed MinKNOW on your computer. 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.

Ending the experiment

Materials

- Flow Cell Wash Kit (EXP-WSH004)

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

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

TIP

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

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

Instructions for returning flow cells can be foundhere.

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

IMPORTANT

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

Issues during DNA/RNA extraction and library preparation

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

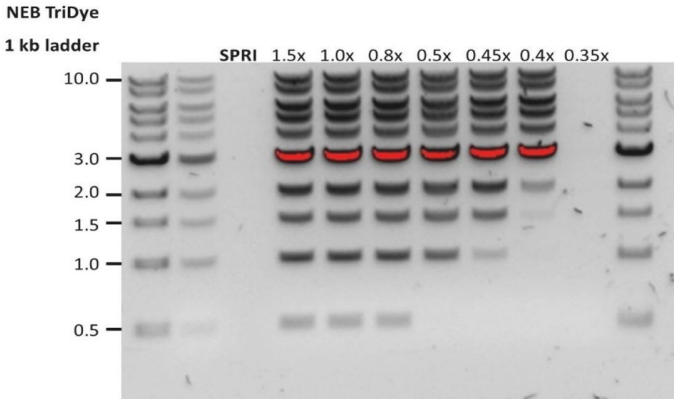
We also have an FAQ section available on theNanopore Community Support section.

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

Low sample quality

Observation	Possible cause	Comments and actions
Low DNA purity (Nanodrop reading for DNA OD 260/280 is <1.8 and OD 260/230 is <2.0–2.2)	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the Contaminants Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover. Consider performing an additional SPRI clean-up step.
Low RNA integrity (RNA integrity number <9.5 RIN, or the rRNA band is shown as a smear on the gel)	The RNA degraded during extraction	Try a different RNA extraction method. For more info on RIN, please see the RNA Integrity Number Know-how piece.
RNA has a shorter than expected fragment length	The RNA degraded during extraction	Try a different RNA extraction method. For more info on RIN, please see the RNA Integrity Number Know-how piece. We recommend working in an RNase-free environment, and to keep your lab equipment RNase-free when working with RNA.

Low DNA recovery after AMPure bead clean-up

Observation	Possible cause	Comments and actions
Low recovery	DNA loss due to a lower than intended AMPure beads-to-sample ratio	1. AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample. 2. When the AMPure beads-to-sample ratio is lower than 0.4:1, DNA fragments of any size will be lost during the clean-up.
Low recovery	DNA fragments are shorter than expected	The lower the AMPure beads-to-sample ratio, the more stringent the selection against short fragments. Please always determine the input DNA length on an agarose gel (or other gel electrophoresis methods) and then calculate the appropriate amount of AMPure beads to use. 
Low recovery after end-prep	The wash step used ethanol <70%	DNA will be eluted from the beads when using ethanol <70%. Make sure to use the correct percentage.

Issues during the sequencing run

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

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

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

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

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

MinKNOW script failed

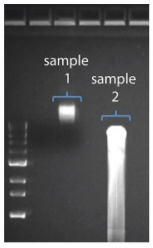
Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support.

Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	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"

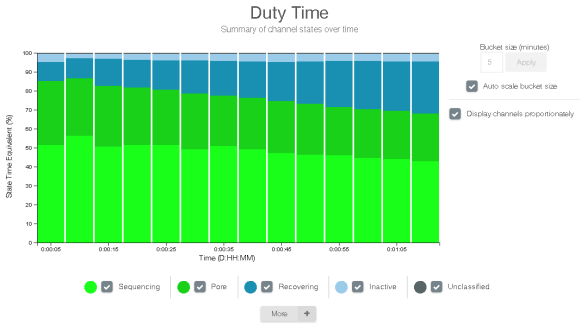
Observation	Possible cause	Comments and actions
Pore occupancy close to 0	The Ligation Sequencing Kit was used, and sequencing adapters did not ligate to the DNA	Make sure to use the NEBNext Quick Ligation Module (E6056) and Oxford Nanopore Technologies Ligation Buffer (LNB, provided in the SQK-LSK109 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
Shorter than expected read length	Unwanted fragmentation of DNA sample	Read length reflects input DNA fragment length. Input DNA can be fragmented during extraction and library prep. 1. Please review the Extraction Methods in the Nanopore Community for best practice for extraction. 2. Visualise the input DNA fragment length distribution on an agarose gel before proceeding to the library prep.  In the image above, Sample 1 is of high molecular weight, whereas Sample 2 has been fragmented. 3. During library prep, avoid pipetting and vortexing when mixing reagents. Flicking or inverting the tube is sufficient.

Large proportion of recovering pores

Observation	Possible cause	Comments and actions

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

Large proportion of inactive pores

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

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

Observation	Possible cause	Comments and actions
Reduction in sequencing speed and q-score later into the run	Fast fuel consumption is typically seen when the flow cell is overloaded with library (please see the appropriate protocol for your DNA library to see the recommendation).	Add more fuel to the flow cell by following the instructions in the MinKNOW protocol. In future experiments, load lower amounts of library to the flow cell.

Temperature fluctuation

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

Failed to reach target temperature

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

Guppy – no input .fast5 was found or basecalled

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

Guppy – no Pass or Fail folders were generated after basecalling

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

Guppy – unusually slow processing on a GPU computer

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

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

Observation	Possible cause	Comments and actions
The MinKNOW interface is not shown in the web browser	Browser compatibility issue	Always use Google Chrome as the browser to view MinKNOW. Alternatively, instead of typing //mt-xxxxxx (x is a number) in the address bar, type in in the generic IP address, 10.42.0.1, which identifies the MinIT Wi-Fi router.
The MinKNOW interface is not shown in the web browser	The MinIT Wi-Fi was not used for connecting to the computer or mobile device	Make sure the computer or mobile device is using the MinIT Wi-Fi. It should be shown as MT-xxxxxx (x is a number) on the underside label on the MinIT:  Disable the Ethernet connection from the computer or mobile device as needed. If necessary, contact your IT department to determine if the MinIT Wi-Fi is blocked (MinIT generic IP: 10.42.0.1). Please white-list MinIT as needed.
The MinKNOW interface is not shown in the web browser	The MinIT was not on the same network that the computer was connected to.	Make sure that the wall sockets used by the Ethernet cables from the MinIT and computer belong to the same local network.

MinIT – the MinIT software cannot be updated

Observation	Possible cause	Comments and actions
The MinIT software cannot be updated	The firewall is blocking IPs for update	Please consult your IT department, as the MinIT software requires access to thefollowing AWS IP ranges. Access to the following IP addresses is also needed: 178.79.175.200 96.126.99.215
The MinIT software cannot be updated	The device already has the latest version of the software	Occassionaly, the MinIT software admin page displays "updates available" even when the software is already up-to-date. Please compare the version listed on the admin page with the one on the Software Downloads page. Alternatively, SSH into the MinIT through a SSH Client (e.g. Bitvise or Putty, as described in the MinIT protocol) on a Windows computer or the terminal window on a Mac, run the command, *dpkg -l | grep minit*, to find out the version of the MinIT software and *sudo apt update* if an update is needed. If the issue still persists, please contact Technical Services with details of the error.