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.

The **PCR tiling of SARS-CoV-2 virus with Rapid Barcoding Kit 96 V14 and Midnight RT PCR Expansion (SQK-RBK114.96 and EXP-MRT001)** protocol is an updated version of the PCR tiling of SARS-CoV-2 virus with rapid barcoding and Midnight RT PCR Expansion (SQK-RBK110.96 and EXP-MRT001) using our most recent Kit 14 chemistry and an updated downstream analysis.

Introduction to the protocol

To enable support for the rapidly expanding user requests, the team at Oxford Nanopore Technologies have put together an updated workflow based on the ARTIC Network protocols and analysis methods. The protocol uses Oxford Nanopore Technologies' Rapid Barcoding Kit 96 V14 (SQK-RBK114.96) and Midnight RT PCR Expansion (EXP-MRT001) for barcoding and library preparation.

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 similar to the ARTIC amplicon sequencing protocol for MinION for SARS-CoV-2 v3 (LoCost) by Josh Quick and the method used in Freed et al., 2020. The protocol generates amplicons in a tiled fashion across the whole SARS-CoV-2 genome.

To generate tiled PCR amplicons from the SARS-CoV-2 viral cDNA for use with the Rapid Barcoding Kit 96 V14 (SQK-RBK114.96), primers were designed by Freed *et al.*, 2020 using Primal Scheme. These primers are in the Midnight RT PCR Expansion (EXP-MRT001) and are designed to generate 1.2 kb amplicons. Primer sequences can be found here.

Steps in the sequencing workflow:

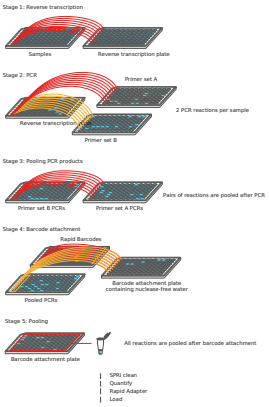
Prepare for your experiment
you will need to:

- Extract your RNA
- Ensure you have your sequencing kit, the correct equipment and 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
- Attach Rapid Barcodes supplied in the kit to the DNA ends, pool the samples and SPRI purify

- 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, selecting SQK-RBK114.96 in kit selection, which will collect raw data from the device and convert it into basecalled reads
- **(Optional)**: Perform downstream analysis of the data using the wf-artic analysis workflow integrated within the EPI2ME Labs application

Before starting

This protocol outlines how to carry out PCR tiling of SARS-CoV-2 viral RNA samples on a 96-well plate using the Rapid Barcoding Kit 96 V14 (SQK-RBK114.96) with the Midnight RT PCR Expansion (EXP-MRT001).

It is required to use total RNA extracted from samples that have been screened by a suitable qPCR assay.

When processing multiple samples at once, we recommend making master mixes with an additional 10% of the volume. We also recommend using a template-free pre-PCR hood for making up the master mixes, and a separate template pre-PCR hood for handling the 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.

All post-PCR procedures must be carried out in a separate area to the pre-PCR preparation, with dedicated equipment for liquid handling in each area.

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Rapid Barcoding Kit 96 V14 (SQK-RBK114.96)
- Midnight RT PCR Expansion (EXP-MRT001)
- R10.4.1 flow cells (FLO-MIN114)
- Flow Cell Wash Kit (EXP-WSH004)

Equipment and consumables

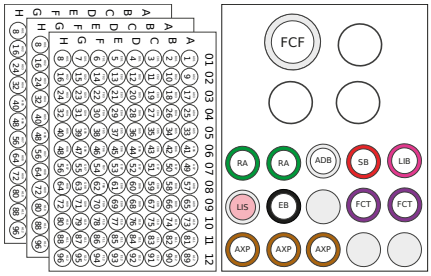
Materials	• Input RNA in 10 mM Tris-HCl, pH 8.0 • Rapid Barcoding Kit 96 V14 (SQK-RBK114.96) • Midnight RT PCR Expansion (EXP-MRT001)
Consumables	• Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • Freshly prepared 80% ethanol in nuclease-free water • Qubit dsDNA HS Assay Kit (Invitrogen, Q32851) • Qubit™ Assay Tubes (Invitrogen, Q32856) • 1.5 ml Eppendorf DNA LoBind tubes • 2 ml Eppendorf DNA LoBind tubes • 5 ml Eppendorf DNA LoBind tubes • Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Cat # 0030129504) with PCR seals • (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)
Equipment	• Hula mixer (gentle rotator mixer) • Magnetic rack • Centrifuge capable of taking 96-well plates • 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 • P100 pipette and tips • P20 pipette and tips • P10 pipette and tips • Ice bucket with ice • Timer • Qubit fluorometer (or equivalent)
Optional equipment	• Eppendorf 5424 centrifuge (or equivalent) • PCR hood with UV steriliser (optional but recommended to reduce cross-contamination) • PCR-Cooler (Eppendorf) • Stepper pipette and tips

For this protocol, you will need your extracted RNA in 8 µl 10 mM Tris-HCl, pH 8.0.

IMPORTANT

The Rapid Adapter (RA) used in this kit and protocol is not interchangeable with other sequencing adapters.

Rapid Barcoding Kit 96 V14 (SQK-RBK114.96) contents

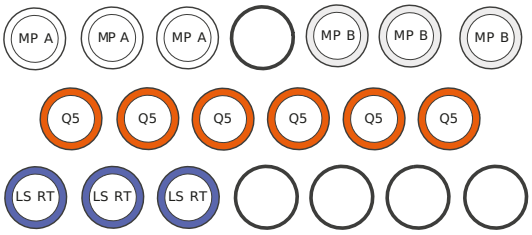


AXP: AMPure XP Beads
FCF: Flow Cell Flush
ADB: Adapter Buffer
RA: Rapid Adapter
LIB: Library Beads
EB: Elution Buffer
LIS: Library Solution
FCT: Flow Cell Tether
SB: Sequencing Buffer

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Rapid Adapter	RA	Green	2	15
Adapter Buffer	ADB	Clear	1	100
AMPure XP Beads	AXP	Amber	3	1,200
Elution Buffer	EB	Black	1	1,500
Sequencing Buffer	SB	Red	1	1,700
Library Beads	LIB	Pink	1	1,800
Library Solution	LIS	White cap, pink label	1	1,800
Flow Cell Flush	FCF	Clear	1	15,500
Flow Cell Tether	FCT	Purple	2	200
Rapid Barcodes	RB01-96	-	3 plates	8 µl per well

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.

Midnight RT PCR Expansion (EXP-MRT001) contents



MP A: Midnight Primer Pool A Q5: Q5 HS Master Mix
MP B: Midnight Primer Pool B LS RT: LunaScript RT SuperMix

Name	Acronym	Cap colour	Number of vials	Fill volume per vial (µl)
LunaScript RT SuperMix	LS RT	Blue	3	500
Q5 HS Master Mix	Q5	Orange	6	1,500
Midnight Primer Pool A	MP A	White	3	15
Midnight Primer Pool B	MP B	Clear	3	15

Midnight Primer sequences

Below are the sequences for the V3 primer scheme used in the Midnight RT PCR Expansion.

Pool A

Primer name	Primer Sequence
SARSCoV_1200_1_LEFT	ACCAACCAACTTTCGATCTCTTGT
SARSCoV_1200_1_RIGHT	GGTTGCATTCATTTGGTGACGC
SARSCoV_1200_3_LEFT	GGCTTGAAGAGAAGTTTAAGGAAGGT
SARSCoV_1200_3_RIGHT	GATTGTCCTCACTGCCGTCTTG
SARSCoV_1200_5_LEFT	ACCTACTAAAAAGGCTGGTGGC
SARSCoV_1200_5_RIGHT	AGCATCTTGTAGAGCAGGTGGA
SARSCoV_1200_7_LEFT	ACCTGGTGTATACGTTGTCTTTGG
SARSCoV_1200_7_RIGHT	GCTGAAATCGGGGCCATTTGTA
SARSCoV_1200_9_LEFT	AGAAGTTACTGGCGATAGTTGTAATAACT
SARSCoV_1200_9_RIGHT	TGCTGATATGTCCAAAGCACCA
SARSCoV_1200_11_LEFT	AGACACCTAAGTATAAGTTTGTTCGCA
SARSCoV_1200_11_RIGHT	GCCCACATGGAAATGGCTTGAT
SARSCoV_1200_13_LEFT	ACCTCTTACAACAGCAGCCAAAC

Primer name	Primer Sequence
SARSCoV_1200_13_RIGHT	CGTCCTTTTCTTGGAAGCGACA
SARSCoV_1200_15_LEFT	TTTTAAGGAATTACTTGTGTATGCTGCT
SARSCoV_1200_15_RIGHT	ACACACAACAGCATCGTCAGAG
SARSCoV_1200_17_LEFT	TCAAGCTTTTTGCAGCAGAAACG
SARSCoV_1200_17_RIGHT	CCAAGCAGGGTTACGTGTAAGG
SARSCoV_1200_19_LEFT	GGCACATGGCTTTGAGTTGACA
SARSCoV_1200_19_RIGHT	CCTGTTGTCCATCAAAGTGTCCC
SARSCoV_1200_21_LEFT	TCTGTAGTTTCTAAGGTTGTCAAAGTGA
SARSCoV_1200_21_RIGHT	GCAGGGGGTAATTGAGTTCTGG
21_right_spike	GTGTATGATTGAGTTCTGGTTGTAAG
SARSCoV_1200_23_LEFT	ACTTTAGAGTCCAACCAACAGAATCT
23_left_spike	ACTTTAGAGTTCAACCAACAGAATCT
SARSCoV_1200_23_RIGHT	TGACTAGCTACACTACGTGCCC
SARSCoV_1200_25_LEFT	TGCTGCTACTAAAATGTCAGAGTGT
SARSCoV_1200_25_RIGHT	CATTTCCAGCAAAGCCAAAGCC
SARSCoV_1200_27_LEFT	TGGATCACCGGTGGAATTGCTA
SARSCoV_1200_27_RIGHT	TGTTCGTTTAGGCGTGACAAGT
SARSCoV_1200_29_LEFT	TGAGGGAGCCTTGAATACACCA
SARSCoV_1200_29_RIGHT	TAGGCAGCTCTCCCTAGCATTG

Pool B

Primer name	Primer sequences
SARSCoV_1200_2_LEFT	CCATAATCAAGACTATTCAACCAAGGGT
SARSCoV_1200_2_RIGHT	ACAGGTGACAATTTGTCCACCG
SARSCoV_1200_4_LEFT	GGAATTTGGTGCCACTTCTGCT
SARSCoV_1200_4_RIGHT	CCTGACCCGGGTAAGTGGTTAT
SARSCoV_1200_6_LEFT	ACTTCTATTAAATGGGCAGATAACAACTG
SARSCoV_1200_6_RIGHT	GATTATCCATTCCCTGCGCGTC
SARSCoV_1200_8_LEFT	CAATCATGCAATTGTTTTTCAGCTATTTTG
SARSCoV_1200_8_RIGHT	TGACTTTTTGCTACCTGCGCAT
SARSCoV_1200_10_LEFT	TTTACCAGGAGTTTTCTGTGGTGT
SARSCoV_1200_10_RIGHT	TGGGCCTCATAGCACATTGGTA
SARSCoV_1200_12_LEFT	ATGGTGCTAGGAGAGTGTGGAC

Primer name	Primer sequences
SARSCoV_1200_12_RIGHT	GGATTTCCCACAATGCTGATGC
SARSCoV_1200_14_LEFT	ACAGGCACTAGTACTGATGTCGT
SARSCoV_1200_14_RIGHT	GTGCAGCTACTGAAAAGCACGT
SARSCoV_1200_16_LEFT	ACAACACAGACTTTATGAGTGTCTCT
SARSCoV_1200_16_RIGHT	CTCTGTCAGACAGCACTTCACG
SARSCoV_1200_18_LEFT	GCACATAAAGACAAATCAGCTCAATGC
SARSCoV_1200_18_RIGHT	TGTCTGAAGCAGTGGAAAAGCA
SARSCoV_1200_20_LEFT	ACAATTTGATACTTATAACCTCTGGAACAC
SARSCoV_1200_20_RIGHT	GATTAGGCATAGCAACACCCGG
SARSCoV_1200_22_LEFT	GTGATGTTCTTGTTAACAACTAAACGAACA
SARSCoV_1200_22_RIGHT	AACAGATGCAAATCTGGTGGCG
22_right_spike	AACAGATGCAAATTTGGTGGCG
SARSCoV_1200_24_LEFT	GCTGAACATGTCAACAACTCATATGA
24_left_spike	GCTGAATATGTCAACAACTCATATGA
SARSCoV_1200_24_RIGHT	ATGAGGTGCTGACTGAGGGAAG
SARSCoV_1200_26_LEFT	GCCTTGAAGCCCCTTTTCTCTA
SARSCoV_1200_26_RIGHT	AATGACCACATGGAACGCGTAC
SARSCoV_1200_28_LEFT	TTTGTGCTTTTTAGCCTTTCTGCT
SARSCoV_1200_28_RIGHT	GTTTGGCCTTGTTGTTGTTGGC
SARSCoV_1200_28_LEFT_27837T	TTTGTGCTTTTTAGCCTTTCTGTT

Rapid barcode sequences

Component	Sequence
RB01	AAGAAAGTTGTCGGTGTCTTTGTG
RB02	TCGATTCCGTTTGTAGTCGTCTGT
RB03	GAGTCTTGTGTCCCAGTTACCAGG
RB04	TTCGGATTCTATCGTGTTTCCCTA
RB05	CTTGTCCAGGGTTTGTGTAACCTT
RB06	TTCTCGCAAAGGCAGAAAGTAGTC
RB07	GTGTTACCGTGGGAATGAATCCTT
RB08	TTCAGGGAACAAACCAAGTTACGT
RB09	AACTAGGCACAGCGAGTCTTGGTT

Component	Sequence
RB10	AAGCGTTGAAACCTTTGTCCTCTC
RB11	GTTTCATCTATCGGAGGGAATGGA
RB12	CAGGTAGAAAGAAGCAGAATCGGA
RB13	AGAACGACTTCCATACTCGTGTGA
RB14	AACGAGTCTCTTGGGACCCATAGA
RB15	AGGTCTACCTCGCTAACACCACTG
RB16	CGTCAACTGACAGTGGTTCGTACT
RB17	ACCCTCCAGGAAAGTACCTCTGAT
RB18	CCAAACCCAACAACCTAGATAGGC
RB19	GTTCCTCGTGCAGTGTCAAGAGAT
RB20	TTGCGTCCTGTTACGAGAACTCAT
RB21	GAGCCTCTCATTGTCCGTTCTCTA
RB22	ACCACTGCCATGTATCAAAGTACG
RB23	CTTACTACCCAGTGAACCTCCTCG
RB24	GCATAGTTCTGCATGATGGGTTAG
RB25	GTAAGTTGGGTATGCAACGCAATG
RB26	CATACAGCGACTACGCATTCTCAT
RB27	CGACGGTTAGATTCACCTCTTACA
RB28	TGAAACCTAAGAAGGCACCGTATC
RB29	CTAGACACCTTGGGTTGACAGACC
RB30	TCAGTGAGGATCTACTTCGACCCA
RB31	TGCGTACAGCAATCAGTTACATTG
RB32	CCAGTAGAAGTCCGACAACGTCAT
RB33	CAGACTTGGTACGGTTGGGTAACT
RB34	GGACGAAGAACTCAAGTCAAAGGC
RB35	CTACTTACGAAGCTGAGGGACTGC
RB36	ATGTCCCAGTTAGAGGAGGAAACA
RB37	GCTTGCGATTGATGCTTAGTATCA
RB38	ACCACAGGAGGACGATACAGAGAA
RB39	CCACAGTGTCAACTAGAGCCTCTC
RB40	TAGTTTGGATGACCAAGGATAGCC
RB41	GGAGTTCGTCCAGAGAAGTACACG

Component	Sequence
RB42	CTACGTGTAAGGCATACCTGCCAG
RB43	CTTTCGTTGTTGACTCGACGGTAG
RB44	AGTAGAAAGGGTTCCTTCCCACTC
RB45	GATCCAACAGAGATGCCTTCAGTG
RB46	GCTGTGTTCCACTTCATTCTCCTG
RB47	GTGCAACTTTCCCACAGGTAGTTC
RB48	CATCTGGAACGTGGTACACCTGTA
RB49	ACTGGTGCAGCTTTGAACATCTAG
RB50	ATGGACTTTGGTAACTTCCTGCGT
RB51	GTTGAATGAGCCTACTGGGTCCTC
RB52	TGAGAGACAAGATTGTTCGTGGAC
RB53	AGATTCAGACCGTCTCATGCAAAG
RB54	CAAGAGCTTTGACTAAGGAGCATG
RB55	TGGAAGATGAGACCCTGATCTACG
RB56	TCACTACTCAACAGGTGGCATGAA
RB57	GCTAGGTCAATCTCCTTCGGAAGT
RB58	CAGGTTACTCCTCCGTGAGTCTGA
RB59	TCAATCAAGAAGGGAAAGCAAGGT
RB60	CATGTTCAACCAAGGCTTCTATGG
RB61	AGAGGGTACTATGTGCCTCAGCAC
RB62	CACCCACACTTACTTCAGGACGTA
RB63	TTCTGAAGTTCCTGGGTCTTGAAC
RB64	GACAGACACCGTTCATCGACTTTC
RB65	TTCTCAGTCTTCCTCCAGACAAGG
RB66	CCGATCCTTGTGGCTTCTAACTTC
RB67	GTTTGTCATACTCGTGTGCTCACC
RB68	GAATCTAAGCAAACACGAAGGTGG
RB69	TACAGTCCGAGCCTCATGTGATCT
RB70	ACCGAGATCCTACGAATGGAGTGT
RB71	CCTGGGAGCATCAGGTAGTAACAG
RB72	TAGCTGACTGTCTTCCATACCGAC
RB73	AAGAAACAGGATGACAGAACCCTC

Component	Sequence
RB74	TACAAGCATCCCAACACTTCCACT
RB75	GACCATTGTGATGAACCCTGTTGT
RB76	ATGCTTGTTACATCAACCCTGGAC
RB77	CGACCTGTTTCTCAGGGATACAAC
RB78	AACAACCGAACCTTTGAATCAGAA
RB79	TCTCGGAGATAGTTCTCACTGCTG
RB80	CGGATGAACATAGGATAGCGATTC
RB81	CCTCATCTTGTGAAGTTGTTTCGG
RB82	ACGGTATGTCGAGTTCCAGGACTA
RB83	TGGCTTGATCTAGGTAAGGTCGAA
RB84	GTAGTGGACCTAGAACCTGTGCCA
RB85	AACGGAGGAGTTAGTTGGATGATC
RB86	AGGTGATCCCAACAAGCGTAAGTA
RB87	TACATGCTCCTGTTGTTAGGGAGG
RB88	TCTTCTACTACCGATCCGAAGCAG
RB89	ACAGCATCAATGTTTGGCTAGTTG
RB90	GATGTAGAGGGTACGGTTTGAGGC
RB91	GGCTCCATAGGAACTCACGCTACT
RB92	TTGTGAGTGGAAAGATACAGGACC
RB93	AGTTTCCATCACTTCAGACTTGGG
RB94	GATTGTCCTCAAACTGCCACCTAC
RB95	CCTGTCTGGAAGAAGAATGGACTT
RB96	CTGAACGGTCATAGAGTCCACCAT

Computer requirements and software

MinION Mk1B IT requirements

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

MinION Mk1C IT requirements

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

Software for nanopore sequencing

MinKNOW

The MinKNOW software controls the nanopore sequencing device, collects sequencing data and basecalls in real time. You will be using MinKNOW for every sequencing experiment to sequence, basecall and demultiplex if your samples were barcoded.

For instructions on how to run the MinKNOW software, please refer to 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.

For instructions on how to create an EPI2ME account and install the EPI2ME Desktop Agent, please refer to the EPI2ME Platform 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 or within four weeks of purchasing 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 in 10 mM Tris-HCl, pH 8.0 • LunaScript RT SuperMix (LS RT)
Consumables	• Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

	• Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Cat # 0030129504) with PCR seals
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

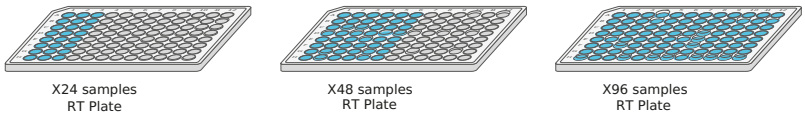
IMPORTANT

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, place a fresh 96-well plate (RT plate) into a PCR Cooler (if using). Using a stepper pipette, or multichannel pipette, add 2 µl of LunaScript RT SuperMix (LS RT) per well.

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



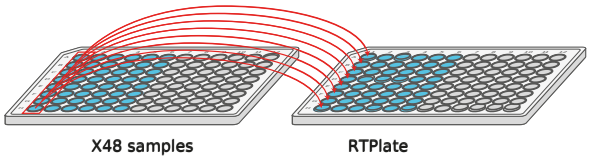
X24 samples RT Plate

X48 samples RT Plate

X96 samples RT Plate

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

Example for X48 samples:



IMPORTANT

We recommend having a negative control and a positive control for every plate of samples.

3 **Seal the RT plate and spin down.**

4 **Incubate the samples in the thermal cycler using the following program:**

Step	Temperature	Time	Cycles
Primer annealing	25°C	2 min	1
cDNA synthesis	55°C	10 min	1
Heat inactivation	95°C	1 min	1
Hold	4°C	∞	

END OF STEP

While the reverse transcription reaction is running, prepare the master mixes as described in the next section.

PCR

~235 minutes

Materials

- Q5 HS Master Mix (Q5)
- Midnight Primer Pool A (MP A)

	• Midnight Primer Pool B (MP B)
Consumables	• Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • 1.5 ml Eppendorf DNA LoBind tubes • Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted (Cat # 0030129504) with PCR seals
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 Freed et al., 2020 using Primal Scheme. These primers are designed to generate 1200 bp amplicons that overlap by approximately 20 bp. These primer sequences can be found here.

IMPORTANT

We recommend handling the primers in a clean template-free PCR hood.

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

Volume per sample:

Reagent	Pool A	Pool B
Nuclease-free water	3.7 µl	3.7 µl
Midnight Primer Pool A (MP A)	0.05 µl	-
Midnight Primer Pool B (MP B)	-	0.05 µl
Q5 HS Master Mix (Q5)	6.25 µl	6.25 µl
Total	**10 µl**	**10 µl**

For **x24** samples:

Reagent	Pool A	Pool B
Nuclease-free water	102 µl	102 µl
Midnight Primer Pool A (MP A)	2 µl	-
Midnight Primer Pool B (MP B)	-	2 µl
Q5 HS Master Mix (Q5)	172 µl	172 µl
Total	**276 µl**	**276 µl**

For **x48** samples:

Reagent	Pool A	Pool B
Nuclease-free water	203 µl	203 µl
Midnight Primer Pool A (MP A)	3 µl	-
Midnight Primer Pool B (MP B)	-	3 µl
Q5 HS Master Mix (Q5)	344 µl	344 µl
Total	**550 µl**	**550 µl**

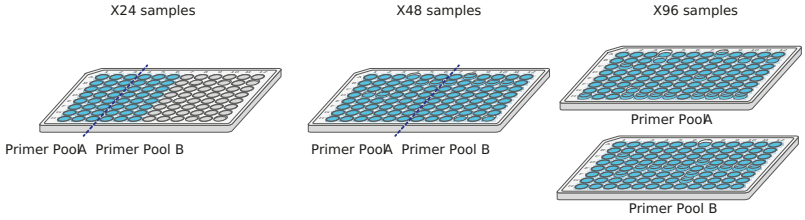
For **x96** samples:

Reagent	Pool A	Pool B
Nuclease-free water	407 µl	407 µl
Midnight Primer Pool A (MP A)	6 µl	-
Midnight Primer Pool B (MP B)	-	6 µl
Q5 HS Master Mix (Q5)	687 µl	687 µl
Total	**1,100 µl**	**1,100 µl**

2 **Using a stepper pipette or a multichannel pipette, aliquot 10 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

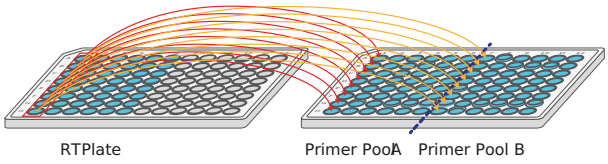
Note: For X96 samples, Pool A is a separate plate to Pool B.



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 in the PCR plate(s), taking care not to cross-contaminate different wells. Mix by pipetting the contents of each well up and down.**

There should be two PCR reactions per sample.

Example for X48 samples:



4 **Mix by pipetting the contents of each well up and down.**

IMPORTANT

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

We recommend having a negative control and a positive control for every plate of samples.

5 Seal the plate(s) and spin down briefly.

6 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 Annealing and extension	98°C 61°C 65°C	15 sec 2 min 3 min	35
Hold	4°C	∞	

Optional action
If necessary, the protocol can be paused at this point. The samples should be kept at 4°C and can be stored overnight.

Addition of rapid barcodes

~10 minutes

Materials
- Rapid Barcode Plate (RB01-96)

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

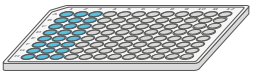
Equipment
- Multichannel pipettes suitable for dispensing 2–20 µl and 20–200 µl, and tips
- Thermal cycler
- Centrifuge capable of taking 96-well plates

1 Spin down the Rapid Barcode Plate and PCR reactions prior to opening to collect material in the bottom of the wells.

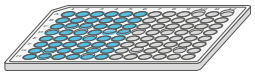
2 Using a multichannel pipette or stepper pipette, transfer 2.5 µl nuclease-free water to the wells of a fresh 96-well plate (Barcode Attachment Plate).

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

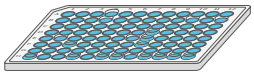
Plate location	X24 samples	X48 samples	X96 samples
Columns	1-3	1-6	1-12



X24 samples
BarcodeAttachment Plate



X48 samples
BarcodeAttachment Plate



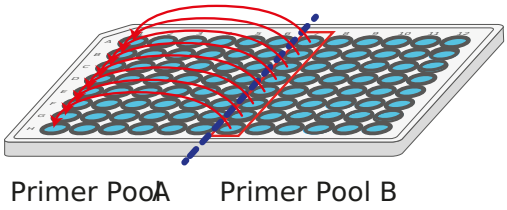
X96 samples
BarcodeAttachment Plate

3 Using a multichannel pipette, transfer the entire contents 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 5 6	1 2 3
X48	7 8 9 10 11 12	1 2 3 4 5 6
X96	1 2 3 4 5 6 7 8 9 10 11 12	1 2 3 4 5 6 7 8 9 10 11 12

Example for X48 samples:

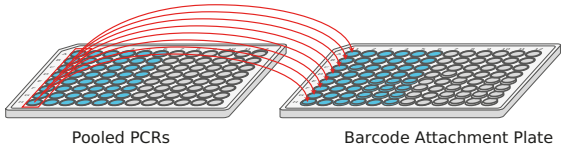


4 Using a multichannel pipette, transfer 5 µl from each well of PCR Pool A (now containing pooled PCR products) to the corresponding well of the Barcode Attachment Plate and mix by pipetting.

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

Example for X48 samples:

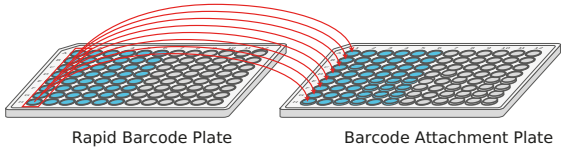


5 Using a multichannel pipette, transfer 2.5 µl from the Rapid Barcode Plate to the corresponding well of the Barcode Attachment Plate, taking care not to cross-contaminate different wells. Mix by pipetting.

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

Example for X48 samples:



IMPORTANT

Samples must be thoroughly mixed.

6 **Seal the Barcode Attachment Plate and spin down.**

7 **Incubate the plate in a thermal cycler at 30°C for 2 minutes and then at 80°C for 2 minutes.**

Pooling samples and clean-up

~30 minutes

Materials

- AMPure XP Beads (AXP)
- Elution Buffer from the Oxford Nanopore kit (EB)
- Rapid Adapter (RA)
- Adapter Buffer (ADB)

Consumables

- Freshly prepared 80% ethanol in nuclease-free water
- 1.5 ml Eppendorf DNA LoBind tubes
- 5 ml Eppendorf DNA LoBind tubes
- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

- Microfuge
- Centrifuge capable of taking 96-well plates
- Hula mixer (gentle rotator mixer)
- Magnetic rack
- Ice bucket with ice
- P1000 pipette and tips
- P200 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- Qubit fluorometer plate reader (or equivalent for QC check)

1 **Briefly spin down the Barcode Attachment Plate to collect the liquid at the bottom of the wells prior to opening.**

2 **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

3 **Mix pooled samples by vortexing.**

> **IMPORTANT**
>
> **Pooled barcoded samples must be thoroughly mixed.**

4 **Transfer half of the barcoded pooled sample to a clean 1.5 ml Eppendorf DNA LoBind tube.**

Per sample, we expect to take forward ~5 µl.

	X24 samples	X48 samples	X96 samples
Example volume	120 µl	240 µl	480 µl

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

6 **To the pooled barcoded sample, add an equal volume of resuspended AMPure XP Beads (AXP, or SPRI) and mix by pipetting.**

Example volume	X24 samples	X48 samples	X96 samples
Volume of 1X AXP	120 µl	240 µl	480 µl

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

8 **Prepare at least 3 ml of fresh 80% ethanol in nuclease-free water.**

9 **Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless.**

10 **Keep the tube on the magnet and wash the beads with 1 ml of freshly-prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

11 **Repeat the previous step.**

12 **Briefly 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.**

13 **Remove the tube from the magnetic rack and resuspend the pellet by pipetting in 15 µl Elution Buffer (EB). Incubate for 10 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.

Quantify DNA concentration by using the Qubit dsDNA HS Assay Kit.

16 Take forward 11 µl of your eluted DNA library.

17 In a fresh 1.5 ml Eppendorf DNA LoBind tube, dilute the Rapid Adapter (RA) as follows and pipette mix:

Reagent	Volume
Rapid Adapter (RA)	1.5 µl
Adapter Buffer (ADB)	3.5 µl
Total	**5 µl**

18 Add 1 µl of the diluted Rapid Adapter (RA) to the barcoded DNA.

19 Mix gently by flicking the tubes, and spin down.

20 Incubate the reaction for 5 minutes at room temperature.

END OF STEP

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

Priming and loading the SpotON flow cell

~10 minutes

Materials

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

Consumables

- 1.5 ml Eppendorf DNA LoBind tubes
- MinION and GridION Flow Cell
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- (Optional) Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen™ UltraPure™ BSA 50 mg/ml, AM2616)

Equipment

- MinION or GridION device
- MinION Flow Cell Light Shield
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

IMPORTANT

Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).

TIP

Priming and loading a flow cell

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

Using the Library Solution

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

1 Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.

IMPORTANT

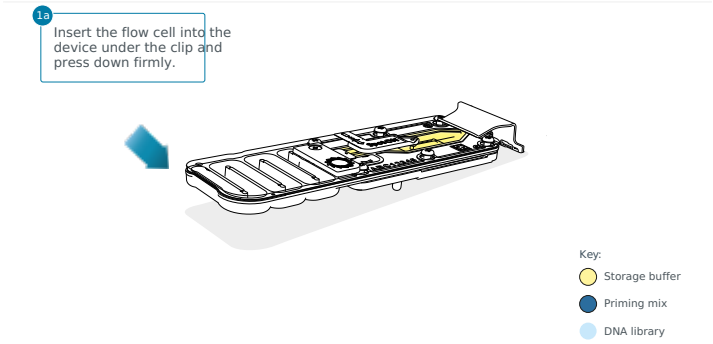
For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), we recommend adding Bovine Serum Albumin (BSA) to the flow cell priming mix at a final concentration of 0.2 mg/ml.

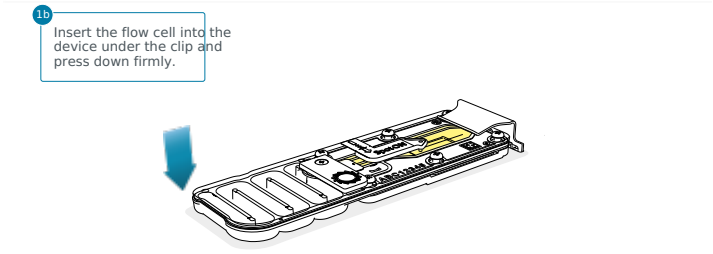
Note: We do not recommend using any other albumin type (e.g. recombinant human serum albumin).

2 Prepare the flow cell priming mix with BSA in a suitable tube for the number of flow cells to flush. Once combined, mix well by pipette mixing.

Reagents	Volume per flow cell
Flow Cell Flush (FCF)	1,170 µl
Bovine Serum Albumin (BSA) at 50 mg/ml	5 µl
Flow Cell Tether (FCT)	30 µl
Total volume	**1,205 µl**

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





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

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

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

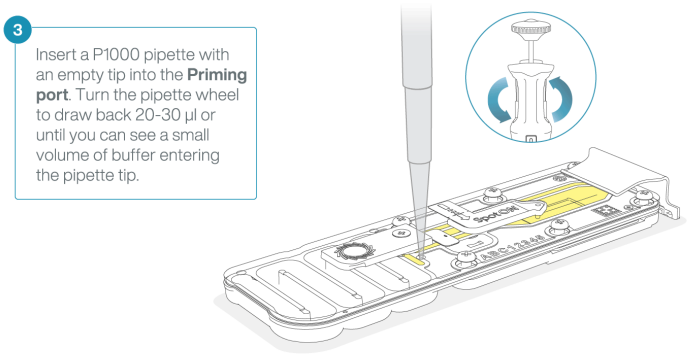
4 Slide the flow cell priming port cover clockwise to open the priming port.

IMPORTANT

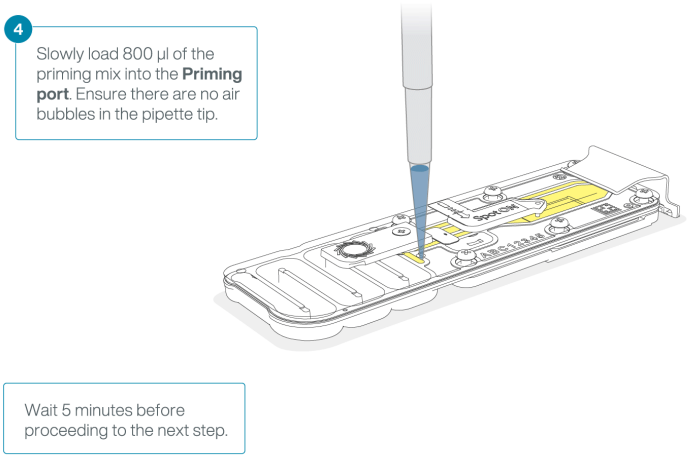
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

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 five minutes. During this time, prepare the library for loading by following the steps below.**



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

IMPORTANT

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

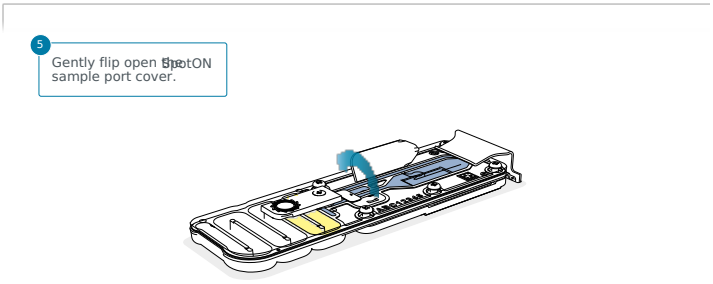
We recommend using the Library Beads (LIB) for most sequencing experiments. However, the Library Solution (LIS) is available for more viscous libraries.

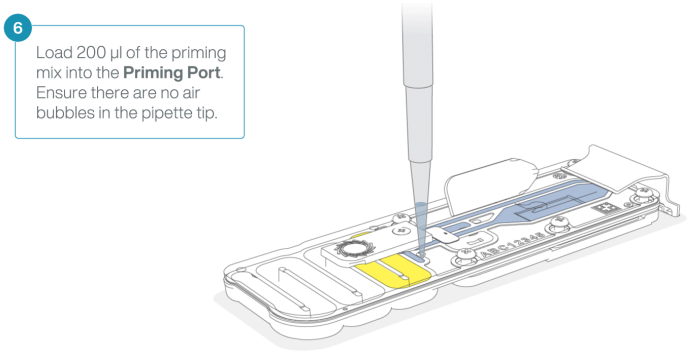
8 **In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:**

Reagent	Volume per flow cell
Sequencing Buffer (SB)	37.5 µl
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using	25.5 µl
DNA library	12 µl
Total	**75 µl**

9 Complete the flow cell priming:

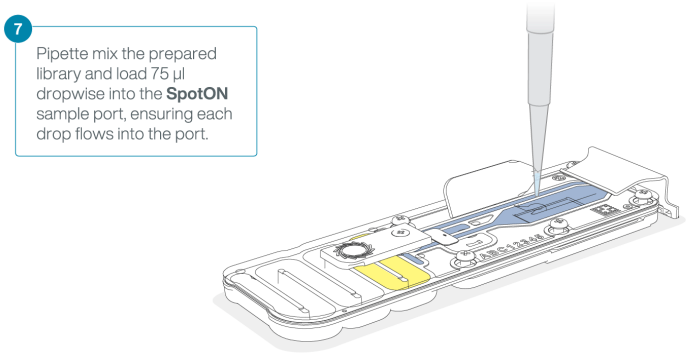
1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load **200 µl** of the priming mix into the flow cell priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.



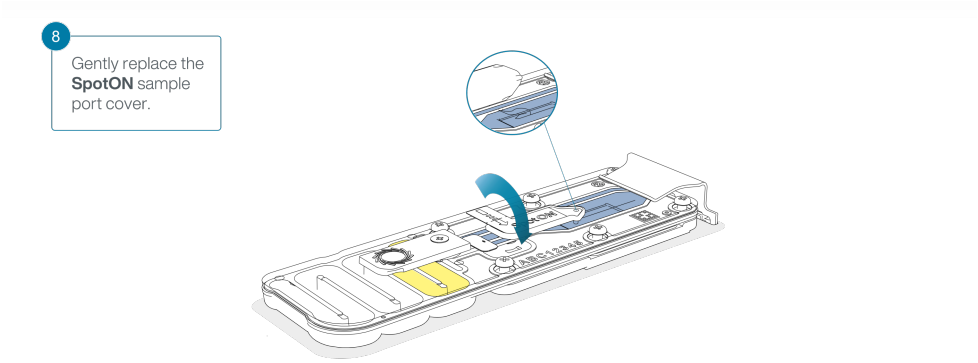


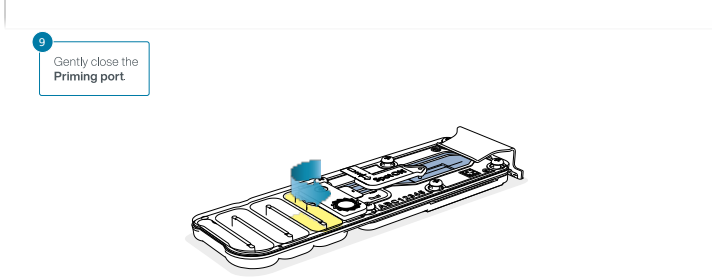
10 Mix the prepared library gently by pipetting up and down just prior to loading.

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 and close the priming port.**





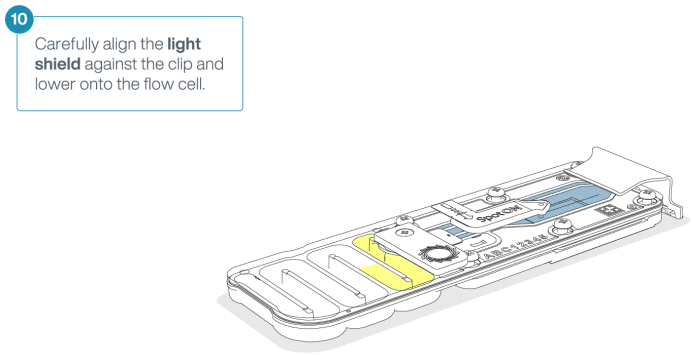
IMPORTANT

Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.

We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

13 Place the light shield onto the flow cell, as follows:

1. Carefully place the leading edge of the light shield against the clip.
   Note: Do not force the light shield underneath the clip.
2. Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.



CAUTION

The MinION Flow Cell Light Shield is not secured to the flow cell and careful handling is required after installation.

END OF STEP

Close the device lid and set up a sequencing run on MinKNOW.

Data acquisition and basecalling

Overview of nanopore data analysis

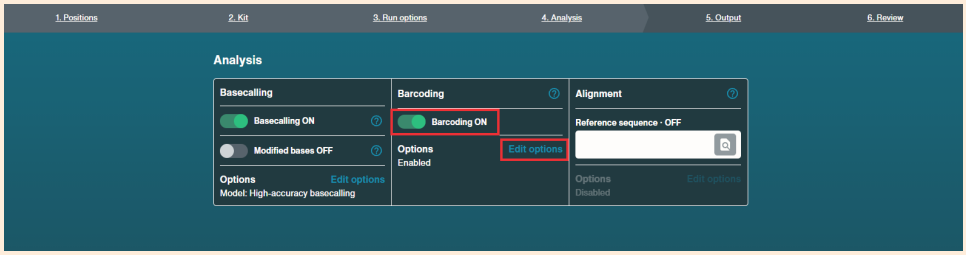
For a full overview of nanopore data analysis, which includes options for basecalling and post-basecalling analysis, please refer to the Data Analysis document.

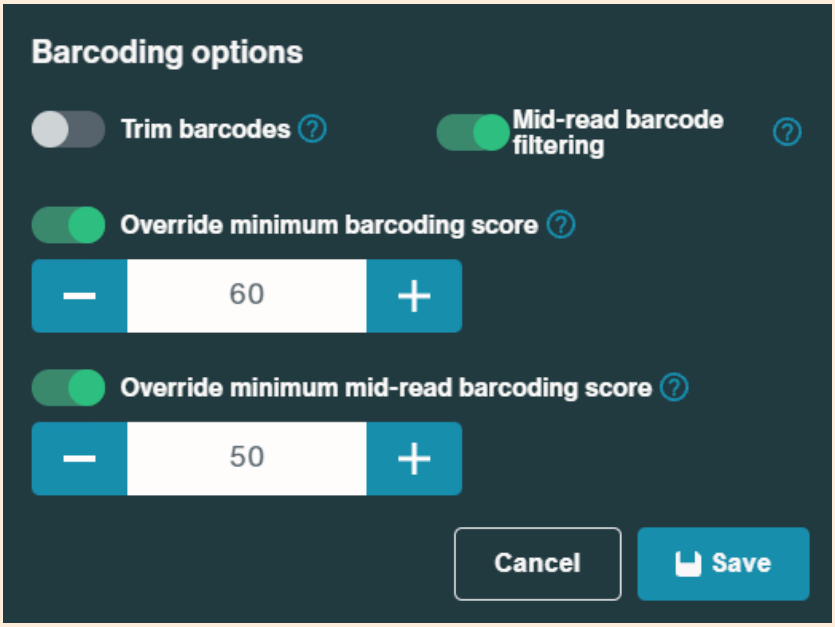
IMPORTANT

Required settings in MinKNOW

The correct barcoding parameters must be set up on MinKNOW prior to the sequencing run. During the run setup, in the Analysis tab:

1. Enable **Barcoding**.
2. Select **Edit options**.
3. Enable **Mid-read barcode filtering**.
4. Enable **Override minimum barcoding score** and set the value to **60**.
5. Enable **Override minimum mid-read barcoding score** and set the value to **50**.





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

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.

Downstream analysis

Recommended pipeline analysis

The wf-artic is a bioinformatics workflow for the analysis of ARTIC sequencing data prepared using the Midnight protocol. The bioinformatics workflow is orchestrated by the Nextflow software. Nextflow is a publicly available and open-source project that enables the execution of scientific workflows in a scalable and reproducible way. The use of the Nextflow software has been integrated into the EPI2ME Labs software that we recommend for running our downstream analysis methods.

Alternative methods for downstream analysis are available using your device terminal or command line, however we only suggest this for experienced users.

Demultiplexed sequence reads are processed using the ARTIC Field Bioinformatics software that has been modified for the analysis of FASTQ sequences prepared using Oxford Nanopore Rapid Sequencing kits. The other modification to the ARTIC workflow is the use of a primer scheme that defines the sequencing primers used by the Midnight protocol and their genomic locations on the SARS-CoV-2 genome.

The wf-artic workflow includes other analytical steps that include cladistic analysis using Nextclade and strain assignment using Pangolin. The data facets included in the report are parameterised and additional information such as plots of depth-of-coverage across the reference genome is optional.

The complete source for wf-artic is linked, and the Nextflow software will download the scripts and logic flow from this location.

The wf-artic workflow needs to be started manually as outlined below in 'Running a Midnight analysis using EPI2ME Labs'.

Software set-up and installation

The EPI2ME Labs application provides a clean interface to accessing bioinformatics workflows, and is our recommended method in performing your post-sequencing analysis.

Follow the instructions in the EPI2ME Labs Installation guide to install the application on your device.

For more information on how to use EPI2ME Labs, refer to the EPI2ME Labs Quick Start guide.

Installing and updating the wf-artic workflow in EPI2ME Labs:

Ensure you have installed the wf-artic workflow prior to the first analysis set-up.

In the EPI2ME Labs home page, scroll down to the "Install workflows" section and click on **epi2me-labs/wf-artic**:

If you have already installed the wf-artic workflow, ensure you are using the latest version.

Updating the workflow can be done directly through EPI2ME Labs by navigating to the **wf-artic** workflow page and clicking **Update Workflow**:

Demultiplexing of multiple barcoded samples

The wf-artic analysis requires FASTQ sequence data that has already been demultiplexed.

Reads will be demultiplexed during sequencing if you are following the recommended "Required settings in MinKNOW". However, demultiplexing can also be done post-sequencing using the MinKNOW software.

For more information and guides on demultiplexing using MinKNOW, refer to the "Post-run analysis" section in our MinKNOW Protocol.

The expected input for wf-artic is a folder of folders as shown below. Each of the barcode folders should contain the FASTQ sequence data and files may either be uncompressed or gzipped.

```
$ tree -d MidnightFastq/

MidnightFastq/

├── barcode01

├── barcode02

├── barcode03

├── barcode04

├── barcode05

├── barcode06

└── unclassified
```

IMPORTANT

Basecalling model

The basecalling model should be specified when setting up the wf-artic analysis. This should reflect the basecalling model selected during your run set-up as follows:

- If using the default model, High-accuracy basecalling (HAC): r1041_e82_400bps_hac_variant_g615
- If you have used Super accurate basecalling (SUP), please use: r1041_e82_400bps_sup_variant_g615
- If you have used FAST basecalling, please use: r1041_e82_400bps_fast_variant_g615

Running a Midnight analysis using EPI2ME Labs

1 Open the EPI2ME Labs application on your device.

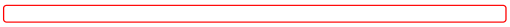
2 **Open the "Workflows" tab in the EPI2ME Labs application and click on the "wf-artic" workflow:**

3 **In the "wf-artic" workflow page, select "Run this workflow" to open analysis set-up:**

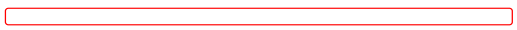
4 **Complete the wf-artic run set-up:**

Select your data input file location. Please note, this folder must contain the demultiplexed FASTQ files of your sequencing run.

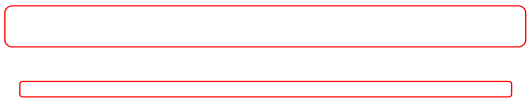
Expand the **Primer Scheme Selection** tab and set the **Scheme version** to **Midnight-ONT/V3**.



Expand the **Advanced Options** tab and set the **Medaka model** to the basecalling model used in your sequencing run.



Expand the **Extra configuration** tab and set the **Run name** for your wf-artic analysis.



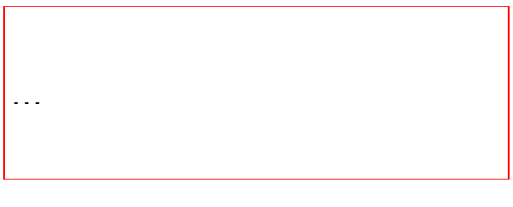
Click **Launch workflow** at the bottom of the page to begin your analysis.

5 Navigate to the "Analysis" tab in the EPI2ME Labs application to monitor your run:

Completed analysis and result files

The wf-artic analysis outputs will be written to the**Working Directory** folder specified in the EPI2ME Labs **Settings** tab.
The location of this folder is specified in the wf-artic run **Instance parameters** preceeded by out_dir.

However, these files can also be accessed directly in the EPI2ME Labs application from the completed analysis page for your run:



These outputs include:

- all_consensus.fasta
  A multi-FASTA format sequence file containing the consensus sequence for each of the samples investigated. This consensus sequence has been prepared for the whole SARS-CoV-2 genome, not just the spike protein region. The consensus sequence masks the non-spike regions and regions of low sequence coverage with N residues.
- all_variants.vcf.gz
  A gzipped VCF file that describes all high-quality genetic variants called by medaka from the sequenced samples.
- all_variants.vcf.gz.tbi
  An index file for the gzipped VCF file.
- consensus_status.txt
  A tab delimited file that reports whether a consensus sequence has been successfully prepared for a sample, or not.
- wf-artic-report.html
  A report summarising these data. This HTML format report also includes the output of the Nextclade software that can be used for a visual inspection of, for example, primer drop out or other qualitative consensus sequence aspects.

Other files are included in the work-directory. This includes per sample VCF files of all genetic variants prior to filtering and other sequences.

Housekeeping and disk usage

The "Working Directory" can be specified in the EPI2ME Labs "Settings" tab and defines where the workflow intermediate files and outputs are stored.

This folder will accumulate a significant number of files that correspond to raw BAM files, other larger intermediates and analysis results files. We recommend this folder to be routinely cleared.

Flow cell reuse and returns

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 Flow Cell Wash Kit protocol and store the washed flow cell at 2-8°C.

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 **Alternatively, 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.

Note: 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.

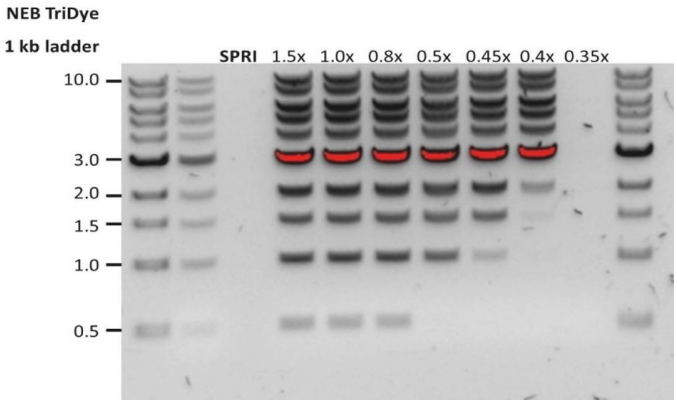
We also have an FAQ section available on theNanopore Community Support section.

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 document. 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 document. Further information can be found in the DNA/RNA Handling page.
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 document. Further information can be found in the DNA/RNA Handling page. 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.

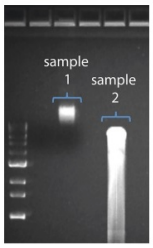
MinKNOW script failed

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. If you do not have another sequencing device available, we recommend storing the flow cell and the loaded library at 4°C and contact Technical Support for further storage guidance.

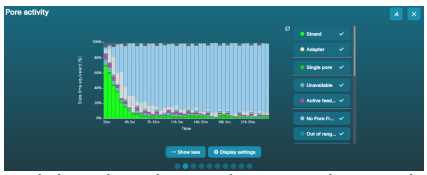
Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	Ensure you load the recommended amount of good quality library in the relevant library prep protocol onto your 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 sequencing 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/FCT tube). Make sure FLT/FCT was added to FB/FCF 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 unavailable pores

Observation	Possible cause	Comments and actions
Large proportion of unavailable pores (shown as blue in the channels panel and pore activity plot)  The pore activity plot above shows an increasing proportion of "unavailable" pores over time.	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 "sequencing pore". If the portion of unavailable pores 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.

Large proportion of inactive pores

Observation	Possible cause	Comments and actions
Large proportion of inactive/unavailable pores (shown as light blue in the channels panel and pore activity 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/unavailable 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/unavailable 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	For Kit 9 chemistry (e.g. SQK-LSK109), 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.