

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Introduction to the protocol

Version: PTCE_9122_v109_revH_10Feb2021

Overview of the protocol

IMPORTANT

This protocol is a work in progress, and some details are expected to change over time. Please make sure you always use the most recent version of the protocol.

This protocol is an update of the [PCR tiling of SARS-CoV-2 virus](#) and [PCR tiling of SARS-CoV-2 with Native Barcoding Expansion 96 \(EXP-NBD196\)](#).

We have updated the protocol to shorten hands-on time during library preparation and to reduce reagent costs. The update removed the PCR SPRI clean-up, quantification, and normalisation steps to shorten and simplify library preparation. Instructions have been re-written to clearly illustrate how to prepare samples in a plate format for x24, x48 and x96 samples per run.

This protocol is based on the [ARTIC amplicon sequencing protocol for MinION for SARS-CoV-2 v3 \(LoCost\) by Josh Quick](#). In the table below, we have highlighted which steps are different between the protocols.

Step change	Oxford Nanopore Technologies protocol PCR tiling of SARS-CoV-2 virus	Oxford Nanopore Technologies protocol Eco PCR tiling of SARS-CoV-2 virus	ARTIC amplicon sequencing protocol for SARS-CoV-2 v3 (LoCost) by Josh Quick
Reverse transcription	LunaScript RT SuperMix (5X): 4 µl RNA sample: 16 µl Total: 20 µl	LunaScript RT SuperMix (5X): 2 µl RNA sample: 8 µl Total: 10 µl	LunaScript RT SuperMix (5X): 2 µl RNA sample: 8 µl Total: 10 µl
PCR	Q5 Hot Start High-Fidelity 2X Master Mix: 12.5 µl Primer pool A/B (10 µM): 3.7 µl Nuclease-free water: 3.8 µl Total: 20 µl cDNA: 5 µl 1.0X SPRI clean-up after PCR	Q5 Hot Start High-Fidelity 2X Master Mix: 12.5 µl Primer pool A/B (100 µM): 0.37 µl Nuclease-free water: 9.63 µl Total: 22.5 µl cDNA: 2.5 µl The clean-up, quantification and normalisation steps have been removed.	Q5 Hot Start High-Fidelity 2X Master Mix: 12.5 µl Primer pool A/B (10 µM): 4 µl Nuclease-free water: 6 µl Total: 22.5 µl cDNA: 2.5 µl
End-prep	DNA in nuclease-free water: 12.5 µl Ultra II End Prep Reaction Buffer: 1.75 µl Ultra II End Prep Enzyme Mix: 0.75 µl Total: 15 µl	DNA: 3.3 µl Nuclease-free water: 5 µl Ultra II End Prep Reaction Buffer: 1.2 µl Ultra II End Prep Enzyme Mix: 0.5 µl Total: 10 µl	DNA: 3.3 µl Nuclease-free water: 5 µl Ultra II End Prep Reaction Buffer: 1.2 µl Ultra II End Prep Enzyme Mix: 0.5 µl Total: 10 µl
Native barcode ligation x24	Nuclease-free water: 6 µl DNA 1.5 µl µl Native barcode: 2.5 µl Blunt/TA Ligase Master Mix: 10 µl Total: 20 µl	Nuclease-free water: 6 µl DNA 1.5 µl µl Native barcode: 2.5 µl Blunt/TA Ligase Master Mix: 10 µl Total: 20 µl	Nuclease-free water: 3 µl DNA 0.75 µl Native barcode: 1.25 µl Blunt/TA Ligase Master Mix: 5 µl Total: 10 µl

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Native barcode ligation for x48 and x96	Nuclease-free water: 3 µl DNA: 0.75 µl Native barcode: 1.25 µl Blunt/TA Ligase Master Mix: 5 µl Total: 10 µl	Nuclease-free water: 3 µl DNA: 0.75 µl Native barcode: 1.25 µl Blunt/TA Ligase Master Mix: 5 µl Total: 10 µl	Same as Native barcode ligation for x24 (above)
Pooled barcoded samples	480 µl	480 µl	Maximum 240 µl
Native barcode ligation clean-up	SFB: 700 µl 80% ethanol wash	SFB: 700 µl 80% ethanol wash	SFB: 250 µl 70% ethanol wash
Adapter ligation clean-up	0.4X SPRI bead clean-up SFB: 125 µl	0.4X SPRI bead clean-up SFB: 125 µl	1.0X SPRI bead clean-up SFB: 250 µl

Introduction to the protocol

To enable support for the rapidly expanding user requests, the team at Oxford Nanopore Technologies have put together an updated, lower cost end-to-end workflow based on the ARTIC Network protocols and analysis methods.

While this protocol is available in the Nanopore Community, we kindly ask users to ensure they are citing the members of the ARTIC network who have been behind the development of these methods.

This protocol is based on the [ARTIC amplicon sequencing protocol for MinION for SARS-CoV-2 v3 \(LoCost\) by Josh Quick](#). The protocol generates 400 bp amplicons in a tiled fashion across the whole SARS-CoV-2 genome. Some example data is shown in the [Downstream analysis and expected results](#) section, this is generated using human coronavirus 229E to show what would be expected when running this protocol with SARS-CoV-2 samples.

Primers were designed by Josh Quick using Primal Scheme; the primer sequences can be found [here](#).

Steps in the sequencing workflow:

Prepare for your experiment

You will need to:

- Extract your RNA
- 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:

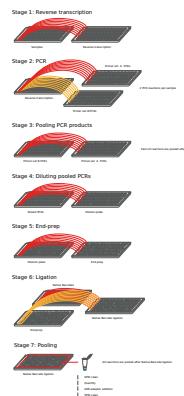
- Reverse transcribe your RNA samples with random hexamers
- Amplify the samples by tiled PCR using separate primer pools
- Combine the primer pools, purify and quantify the PCR products
- Prepare the DNA ends for adapter attachment
- Ligate native barcodes supplied in the kit to the DNA ends and pool the samples

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- Ligate the sequencing adapters supplied in the kit to the DNA ends
- Prime the flow cell and load your DNA library into the flow cell



Sequencing and analysis

You will need to:

- Start a sequencing run using the MinKNOW software, which will collect raw data from the device and convert it into basecalled reads

Before starting

This protocol outlines how to carryout PCR tiling of SARS-CoV-2 viral RNA samples on a 96-well plate using the Native Barcoding 96 Expansion (EXP-NBD196) or Native Barcoding Expansions 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114).

It is required to use total RNA extracted from samples that have been screened by a suitable qPCR assay. Here, we demonstrate the level of sensitivity and specificity by titrating total RNA extracted from cell culture infected with Human coronavirus 229E spiked into 100 ng human RNA extracted from GM12878 to give approximate figures.

Although not tested here, work performed by Josh Quick *et al.* on the Zika virus gives approximate dilution factors that may help the reduction of inhibiting compounds that can be co-extracted from samples.

Note: this is a guideline and not currently tested for COVID-19.

qPCR ct	Dilution factor
18–35	none
15–18	1:10
12–15	1:100

When processing multiple samples at once, we recommend making master mixes with an additional 10% of the volume. We also recommend using pre- and post-PCR hoods when handling master mixes and samples. It is important to clean and/or UV irradiate these hoods between sample batches. Furthermore, to track and monitor cross-contamination events, it is important to run a negative control reaction at the reverse transcription stage using nuclease-free water instead of sample, and carrying this control through the rest of the prep.

To minimise the chance of pipetting errors when preparing primer mixes, we recommend ordering the tiling primers from IDT in a *lab-ready* format at 100 µM.

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Equipment and consumables

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IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Native Barcoding Expansions 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114)
- Native Barcoding Expansion 96 (EXP-NBD196)
- Ligation Sequencing Kit (SQK-LSK109)
- FLO-MIN106D, FLO-MINSP6 or FLO-MIN111 flow cells
- Flow Cell Wash Kit (EXP-WSH004)
- Sequencing Auxiliary Vials (EXP-AUX001)
- SFB Expansion (EXP-SFB001)

Equipment and consumables

Materials

- Input RNA
- Native Barcoding Expansion 96 (EXP-NBD196)
- Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) if multiplexing 24 samples
- Flow Cell Priming Kit (EXP-FLP002)
- SFB Expansion (EXP-SFB001)
- Sequencing Auxiliary Vials (EXP-AUX001)
- Adapter Mix II Expansion (EXP-AMII001)

Consumables

- LunaScript™ RT SuperMix Kit
- Q5® Hot Start High-Fidelity 2X Master Mix (NEB, M0494)
- SARS-CoV-2 primers (lab-ready at 100 µM, IDT)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Agencourt AMPure XP beads
- Freshly-prepared 80% ethanol in nuclease-free water
- Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)
- NEB Blunt/TA Ligase Master Mix (M0367)
- NEBNext Ultra II End repair / dA-tailing Module (E7546)
- NEBNext Quick Ligation Module (E6056)
- DNA 12000 Kit & Reagents - optional (Agilent Technologies)
- 1.5 ml Eppendorf DNA LoBind tubes
- 5 ml Eppendorf DNA LoBind tubes
- Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Cat # 0030129504) with heat seals

Equipment

- Hula mixer (gentle rotator mixer)
- Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- Centrifuge capable of taking 96-well plates

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Equipment and consumables

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- Microfuge
- Vortex mixer
- Thermal cycler
- Multichannel pipettes suitable for dispensing 0.5–10 µl, 2–20 µl and 20–200 µl, and tips
- P1000 pipette and tips
- P200 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)
- PCR hood with UV steriliser (optional but recommended to reduce cross-contamination)
- PCR-Cooler (Eppendorf)
- Stepper pipette and tips

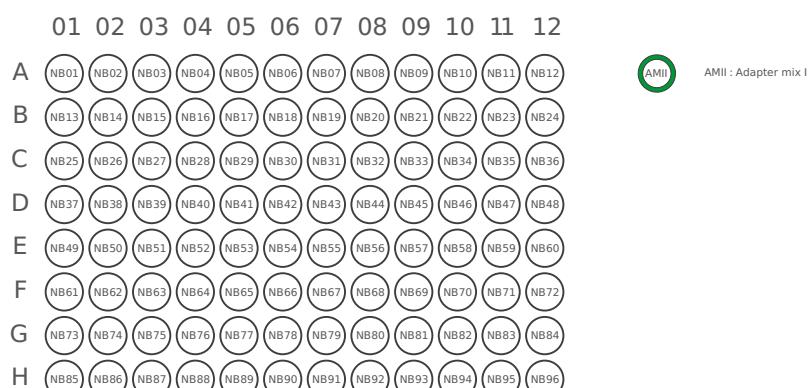
Input RNA guidelines

Where sample RNA is added to the below reaction, it is likely advantageous to follow the dilution guidelines proposed by Josh Quick:

qPCR Ct	Dilution factor
18–35	none
15–18	1:10
12–15	1:100

If the sample has a low copy number (ct 18–35), use up to 16 µl of sample. Use nuclease-free water to make up any remaining volume. Take note to be aware that co-extracted compounds may inhibit reverse transcription and PCR.

Native Barcoding Expansion 96 (EXP-NBD196) contents



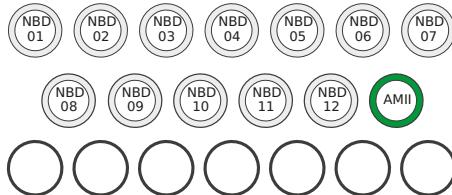
Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) contents

EXP-NBD104 kit contents

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

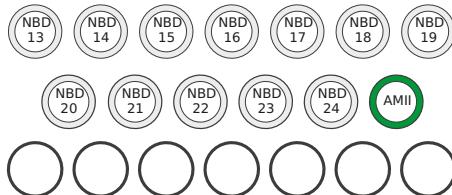
Equipment and consumables

Version: PTCE_9122_v109_revH_10Feb2021



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

EXP-NBD114 kit contents



NBD13: Native barcode 13
 NBD14: Native barcode 14
 NBD15: Native barcode 15
 NBD16: Native barcode 16
 NBD17: Native barcode 17
 NBD18: Native barcode 18
 NBD19: Native barcode 19
 NBD20: Native barcode 20
 NBD21: Native barcode 21
 NBD22: Native barcode 22
 NBD23: Native barcode 23
 NBD24: Native barcode 24
 AMII : Adapter mix II

Flow Cell Priming Kit contents (EXP-FLP002)



FLB : Flush buffer
 FLT : Flush tether

SFB Expansion contents (EXP-SFB001)



SFB : S fragment buffer

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Equipment and consumables

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Sequencing Auxiliary Vials contents (EXP-AUX001)



SQB : Sequencing buffer

EB : Elution buffer

LB : Loading beads

Adapter Mix II Expansion contents (EXP-AMII001)



AMII : Adapter Mix II

Name	Acronym	Cap colour	No. of tubes	Fill volume
Adapter Mix II	AMII	Green	2	30 µl

Adapter Mix II Expansion use

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

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

Native Barcoding Expansion 96 barcode sequences

Component	Forward sequence	Reverse sequence
BC01	CACAAAGACACCGACAACTTCTT	AAGAAAGTTGTCGGTGCTTTGTG
BC02	ACAGACGACTACAAACGGAATCGA	TCGATTCCGTTGTAGTCGTCTGT

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Equipment and consumables

Version: PTCE_9122_v109_revH_10Feb2021

Component	Forward sequence	Reverse sequence
BC03	CCTGGTAAC TGGGACACAAGACTC	GAGTCTTG TGCCCAGTT ACCAGG
BC04	TAGGGAAACACGATAGAATCCGAA	TTCGGATTCTATCGTGTTCCTA
BC05	AAGGTTACACAAACCCCTGGACAAG	CTTGTCCAGGGTTGTGAACCTT
BC06	GACTACTTCTGCCTTGCGAGAA	TTCTCGCAAAGGCAGAAAGTAGTC
BC07	AAGGATTCA TCCCACGGTAACAC	GTGTTACCGTGGGAATGAATCCTT
BC08	ACGTAAC TGGTTGTTCCCTGAA	TTCAGGGAAACAAACCAAGTTACGT
BC09	AACCAAGACTCGCTGTGCCTAGTT	AACTAGGCACAGCGAGTCTGGTT
BC10	GAGAGGACAAAGGTTCAACGCTT	AAGCGTTGAAACCTTGTCCCTTC
BC11	TCCATTCCCTCCGATAGATGAAAC	GTTTCATCTATCGGAGGGAATGGA
BC12	TCCGATTCTGCTTCTTCTACCTG	CAGGTAGAAAAGAACGAGAATCGGA
BC13	AGAACGACTTCCATACTCGTGTGA	TCACACGAGTATGGAAGTCGTTCT
BC14	AACGAGTCTCTGGGACCCATAGA	TCTATGGGTC CCAAGAGACTCGTT
BC15	AGGTCTACCTCGCTAACACCACTG	CAGTGGTGTAGCGAGGTAGACCT
BC16	CGTCAACTGACAGTGGTCGTACT	AGTACGAACCACTGTCA GTGACCG
BC17	ACCCTCCAGGAAAGTACCTCTGAT	ATCAGAGGTACTT CCTGGAGGGT
BC18	CCAAACCCAACAAACCTAGATAGGC	GCCTATCTAGGTTGTTGGTTGG
BC19	GTTCCCTCGTGCAGTGTCAAGAGAT	ATCTCTTGACACTGCACGAGGAAC
BC20	TTGCGTCCTGTTACGAGAACTCAT	ATGAGTTCTCGTAACAGGACGCAA
BC21	GAGCCTCTCATTGCCGTTCTCA	TAGAGAACGGACAATGAGAGGCTC
BC22	ACCACTGCCATGTATCAAAGTACG	CGTACTTGTACATGGCAGTGGT
BC23	CTTACTACCCAGTGAACCTCCTCG	CGAGGAGGTTCACTGGTAGTAAG
BC24	GCATAGTTCTGCATGATGGTTAG	CTAACCCATCATGCAGAACTATGC
BC25	GTAAGTTGGGTATGCAACGCAATG	CATTGCGTTGCATACCCAACTTAC
BC26	CATACAGCGACTACGCATTCTCAT	ATGAGAAATGCGTAGTCGCTGTATG
BC27	CGACGGTTAGATTCACCTCTTACA	TGTAAGAGGTGAATCTAACCGTCG
BC28	TGAAACCTAAGAAGGCACCGTATC	GATACGGTGCCTTCTTAGGTTCA
BC29	CTAGACACCTGGGTTGACAGACC	GGTCTGTCAACCCAAGGTGTCTAG
BC30	TCAGTGAGGATCTACTCGACCCA	TGGGTCGAAGTAGATCCTCACTGA
BC31	TGCGTACAGCAATCAGTTACATTG	CAATGTAAC TGATTGCTGTACGCA
BC32	CCAGTAGAAGTCCGACAACGTCAT	ATGACGTTGCGGACTTCTACTGG
BC33	CAGACTTGGTACGGTTGGTACT	AGTTACCCAACCGTACCAAGTCTG
BC34	GGACGAAGAACTCAAGTCAAAGGC	GCCTTGACTTGAGTTCTCGTCC
BC35	CTACTTACGAAGCTGAGGGACTGC	GCAGTCCCTCAGCTTCGTAAGTAG

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Equipment and consumables

Version: PTCE_9122_v109_revH_10Feb2021

Component	Forward sequence	Reverse sequence
BC36	ATGTCCCAGTTAGAGGAGGAACA	TGTTTCCTCCTCTAACTGGGACAT
BC37	GCTTGCATTGATGCTTAGTATCA	TGATACTAACATCAATCGCAAGC
BC38	ACCACAGGAGGACGATACAGAGAA	TTCTCTGTATCGTCCTCCTGTGGT
BC39	CCACAGTGTCAACTAGAGCCTCTC	GAGAGGCTCTAGTTGACACTGTGG
BC40	TAGTTGGATGCCAAGGATAGCC	GGCTATCCTGGTCATCCAAACTA
BC41	GGAGTTCGTCCAGAGAAGTACACG	CGTGTACTTCTCTGGACGAACCTCC
BC42	CTACGTGTAAGGCATACCTGCCAG	CTGGCAGGTATGCCCTACACGTAG
BC43	CTTCGTTGTTGACTCGACGGTAG	CTACCGTGGAGTCACAAACGAAAG
BC44	AGTAGAAAGGGTCCCTCCACTC	GAGTGGGAAGGAACCCCTTCTACT
BC45	GATCCAACAGAGATGCCCTCAGTG	CACTGAAGGCATCTGTGGATC
BC46	GCTGTGTTCCACTTCATTCTCCTG	CAGGAGAATGAAGTGGAACACAGC
BC47	GTGCAACTTCCCACAGGTAGTTC	GAACATACCTGTGGAAAGTTGCAC
BC48	CATCTGGAACGTGGTACACCTGTA	TACAGGTGTACCACGTTCCAGATG
BC49	ACTGGTGCAGCTTGAACATCTAG	CTAGATGTTCAAAGCTGCACCACT
BC50	ATGGACTTGGTAACCTCCTCGT	ACGCAGGAAGTTACCAAAGTCCAT
BC51	GTTGAATGAGCCTACTGGTCCTC	GAGGACCCAGTAGGCTCATTCAC
BC52	TGAGAGACAAGATTGTCGTGGAC	GTCCACGAACAATCTGTCTCTCA
BC53	AGATTCAACCGTCTCATGCAAAG	CTTGCATGAGACGGTCTGAATCT
BC54	CAAGAGCTTGACTAAGGAGCATG	CATGCTCCTAGTCACAGCTCTTG
BC55	TGGAAGATGAGACCCCTGATCTACG	CGTAGATCAGGGTCTCATCTCCA
BC56	TCACTACTCAACAGGTGGCATGAA	TTCATGCCACCTGTTGAGTAGTGA
BC57	GCTAGGTCAATCCTTCGGAAAGT	ACTTCCGAAGGAGATTGACCTAGC
BC58	CAGGTTACTCCTCCGTGAGTCTGA	TCAGACTCACGGAGGAGTAACCTG
BC59	TCAATCAAGAAGGGAAAGCAAGGT	ACCTTGCTTCCCTCTTGATTGA
BC60	CATGTTCAACCAAGGCTTCTATGG	CCATAGAACGCTTGGTTGAACATG
BC61	AGAGGGTACTATGTGCCTCAGCAC	GTGCTGAGGCACATAGTACCTCT
BC62	CACCCACACTTACTCAGGACGTA	TACGTCCTGAAGTAAGTGTGGGTG
BC63	TTCTGAAGTTCCTGGGTCTTGAAC	GTTCAAGACCCAGGAACCTCAGAA
BC64	GACAGACACCGTTCATGACTTC	GAAAGTCGATGAACGGTGTCTGTC
BC65	TTCTCAGTCTCCTCCAGACAAGG	CCTTGCTGGAGGAAGACTGAGAA
BC66	CCGATCCTGTGGCTTCTAACTTC	GAAGTTAGAACGCCACAAGGATCGG
BC67	GTTTGTCTACTCGTGTGCTCACC	GGTGAGCACACGAGTATGACAAAC
BC68	GAATCTAACGAAACACGAAGGTGG	CCACCTCGTGTGCTTAGATTC

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Equipment and consumables

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Component	Forward sequence	Reverse sequence
BC69	TACAGTCCGAGCCTCATGTGATCT	AGATCACATGAGGCTCGGACTGTA
BC70	ACCGAGATCCTACGAATGGAGTGT	ACACTCCATTCTGAGGATCTCGGT
BC71	CCTGGGAGCATCAGGTAGTAACAG	CTGTTACTACCTGATGCTCCCAGG
BC72	TAGCTGACTGTCTTCCATACCGAC	GTCGGTATGGAAGACAGTCAGCTA
BC73	AAGAAACAGGATGACAGAACCCCTC	GAGGGTTCTGTCATCCTGTTCTT
BC74	TACAAGCATCCCAACACTCCACT	AGTGGAACTGTTGGATGCTTGTA
BC75	GACCATTGTGATGAACCCCTGTTGT	ACAACAGGGTTCATCACAATGGTC
BC76	ATGCTTGTACATCAACCCCTGGAC	GTCCAGGGTTGATGTAACAAGCAT
BC77	CGACCTGTTCTCAGGGATAACAC	GTTGTATCCCTGAGAACAGGTCG
BC78	AACAACCGAACCTTGAATCAGAA	TTCTGATTCAAAGGTTGGTTGTT
BC79	TCTCGGAGATAGTTCTCACTGCTG	CAGCAGTGAGAACTATCTCCGAGA
BC80	CGGATGAACATAGGATAGCGATT	GAATCGCTATCCTATGTTCATCCG
BC81	CCTCATTTGTGAAGTTGTTGG	CCGAAACAACCTCACAGATGAGG
BC82	ACGGTATGTCGAGTTCCAGGACTA	TAGTCCTGGAACTCGACATACCGT
BC83	TGGCTTGATCTAGGTAAAGTCGAA	TTCGACCTTACCTAGATCAAGCCA
BC84	GTAGTGGACCTAGAACCTGTGCCA	TGGCACAGGTTCTAGGTCCACTAC
BC85	AACGGAGGAGTTAGTTGGATGATC	GATCATCCAACTAACCTCCCGTT
BC86	AGGTGATCCCAACAAGCGTAAGTA	TACTTACGTTGTTGGATCACCT
BC87	TACATGCTCCTGTTAGGGAGG	CCTCCCTAACACAGGAGCATGTA
BC88	TCTTCACTACCGATCCGAAGCAG	CTGCTTCGGATCGGTAGTAGAAGA
BC89	ACAGCATCAATGTTGGCTAGTTG	CAACTAGCCAAACATTGATGCTGT
BC90	GATGTAGAGGGTACGGTTGAGGC	GCCTCAAACCGTACCCCTACATC
BC91	GGCTCCATAGGAACCTACCGCTACT	AGTAGCGTGAGTTCTATGGAGCC
BC92	TTGTGAGTGGAAAGATAACAGGACC	GGTCCTGTATCTTCCACTCACAA
BC93	AGTTTCCATCACTTCAGACTGGG	CCCAAGTCTGAAGTGTGGAAACT
BC94	GATTGCCTCAAACGCCACCTAC	GTAGGTGGCAGTTGAGGACAATC
BC95	CCTGTCTGGAAGAAGAATGGACTT	AAGTCCATTCTTCTCCAGACAGG
BC96	CTGAACGGTCATAGAGTCCACCAT	ATGGTGGACTCTATGACCCTCAG

Native barcode sequences

The native barcode sequences are the reverse complement of the corresponding barcode sequence in other kits:

Native Barcoding Expansion 1-12

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Computer requirements and software

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Component	Sequence
NB01	CACAAAGACACCGACAACCTTCTT
NB02	ACAGACGACTACAAACGGAATCGA
NB03	CCTGGTAACGGACACAAGACTC
NB04	TAGGGAAACACGATAGAACATCCGAA
NB05	AAGGTTACACAAACCCCTGGACAAG
NB06	GACTACTTCTGCCTTGCGAGAA
NB07	AAGGATTCAATTCCCACGGTAACAC
NB08	ACGTAACTTGGTTGTTCCCTGAA
NB09	AACCAAGACTCGCTGTGCCTAGTT
NB10	GAGAGGACAAAGGTTCAACGCTT
NB11	TCCATTCCCTCCGATAGATGAAAC
NB12	TCCGATTCTGCTTCTTCTACCTG

Native Barcoding Expansion 13-24

Component	Sequence
NB13	AGAACGACTTCCATACTCGTGTGA
NB14	AACGAGTCTCTGGGACCCATAGA
NB15	AGGTCTACCTCGCTAACACCCTG
NB16	CGTCAACTGACAGTGGTCGTACT
NB17	ACCCCTCAGGAAAGTACCTCTGAT
NB18	CCAAACCCAACAACCTAGATAGGC
NB19	GTTCCCTCGTGCAGTGTCAAGAGAT
NB20	TTGCGTCTGTTACGAGAACTCAT
NB21	GAGCCTCTCATTGTCCGTTCTA
NB22	ACCACTGCCATGTATCAAAGTACG
NB23	CTTACTACCCAGTGAACCTCCTCG
NB24	GCATAGTTCTGCATGATGGGTTAG

Computer requirements and software

MinION Mk1B IT requirements

Unless you are using a MinIT device, sequencing on a MinION Mk1B requires a high-spec computer or laptop to keep up with the rate of data acquisition.

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Library preparation

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Read more in the [MinION IT Requirements document](#).

Software for nanopore sequencing

MinKNOW

The MinKNOW software controls the nanopore sequencing device, collects sequencing data in real time and processes it into basecalls. You will be using MinKNOW for every sequencing experiment. MinKNOW can also demultiplex reads by barcode, and basecall/demultiplex data after a sequencing run has completed.

MinKNOW use

For instructions on how to run the MinKNOW software, please refer to the relevant section in the [MinKNOW protocol](#).

EPI2ME (optional)

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

EPI2ME installation and use

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

Guppy (optional)

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

Guppy installation and use

If you would like to use the Guppy software, please refer to the [Guppy protocol](#).

Check your flow cell

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

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

Reverse transcription

~30 minutes

Materials

- Input RNA

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Library preparation

Version: PTCE_9122_v109_revH_10Feb2021

Consumables

- LunaScript™ RT SuperMix Kit
- Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Cat # 0030129504) with heat seals
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

Equipment

- Multichannel pipettes suitable for dispensing 0.5–10 µl, 2–20 µl and 20–200 µl, and tips
- Thermal cycler
- Centrifuge capable of taking 96-well plates
- Ice bucket with ice

Optional Equipment

- PCR-Cooler (Eppendorf)
- PCR hood with UV steriliser (optional but recommended to reduce cross-contamination)
- Stepper pipette and tips

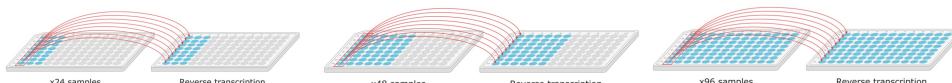
IMPORTANT

Keep the RNA sample on ice as much as possible to prevent nucleolytic degradation, which may affect sensitivity.

1 In a clean pre-PCR hood, using a stepper pipette, or a multichannel pipette, add 2 µl of LunaScript™ RT SuperMix to a fresh 96-well plate (RT Plate).

Depending on the number of samples, fill each well per column as follows:

Plate location	X24 samples	X48 samples	X96 samples
Columns	1-3	1-6	1-12



2 To each well containing LunaScript reagent of the RT plate, add 8 µl of sample and gently mix by pipetting. If adding less than 8 µl, make up the rest to the volume with nuclease-free water.

3 Seal the RT plate and spin down. Return the plate to ice.

4 Preheat the thermal cycler to 25°C.

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

PCR

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5 Incubate the samples in the thermal cycler using the following program:

Temperature	Time
25°C	2 minutes
55°C	10 minutes
95°C	1 minute
4°C	hold

END OF STEP

While the reverse transcription reaction is running, prepare the primer pools as described in the next section.

PCR

~235 minutes

Consumables

- SARS-CoV-2 primers (lab-ready at 100 µM, IDT)
- Q5® Hot Start High-Fidelity 2X Master Mix (NEB, M0494)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Cat # 0030129504) with heat seals
- 5 ml Eppendorf DNA LoBind tubes
- 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- Multichannel pipettes suitable for dispensing 0.5–10 µl, 2–20 µl and 20–200 µl, and tips
- P1000 pipette and tips
- P200 pipette and tips
- Thermal cycler
- Microfuge
- Centrifuge capable of taking 96-well plates
- Ice bucket with ice

Optional Equipment

- PCR-Cooler (Eppendorf)
- PCR hood with UV steriliser (optional but recommended to reduce cross-contamination)
- Stepper pipette and tips

Primer design

To generate tiled PCR amplicons from the SARS-CoV-2 viral cDNA, primers were designed by Josh Quick using Primal Scheme. These primers are designed to generate 400 bp amplicons that overlap by approximately 20 bp. These primer sequences can be found [here](#). Where we show example data outputs in this protocol, the same parameters were used to design primers to the human coronavirus 229E to provide guideline statistics.

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

PCR

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IMPORTANT

We recommend ordering the required primers from IDT in a lab-ready format at 100 µM. However, if primers have been ordered lyophilised, they should be resuspended in water or low-EDTA TE buffer to a final concentration of 100 µM.

IMPORTANT

We recommend handling the primer stocks and derivatives in a clean pre-PCR hood.

1 In the pre-PCR hood, prepare the following master mixes in Eppendorf DNA LoBind tubes and mix thoroughly as follows:

For **x24** samples:

Reagent	Pool A	Pool B
RNase-free water	270 µl	270 µl
Primer pool A (100 µM)	10.4 µl	-
Primer pool B (100 µM)	-	10.4 µl
Q5® Hot Start HF 2x Master Mix	350 µl	350 µl
Total	630.4 µl	630.4 µl

For **x48** samples:

Reagent	Pool A	Pool B
RNase-free water	530 µl	530 µl
Primer pool A (100 µM)	20.5 µl	-
Primer pool B (100 µM)	-	20.5 µl
Q5® Hot Start HF 2x Master Mix	690 µl	690 µl
Total	1240.5 µl	1240.5 µl

For **x96** samples:

Reagent	Pool A	Pool B
RNase-free water	1060 µl	1060 µl
Primer pool A (100 µM)	41 µl	-
Primer pool B (100 µM)	-	41 µl
Q5® Hot Start HF 2x Master Mix	1375 µl	1375 µl
Total	2476 µl	2476 µl

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

End-prep

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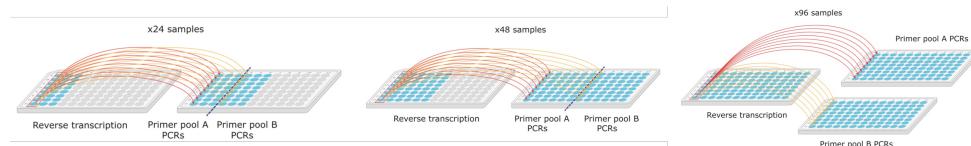
2 Using a stepper pipette or a multichannel pipette, aliquot 22.5 µl of Pool A and Pool B into a clean 96-well plate(s) as follows:

Plate location	X24 samples	X48 samples	X96 samples
Columns	Pool A: 1-3 Pool B: 4-6	Pool A: 1-6 Pool B: 7-12	Pool A: 1-12 Pool B: 1-12

Note: For x96 samples, Pool A is a separate plate to Pool B.

3 Using a multichannel pipette, transfer 2.5 µl of each RT reaction from the RT plate to the corresponding well for both Pool A and Pool B of the PCR plate(s). Mix by pipetting the contents of each well up and down.

There should be two PCR reactions per sample.



IMPORTANT

Carry forward the negative control from the reverse transcription reaction to monitor cross-contamination events.

We recommend having a single negative for every plate of samples and a standard curve of positive controls.

4 Seal the plate(s) and spin down.

5 Incubate using the following program, with the heated lid set to 105°C:

Step	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	15 sec	25–35
Annealing and extension	65°C	5 min	
Hold	4°C	∞	

Note: Cycle number should be varied for low or high viral load samples. Guidelines provided by Josh Quick suggest that 25 cycles should be used for Ct 18–21 up to a maximum of 35 cycles for Ct 35, however this has not been tested here.

END OF STEP

When PCR reaches 30–35 cycles, assemble the Dilution Plate and End-prep Plate in the next section.

End-prep

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

End-prep

Version: PTCE_9122_v109_revH_10Feb2021

~30 minutes

Consumables

- NEBNext Ultra II End repair/dA-tailing Module (E7546)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Cat # 0030129504) with heat seals
- 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- Multichannel pipettes suitable for dispensing 0.5–10 µl, 2–20 µl and 20–200 µl, and tips
- P1000 pipette and tips
- P200 pipette and tips
- Thermal cycler
- Microfuge
- Centrifuge capable of taking 96-well plates
- Ice bucket with ice

Optional Equipment

- PCR-Cooler (Eppendorf)
- Stepper pipette and tips

IMPORTANT

We recommended carrying the RT negative control through this step until sequencing.

1 Prepare the NEBNext Ultra II End Repair/dA-Tailing Module reagents according to the manufacturer's instructions, and place on ice.

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 s may be required to solubilise all precipitate.

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

2 During the final few cycles of the PCR, prepare the Dilution Plate. Using a stepper pipette, or a multichannel pipette, aliquot 45 µl of nuclease-free water per well of a clean 96-well plate (Dilution Plate).

Depending on the number of samples, aliquot into each well of the columns as follows:

Plate location	x24 samples	x48 samples	x96 samples
Columns	1-3	1-6	1-12

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

End-prep

Version: PTCE_9122_v109_revH_10Feb2021

3 Prepare the following end-prep master mix in a 1.5 ml Eppendorf DNA LoBind tube and mix thoroughly.

Reagent	Volume x24 samples	Volume x48 samples	Volume x96 samples
Nuclease-free water	140 µl	280 µl	560 µl
NEBNext Ultra II End Prep Reaction Buffer	32.7 µl	65.3 µl	130.7 µl
NEBNext Ultra II End Prep Enzyme Mix	14 µl	28 µl	56 µl
Total	186.7 µl	373.3 µl	746.7 µl

4 Using a stepper pipette, or a multichannel pipette, aliquot 6.7 µl of the end-prep master mix per well of a clean 96-well plate (End-prep Plate). Keep on ice.

Depending on the number of samples, aliquot into each well of the columns as follows:

Plate location	x24 samples	x48 samples	x96 samples
Columns	1-3	1-6	1-12

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

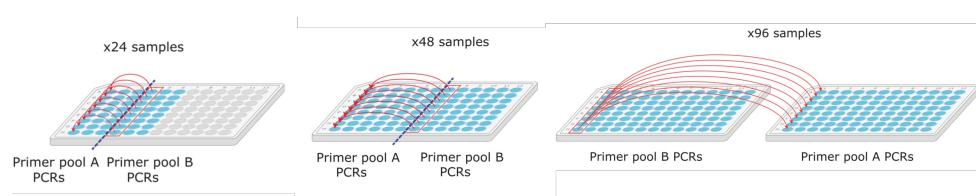
End-prep

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5 Once PCR is complete, using a multichannel pipette, transfer 25 µl of each well of PCR Pool B to the corresponding well of PCR Pool A and mix by pipetting.

Depending on the number of samples, Pool B columns will correspond to different Pool A columns.

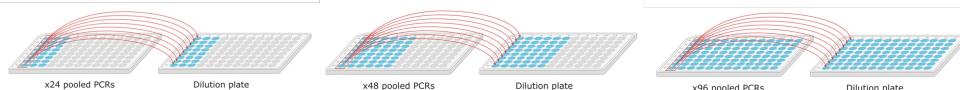
No. of samples	Pool B column	Corresponding Pool A column
x24	4	1
	5	2
	6	3
x48	7	1
	8	2
	9	3
	10	4
	11	5
	12	6
x96	1	1
	2	2
	3	3
	4	4
	5	5
	6	6
	7	7
	8	8
	9	9
	10	10
	11	11
	12	12



6 Using a multichannel pipette, transfer 5 µl from each well of PCR Pool A (now containing pooled PCR products) to the corresponding well of the Dilution Plate and mix by pipetting.

Depending on the number of samples, PCR Pool A will be in each well of the following columns:

Plate location	x24 samples	x48 samples	x96 samples
Columns	1-3	1-6	1-12

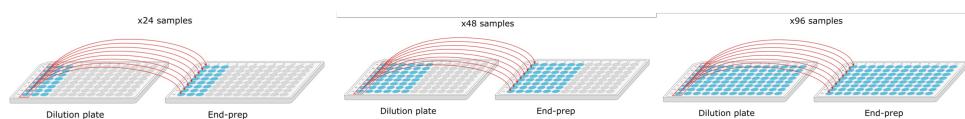


Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Native barcode ligation

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7 Using a multichannel pipette, transfer 3.3 µl from each filled well of the Dilution Plate to the corresponding well of the End-prep Plate and mix by pipetting.



8 Seal the End-prep Plate and spin down.

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

END OF STEP

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

Native barcode ligation

~75 minutes

Materials

- Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) if multiplexing 24 samples
- Native Barcoding Expansion 96 (EXP-NBD196)
- Short Fragment Buffer (SFB)

Consumables

- Freshly-prepared 80% ethanol in nuclease-free water
- NEB Blunt/TA Ligase Master Mix (M0367)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Cat # 0030129504) with heat seals
- 1.5 ml Eppendorf DNA LoBind tubes
- Agencourt RNase-free XP beads

Equipment

- Multichannel pipettes suitable for dispensing 0.5–10 µl, 2–20 µl and 20–200 µl, and tips
- Thermal cycler
- Microfuge
- Centrifuge capable of taking 96-well plates
- Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- Hula mixer (gentle rotator mixer)
- Ice bucket with ice
- Qubit fluorometer (or equivalent for QC check)

Optional Equipment

- PCR-Cooler (Eppendorf)
- Stepper pipette and tips

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Native barcode ligation

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IMPORTANT

To monitor cross-contamination events, we recommend that the RT negative control is carried through this process and a barcode is used to sequence this control.

- 1 Thaw the native barcodes at room temperature, enough for one barcode per sample. Individually mix the barcodes by pipetting, and place them on ice.**
- 2 Thaw the tube of Short Fragment Buffer (SFB) at room temperature, mix by vortexing, spin down and place on ice.**
- 3 Select a unique barcode for every sample to be run.**
- 4 Using a stepper pipette, or a multichannel pipette, make up the NB Ligation Plate in a clean 96-well plate. Add the reagents in the following order per well:**

Reagent	Volume x24 samples	Volume x48 samples	Volume x96 samples
Nuclease-free water	6 µl	3 µl	3 µl
End-prepped DNA Note: Transfer to the corresponding well	1.5 µl	0.75 µl	0.75 µl
Native barcode Note: Transfer to the corresponding well	2.5 µl	1.25 µl	1.25 µl
Blunt/TA Ligation Master Mix	10 µl	5 µl	5 µl
Total	20 µl	10 µl	10 µl

Depending on the number of samples, aliquot to each well of the columns:

Plate location	x24 samples	x48 samples	x96 samples
Columns	1-3	1-6	1-12

- 5 Mix the contents thoroughly by pipetting.**
- 6 Seal the plate and spin down briefly.**
- 7 Using a thermal cycler, incubate at 20°C for 20 mins and at 65°C for 10 mins.**

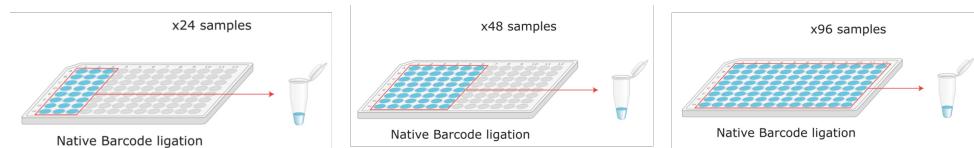
Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Native barcode ligation

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8 Pool the barcoded samples.

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



9 Take forward 480 µl of the pooled barcoded samples.

TIP

For x96 samples, there will be enough pooled reaction remaining for a second library to be prepared. This can be stored at -20°C for later use.

10 Resuspend the AMPure XP beads by vortexing.

11 Add 192 µl of resuspended AMPure XP beads to the 480 µl of pooled reaction and mix by pipetting.

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

13 Prepare 500 µl of fresh 80% ethanol in nuclease-free water.

14 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.

15 Wash the beads by adding 700 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Keep the tube on the magnet until the eluate is clear and colourless. Remove the supernatant using a pipette and discard.

16 Repeat the previous step.

17 Keep the tube on the magnet and wash the beads with 100 µl of freshly-prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

18 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.

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Adapter ligation and clean-up

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19 Remove the tube from the magnetic rack and resuspend the pellet in 35 µl nuclease-free water. Incubate for 2 minutes at room temperature.

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.

22 Quantify 1 µl of eluted sample using a Qubit fluorometer - recovery aim 2 ng/µl.

END OF STEP

Take forward 30-50 ng of pooled barcoded samples in 30 µl into the adapter ligation and clean-up step.

Adapter ligation and clean-up

~20 minutes

Materials

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

Consumables

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

Equipment

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

Optional Equipment

- Qubit fluorometer (or equivalent for QC check)

Adapter Mix II Expansion use

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

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

1 Thaw the Elution Buffer (EB), Short Fragment Buffer (SFB), and NEBNext Quick Ligation Reaction Buffer (5x) at room temperature, mix by vortexing, spin down and place on ice. Check the contents of each tube are clear of any precipitate.

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Adapter ligation and clean-up

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2 Spin down the T4 Ligase and the Adapter Mix II (AMII), and place on ice.

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

Reagent	Volume
Pooled barcoded sample (30-50 ng)	30 µl
Adapter Mix II (AMII)	5 µl
NEBNext Quick Ligation Reaction Buffer (5X)	10 µl
Quick T4 DNA Ligase	5 µl
Total	50 µl

4 Mix gently by flicking the tube, and spin down.

5 Incubate the reaction for 20 minutes at room temperature.

IMPORTANT

The next clean-up step uses SFB (Short Fragment Buffer) and not 80% ethanol to wash the beads. The use of ethanol will significantly damage the sequencing reaction.

6 Resuspend the AMPure XP beads by vortexing.

7 Add 20 µl of resuspended AMPure XP beads to the reaction and mix by pipetting.

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

9 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.

10 Wash the beads by adding 125 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Keep the tube on the magnet until the eluate is clear and colourless. Remove the supernatant using a pipette and discard.

11 Repeat the previous step.

12 Spin down and place the tube back on the magnet. Pipette off any residual supernatant.

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Priming and loading the SpotON flow cell

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13 Remove the tube from the magnetic rack and resuspend the pellet by pipetting in 15 µl Elution Buffer (EB). Spin down and incubate for 5 minutes at room temperature.

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

15 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

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

IMPORTANT

We recommend loading ~15 ng (15-20 ng for R10.3 flow cells) of this final prepared library onto the flow cell. Loading more than 15 ng can have a detrimental effect on throughput. Dilute the library in EB if required.

END OF STEP

The prepared library is used for loading onto the MinION Mk1B flow cell. Store the library on ice until ready to load.

TIP

Library storage recommendations

We recommend storing libraries in Eppendorf 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 LoBind tubes.

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

Optional Action

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

Additional buffer for doing this can be found in the Sequencing Auxiliary Vials expansion (EXP-AUX001), available to purchase separately. This expansion also contains additional vials of Sequencing Buffer (SQB) and Loading Beads (LB), required for loading the libraries onto flow cells.

Priming and loading the SpotON flow cell

~10 minutes

Materials

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

Consumables

- 1.5 ml Eppendorf DNA LoBind tubes

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Priming and loading the SpotON flow cell

Version: PTCE_9122_v109_revH_10Feb2021

- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

Equipment

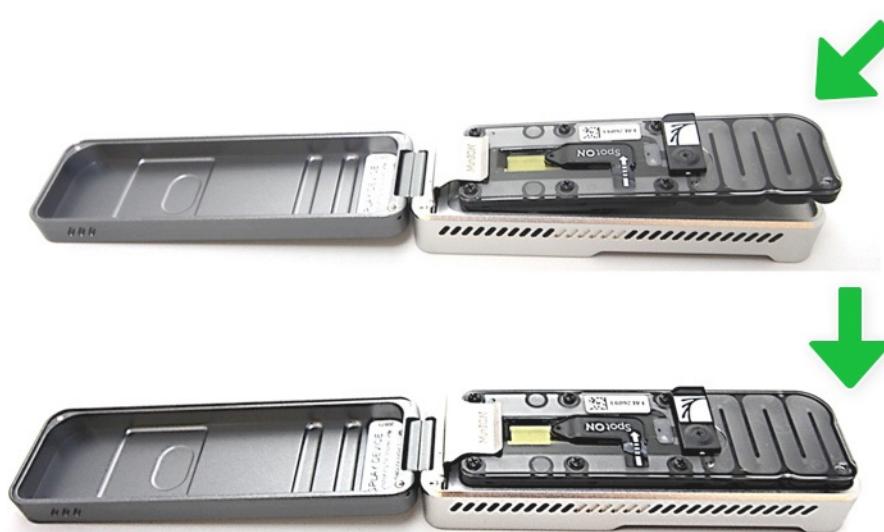
- MinION Mk1B
- SpotCN Flow Cell
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

IMPORTANT

Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.

- 1 Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature.
- 2 Mix the Sequencing Buffer (SQB), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing, spin down at room temperature.
- 3 Open the MinION Mk1B lid and slide the flow cell under the clip.

Press down firmly on the flow cell to ensure correct thermal and electrical contact.



Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Priming and loading the SpotON flow cell

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



How to prime and load the SpotON Flow Cell

Priming and loading: The steps for priming and loading the SpotON Flow Cell. Written instructions are given below. The library is loaded dropwise without putting the pipette tip firmly into the port.

Take care to avoid introducing any air during pipetting.

IMPORTANT

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

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

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

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

6 Prepare the flow cell priming mix

- Add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing.

7 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.

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

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Priming and loading the SpotON flow cell

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IMPORTANT

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

TIP

Using the Loading Beads

Demo of how to use the Loading Beads.

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

Reagent	Volume
Sequencing Buffer (SQB)	37.5 µl
Loading Beads (LB), mixed immediately before use	25.5 µl
DNA library	12 µl
Total	75 µl

10 Complete the flow cell priming:

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

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

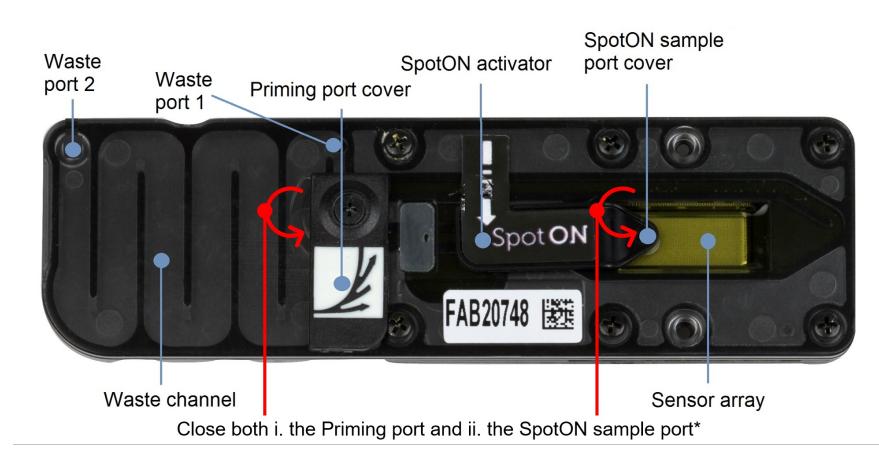
12 Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Sequencing and data analysis

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13 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION Mk1B 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, or that you are using the MinIT device for data acquisition and basecalling. There are three options for how to carry out sequencing:

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

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

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

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

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

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

4. Data acquisition using MinKNOW on a computer and basecalling at a later time using Guppy

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Sequencing and data analysis

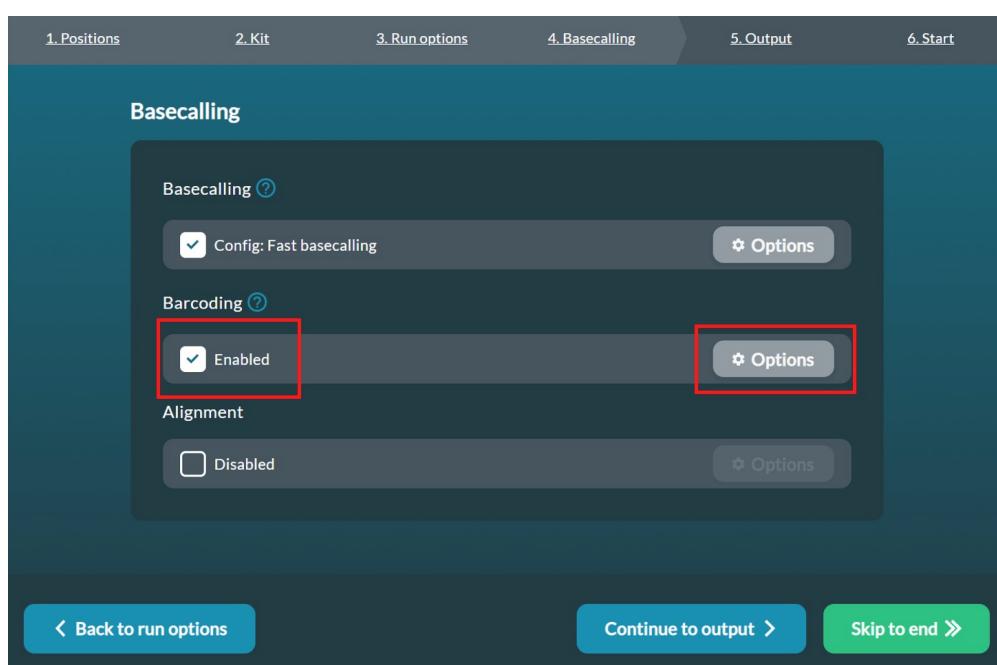
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Follow the instructions in the [MinKNOW protocol](#) beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section. **When setting your experiment parameters, set the **Basecalling** tab to OFF.** After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

Required settings in MinKNOW

When setting the sequencing parameters in MinKNOW, in the **Basecalling** set barcoding as **Enabled**, and in the barcoding options, toggle **Barcode both ends** and **Mid-read barcodes** to ON.

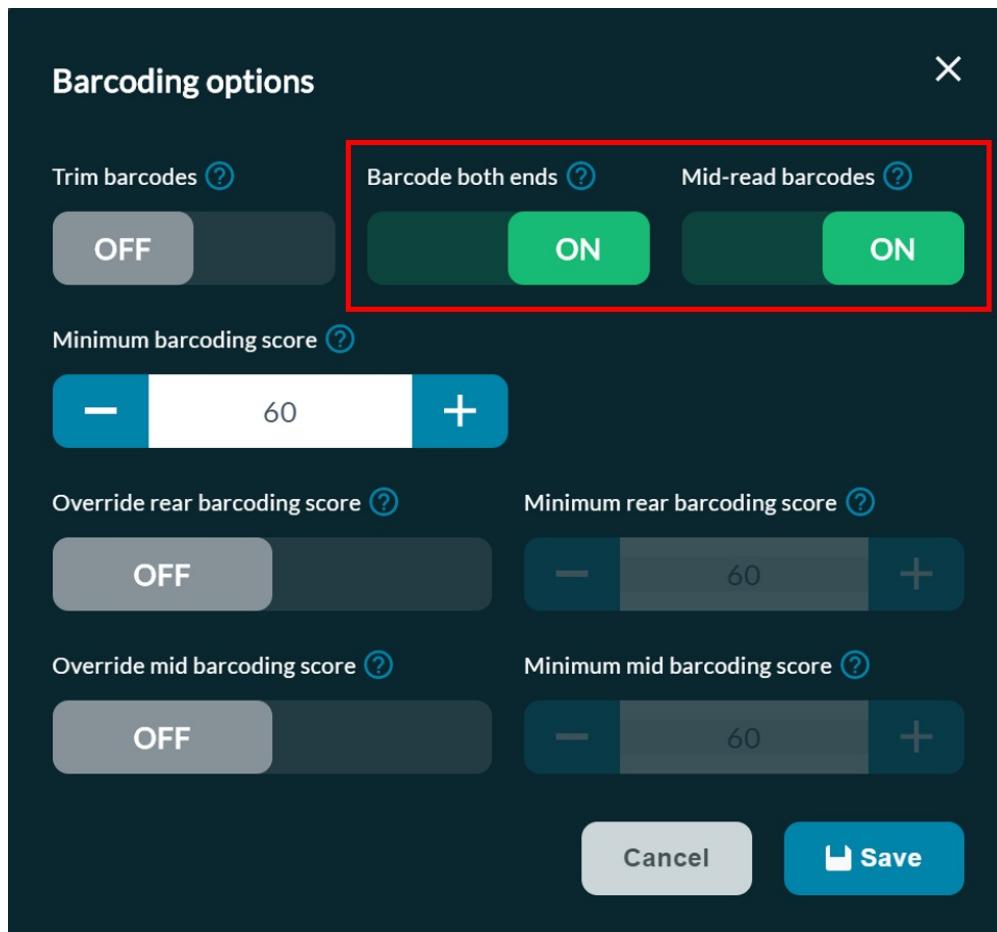
Optional: basecalling and/or demultiplexing of sequences can be performed using the stand-alone Guppy software.



Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Downstream analysis and expected results

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Downstream analysis and expected results

Recommended analysis pipeline

The recommended workflows for the bioinformatics analyses are provided by the ARTIC network and are documented on their web pages at <https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.html>.

The reference guided genome assembly and variant calling are also performed according to the bioinformatics protocol provided by the ARTIC network. Their best practices guide uses the software contained within the [FieldBioinformatics project on GitHub](#).

This workflow uses only the basecalled FASTQ files to perform a high-quality reference-guided assembly of the SARS-CoV-2 genome. Sequenced reads are re-demultiplexed with the requirement that reads must contain a barcode at both ends of the sequence (this only applies to the Classic and Eco PCR tiling of SARS-CoV-2 protocols but not the Rapid Barcoding PCR tiling of SARS-CoV-2), and must not contain internal barcodes. The reads are mapped to the reference genome, primer sequences are excluded and the consensus sequence is polished. The Medaka software is used to call single-nucleotide variants while the ARTIC software reports the high-quality consensus sequence from the workflow.

To further simplify the installation of the coronavirus bioinformatics protocols, the workflows have been packaged into two EPI2ME products

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Downstream analysis and expected results

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The FieldBioinformatics workflow for SARS-CoV-2 sequence analysis is provided as a Jupyter notebook tutorial in the [EPI2ME Labs](#) software. The coronavirus workflow has been augmented to include additional steps that help with the quality control of individual libraries, and aid in the presentation of summary statistics and the final sets of called variants.

The FieldBioinformatics workflow for SARS-CoV-2 sequence analysis is also provided as an [EPI2ME workflow](#) – this provides a more accessible interface to a bioinformatics workflow and the provided cloud-based analysis also performs some secondary interpretation by preparing an additional report using the [Nextclade](#) software.

Expected results

Here, results are shown based on human coronavirus 229E spiked into 100 ng of human RNA derived from GM12878 cell line. 10 pg–0.001 pg of viral RNA obtained from ATCC was spiked into the human RNA and human-only and reverse transcription negative controls were carried through the prep to sequencing. Every sample underwent 30 and 35 cycles of PCR to determine sensitivity and specificity guidelines, as well as the expected amplicon drop-out rate for each sample.

Note: The viral RNA from ATCC is generated from cell lines infected with human coronavirus 229E. The RNA supplied is total RNA extracted from the cell lines and includes both human and viral RNA. Therefore, the levels of sensitivity are likely to be higher than those reported here.

Sample balancing

The graph below shows the expected sequence balancing if the protocol is followed. Here, equal masses went into the end-prep and native barcode ligation prior to pooling by equal mass for adapter ligation.

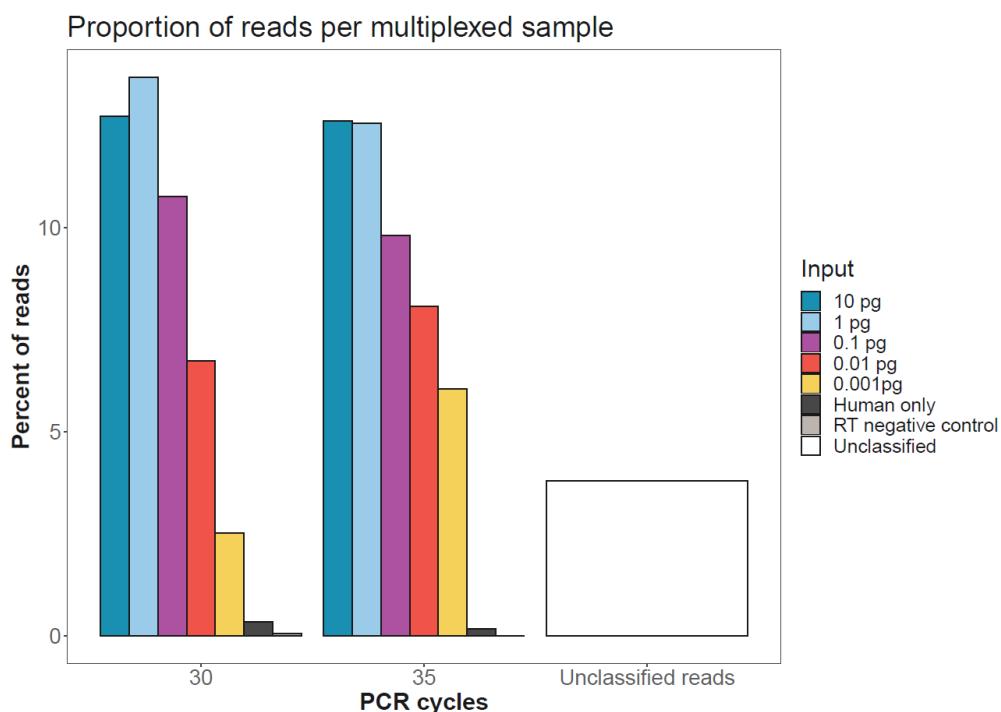


Figure 3. Number of reads per sample after native barcode demultiplexing in MinKNOW. All 14 samples were run on a single flow cell.

On-target rate

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Downstream analysis and expected results

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Sequences from each demultiplexed sample were aligned to the human coronavirus 229E genome using *minimap2*. The proportion of primary alignments per sample are reported below.

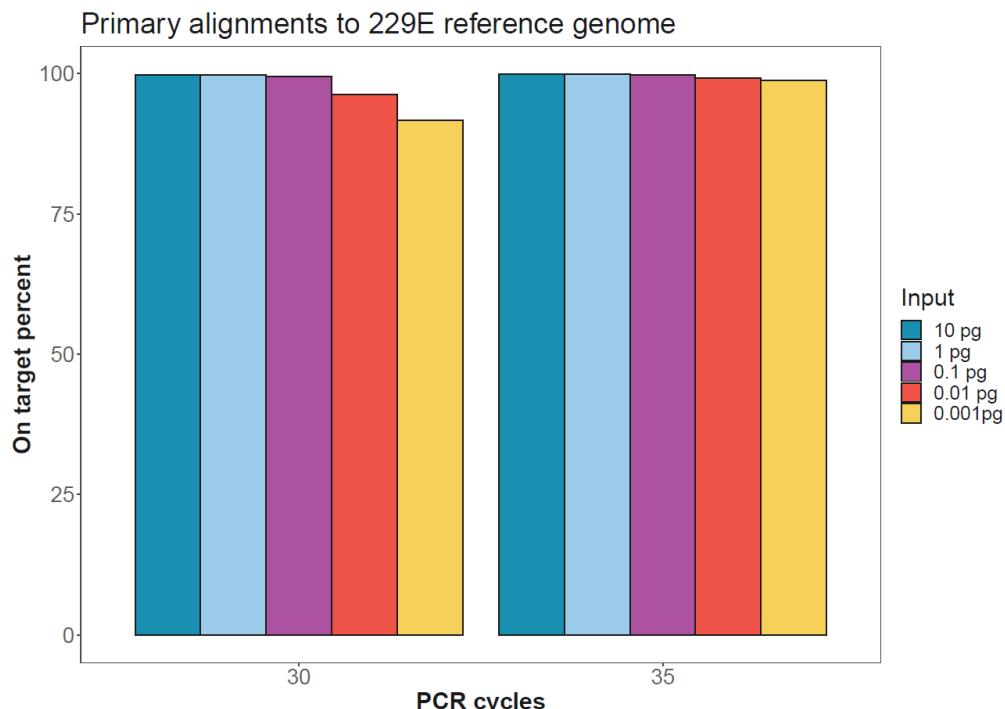


Figure 4. Proportion of reads for each sample aligning to the human coronavirus 229E reference genome.

Assessment of negative controls

After 12 hours of sequencing, the number of reads from the negative control samples aligning to the viral reference genome is shown in the graph below and is compared with the absolute number of sequences aligning to the lowest input (0.001 pg).

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Downstream analysis and expected results

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Figure 5. Absolute number of reads aligning to the human coronavirus 229E reference genome in the negative controls compared with the lowest input of viral RNA. Sequencing was carried out for 12 hours to pick up low levels of sequences assigned to barcodes representing these samples.

Target coverage for different PCR cycles and viral load

To assess the impact of PCR dropout with lowering input viral load and increasing PCR cycles, *Mosdepth* was used to calculate the proportion of the viral genome covered to different depth levels. These numbers were calculated after 12 hours of sequencing with 14 samples multiplexed.

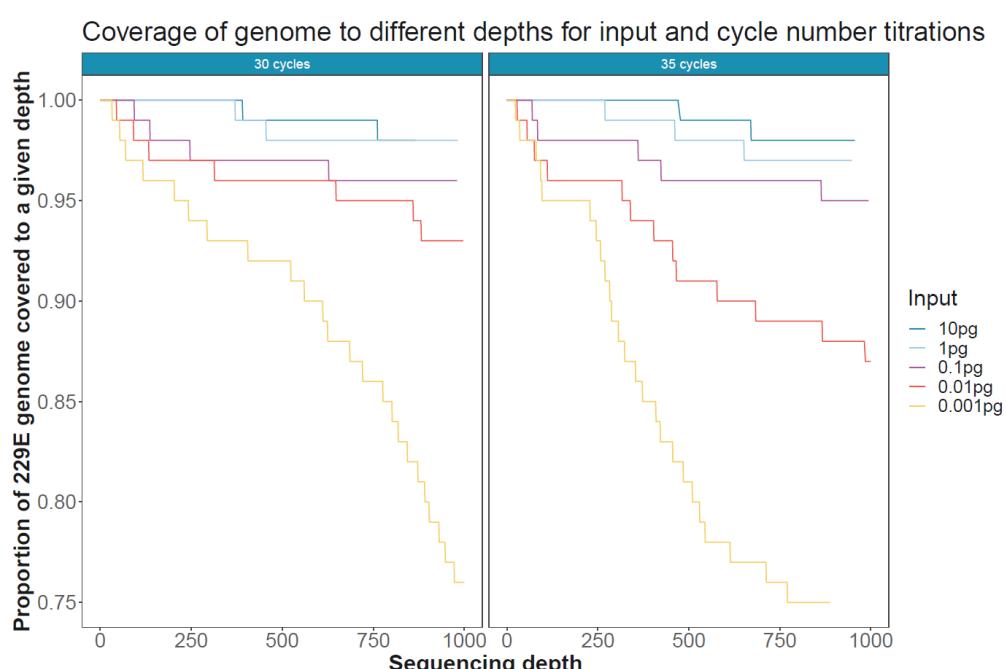


Figure 6. Coverage and depth of the human coronavirus 229E genome for different input quantities of viral RNA and different cycle numbers after 12 hours of

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Ending the experiment

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sequencing on a single flow cell.

How much sequencing is required?

This is unknown in real clinical samples. The graph below can be used to determine the proportion of the genome that could be covered to a given depth with different numbers of reads (30 cycles) at different input amounts in a background of 100 ng human RNA.

Note: this is absolute depth.

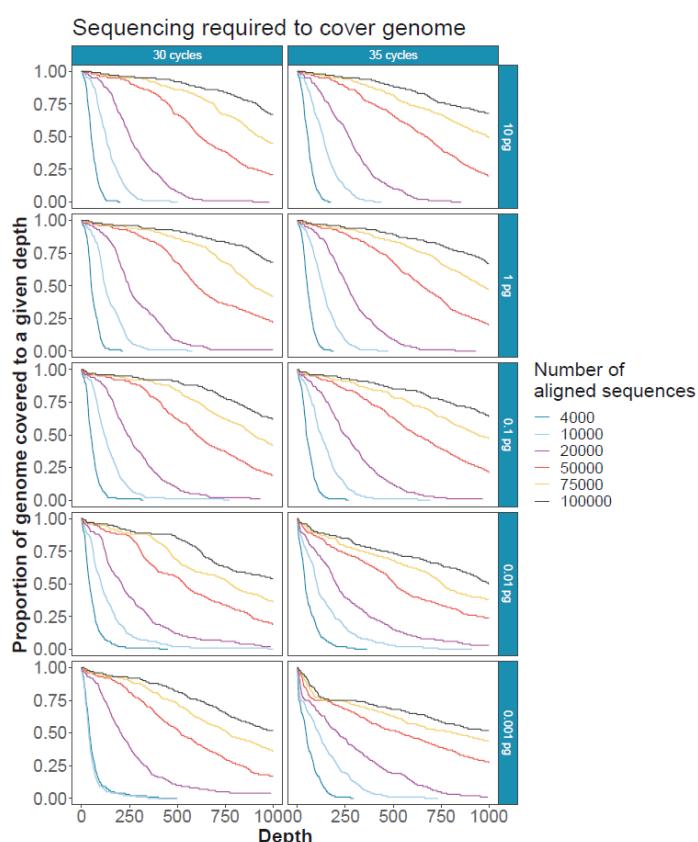


Figure 7. Subsampled sequences to give an indication of the depth of sequencing achievable covering different amounts of the human coronavirus 229E genome. Input quantities and cycle number titrations show that high cycle numbers should be avoided where possible to minimise amplicon drop out.

This protocol provides amplification of low copy number viral genomes in a tiled method with low off-target amplification and minimal cross-contamination between samples. With <60 copies per reaction (0.001 pg viral input) in 100 ng background human RNA, under ideal circumstances, one should expect to cover >75% of the targeted genome at a depth of 200X within under 50,000 reads in the samples with the lowest viral titre and <20,000 reads in those with a higher viral titre.

Ending the experiment

Materials

- Flow Cell Wash Kit (EXP-WSH004)

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Troubleshooting

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- 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.

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

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

IMPORTANT

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

Issues during DNA/RNA extraction and library preparation

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

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

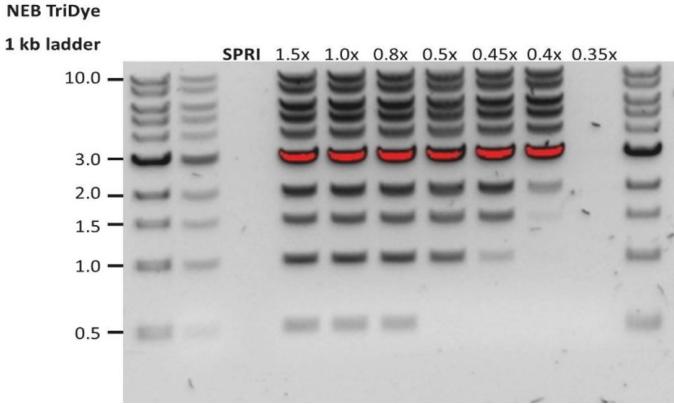
Observation	Possible cause	Comments and actions
Low DNA purity (Nanodrop reading for DNA OD 260/280 is <1.8 and OD 260/230 is <2.0-2.2)	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the Contaminants Know-how piece. Please try an alternative extraction 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

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Troubleshooting

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

The VolTRAX run terminated in the middle of the library prep

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

The VolTRAX software shows an inaccurate amount of reagents loaded

Observation	Possible cause	Comments and actions
The VolTRAX software shows an inaccurate amount of reagents loaded	Pipette tips do not fit the VolTRAX cartridge ports	TRainin 20 µl or 30 µl and Gilson 10 µl, 20 µl or 30 µl pipette tips are compatible with loading reagents into the VolTRAX cartridge. Rainin 20 µl is the most suitable.

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Issues during the sequencing run

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Observation	Possible cause	Comments and actions
The VoITRAX software shows an inaccurate amount of reagents loaded	The angle at which reagents are pipetted into the cartridge is incorrect	The pipetting angle should be slightly greater than the cartridge inlet angle. Please watch the demo video included in the VoITRAX software before loading.

Issues during the sequencing run

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

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

Eco PCR tiling of SARS-CoV-2 virus with native barcoding (EXP-NBD104, EXP-NBD114, and EXP-NBD196)

Issues during the sequencing run

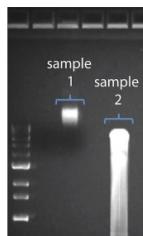
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Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support.

Pore occupancy below 40%

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

Shorter than expected read length

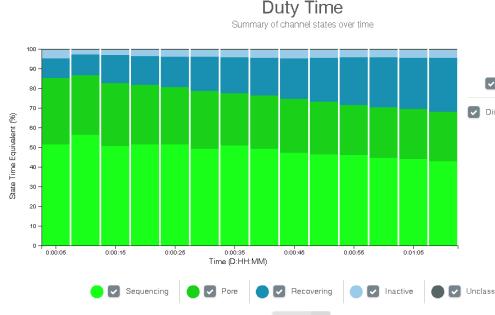
Observation	Possible cause	Comments and actions
Shorter than expected read length	Unwanted fragmentation of DNA sample	<p>Read length reflects input DNA fragment length. Input DNA can be fragmented during extraction and library prep.</p> <ol style="list-style-type: none"> 1. Please review the Extraction Methods in the Nanopore Community for best practice for extraction. 2. Visualise the input DNA fragment length distribution on an agarose gel before proceeding to the library prep.  <p>In the image above, Sample 1 is of high molecular weight, whereas Sample 2 has been fragmented.</p> <ol style="list-style-type: none"> 3. During library prep, avoid pipetting and vortexing when mixing reagents. Flicking or inverting the tube is sufficient.

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Issues during the sequencing run

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Large proportion of recovering pores

Observation	Possible cause	Comments and actions
Large proportion of recovering pores (shown as dark blue in the channels panel and duty time plot)	Contaminants are present in the sample	<p>Some contaminants can be cleared from the pores by the unblocking function built into MinKNOW. If this is successful, the pore status will change to "single pores". If the portion of recovering pores (unavailable pores in the extended view) stays large or increases:</p> <ol style="list-style-type: none"> 1. A nuclease flush can be performed, or 2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.  <p>The duty time plot above shows an increasing proportion of "recovering" pores over the course of a sequencing experiment</p>

Large proportion of inactive pores

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

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

Observation	Possible cause	Comments and actions
Reduction in sequencing speed and q-score later into the run	Fast fuel consumption is typically seen when the flow cell is overloaded with library (~5–50 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.

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Issues during the sequencing run

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Temperature fluctuation

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

Failed to reach target temperature

Observation	Possible cause	Comments and actions
MinKNOW shows "Failed to reach target temperature" (37°C for Flow Cell Check, 34°C for sequencing on MinION Mk 1B/PromethION flow cells, and 35°C for sequencing on Flongle)	The instrument was placed in a location that is colder than normal room temperature, or a location with poor ventilation (which leads to the flow cells overheating)	MinKNOW has a default timeframe for the flow cell to reach the target temperature. Once the timeframe is exceeded, an error message will appear and the sequencing experiment will continue. However, sequencing at an incorrect temperature may lead to a decrease in throughput and lower q-scores. Please adjust the location of the sequencing device to ensure that it is placed at room temperature with good ventilation, then re-start the process in MinKNOW. Please refer to this FAQ for more information on MinION Mk 1B temperature control.

Guppy – no input .fast5 was found or basecalled

Observation	Possible cause	Comments and actions
No input .fast5 was found or basecalled	<i>input_path</i> did not point to the .fast5 file location	The <code>--input_path</code> has to be followed by the full file path to the .fast5 files to be basecalled, and the location has to be accessible either locally or remotely through SSH.
No input .fast5 was found or basecalled	The .fast5 files were in a subfolder at the <i>input_path</i> location	To allow Guppy to look into subfolders, add the <code>--recursive</code> 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 <code>--qscore_filtering</code> flag was not included in the command	The <code>--qscore_filtering</code> flag enables filtering of reads into Pass and Fail folders inside the output folder, based on their strand q-score. When performing live basecalling in MinKNOW, a q-score of 7 (corresponding to a basecall accuracy of ~80%) is used to separate reads into Pass and Fail folders.

Guppy – unusually slow processing on a GPU computer

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Issues during the sequencing run

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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 <code>--device cuda:0 cuda:1</code> , when 2 GPUs are specified to use by the Guppy command.

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

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

MinIT – the MinIT software cannot be updated

Observation	Possible cause	Comments and actions
The MinIT software cannot be updated	The firewall is blocking IPs for update	Please consult your IT department, as the MinIT software requires access to the following AWS IP ranges . Access to the following IP addresses is also needed: <code>178.79.175.200</code> <code>96.126.99.215</code>
The MinIT software cannot be updated	The device already has the latest version of the software	Occassionally, 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, <code>dpkg -l grep minit</code> , to find out the version of the MinIT software and <code>sudo apt update</code> if an update is needed. If the issue still persists, please contact Technical Services with details of the error.