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 the first iteration of the PCR tiling with rapid barcoding protocol Oxford Nanopore Technologies released using third party reagents. This protocol uses the Rapid Barcoding Kit 96 (SQK-RBK110.96) for barcoding and library preparation and the third party reagents are not included in the kit.

For the most up to date and optimised protocol, we recommend using the PCR tiling of SARS-CoV-2 virus with rapid barcoding and Midnight RT PCR Expansion (SQK-RBK110.96 and EXP-MRT001) protocol.

Below, we have highlighted the differences between the protocols.

Step	**PCR tiling of SARS-CoV-2 virus with rapid barcoding (SQK-RBK110.96)** **Version: pctr_9125_v110_reve_24mar2021**	**PCR tiling of SARS-CoV-2 virus with rapid barcoding and Midnight RT PCR Expansion (SQK-RBK110.96 and EXP-MRT001)** **Version: mrt_9127_v110_revh_14jul2021**
Reverse transcription	LunaScript: 4 µl Sample: 16 µl	LunaScript: 2 µl Sample: 8 µl
PCR	IDT lab-ready midnight primers or stock primers PCR mastermix: Nuclease-free water: 9.89 µl Primer pool A/B (100 µM): 0.11 µl Q5® Hot Start HF 2x Master Mix: 12.5 µl **Total:** 22.5 µl per sample RT reaction: 2.5 µl per primer pool	Lab-ready Midnight primers from the kit PCR mastermix: Nuclease-free water : 3.7 µl Midnight Primer Pool A/B (MP A/MP B) (100 µM): 0.05 µl Q5 HS Master Mix (Q5): 6.25 µl **Total:** 10 µl RT reaction: 2.5 µl per primer pool
Addition of rapid barcodes	25 µl pool B transferred into pool A	12.5 µl pool B transferred into pool A
Pooling samples and clean-up	Full volume of pooled samples taken forward for SPRI clean Washed in 1.5 ml of 80% ethanol Eluate retained: 30 µl 600-800 ng library in 11 µl EB	Half volume of pooled samples taken forward for SPRI clean Washed in 1 ml of 80% ethanol Eluate retained: 15 µl 800 ng library in 11 µl EB

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 (SQK-RBK110.96) 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 . The protocol generates 1200 bp

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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 the Twist Synthetic SARS-CoV-2 RNA controls to show what would be expected when running this protocol with SARS-CoV-2 samples.

To generate tiled PCR amplicons from the SARS-CoV-2 viral cDNA for use with the Rapid Barcoding Kit 96, primers were designed by Freed *et al.*, 2020 using Primal Scheme. These primers are designed to generate 1.2 kb amplicons that overlap by approximately 20 bp. Primer sequences can be found here.

Primers can be ordered pre-pooled directly from IDT under the name SARS-Cov2-Midnight-1200, 500 rxn at a concentration of 100 µM per pool.

Steps in the sequencing workflow:

Prepare for your experiment

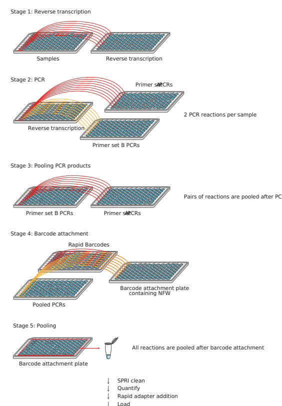
you will need to:

- 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
- 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, which will collect raw data from the device and convert it into basecalled reads

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 (SQK-RBK110.96).

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.

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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.

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Rapid Barcoding Kit 96 (SQK-RBK110.96)
- FLO-MIN106D, FLO-MINSP6 or FLO-MIN111 flow cells
- Flow Cell Wash Kit (EXP-WSH004)

Equipment and consumables

Materials

- Input RNA
- Rapid Barcoding Kit 96 (SQK-RBK110.96)

Consumables

- LunaScript™ RT SuperMix Kit
- Q5® Hot Start High-Fidelity 2X Master Mix (NEB, M0494)
- SARS-CoV-2 midnight primers (lab-ready at 100 µM, IDT cat # 10007184)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly-prepared 80% ethanol in nuclease-free water
- Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)
- DNA 12000 Kit & Reagents - optional (Agilent Technologies)
- 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 heat seals

Equipment

- Hula mixer (gentle rotator mixer)
- Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- 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

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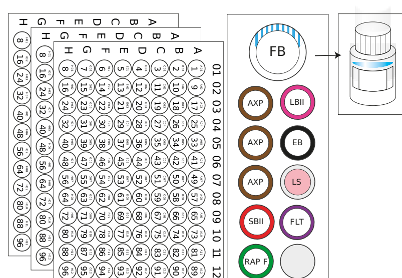
Optional Equipment

- 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

Rapid Barcoding Kit 96 (SQK-RBK110.96) contents



FB : Flush Buffer
AXP : AMPure XP Beads
SBII : Sequencing Buffer II
RAP F : Rapid Adapter F
LBII : Loading Beads II
EB : Elution Buffer
LS : Loading Solution
FLT : Flush Tether

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Rapid Barcode plate	RB96	-	3 plates	8 µl per well
AMPure XP Beads	AXP	Brown	3	1,200
Sequencing Buffer II	SBII	Red	1	500
Rapid Adapter F	RAP-F	Green	1	25
Elution Buffer	EB	Black	1	500
Loading Beads II	LBII	Pink	1	360
Loading Solution	LS	White cap, pink label	1	400
Flush Tether	FLT	Purple	1	400
Flush Buffer	FB	White	1 bottle	15,500

This product contains AMPure XP reagent manufactured by Beckman Coulter, Inc.

Rapid barcode sequences

Component	Sequence
RB01	AAGAAAGTTGTCGGTGTCTTTGTG
RB02	TCGATTCCGTTTGTAGTCGTCTGT
RB03	GAGTCTTGTGTCCCAGTTACCAGG
RB04	TTCGGATTCTATCGTGTTTCCCTA
RB05	CTTGTCCAGGGTTTGTGTAACCTT

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Component	Sequence
RB06	TTCTCGCAAAGGCAGAAAGTAGTC
RB07	GTGTTACCGTGGGAATGAATCCTT
RB08	TTCAGGGAACAAACCAAGTTACGT
RB09	AACTAGGCACAGCGAGTCTTGGTT
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

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Component	Sequence
RB40	TAGTTTGGATGACCAAGGATAGCC
RB41	GGAGTTCGTCCAGAGAAGTACACG
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

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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

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. 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

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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
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

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Optional Equipment

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

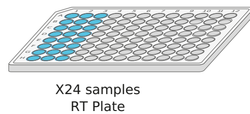
IMPORTANT

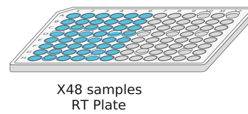
Keep the RNA sample on ice as much as possible to prevent nucleolytic degradation, which may affect sensitivity.

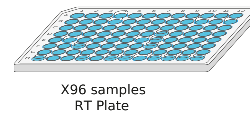
1 **In a clean pre-PCR hood, using a stepper pipette, or a multichannel pipette, add 4 µl of LunaScript™ RT SuperMix to a fresh 96-well plate (RT Plate).**

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

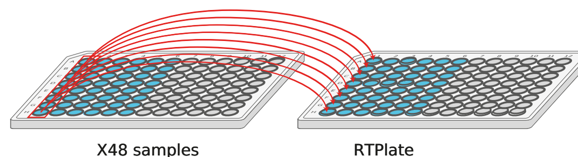






2 **To each well containing LunaScript reagent of the RT plate, add 16 µl of sample and gently mix by pipetting. If adding less than 16 µl, make up the rest to the volume with nuclease-free water.**

Example for X48 samples:



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IMPORTANT

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

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

4 **Preheat the thermal cycler to 25°C.**

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 midnight primers (lab-ready at 100 µM, IDT cat # 10007184)
- 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

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PCR

Version: PCTR_9125_v110_revE_24Mar2021

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 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 template-free PCR hood.

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PCR

Version: PCTR_9125_v110_revE_24Mar2021

1 In the 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
RNase-free water	9.89 µl	9.89 µl
Primer pool A (100 µM)	0.11 µl	-
Primer pool B (100 µM)	-	0.11 µl
Q5® Hot Start HF 2x Master Mix	12.5 µl	12.5 µl
Total	**22.5 µl**	**22.5 µl**

For **x24** samples:

Reagent	Pool A	Pool B
RNase-free water	269.7 µl	269.7 µl
Primer pool A (100 µM)	3 µl	-
Primer pool B (100 µM)	-	3 µl
Q5® Hot Start HF 2x Master Mix	341 µl	341 µl
Total	**613.7 µl**	**613.7 µl**

For **x48** samples:

Reagent	Pool A	Pool B
RNase-free water	548.5 µl	548.5 µl
Primer pool A (100 µM)	6.1 µl	-
Primer pool B (100 µM)	-	6.1 µl
Q5® Hot Start HF 2x Master Mix	693.2 µl	693.2 µl
Total	**1247.8 µl**	**1247.8 µl**

For **x96** samples:

Reagent	Pool A	Pool B
RNase-free water	1088 µl	1088 µl
Primer pool A (100 µM)	12.1 µl	-
Primer pool B (100 µM)	-	12.1 µl
Q5® Hot Start HF 2x Master Mix	1375 µl	1375 µl
Total	**2475.1 µl**	**2475.1 µl**

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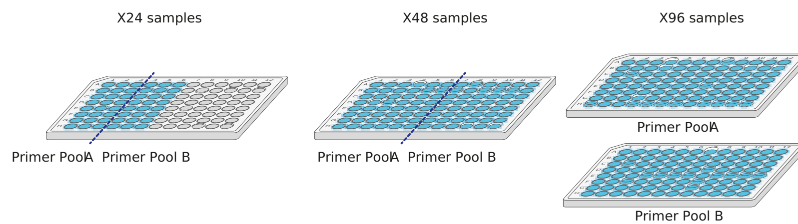
2 **Using a stepper pipette or a multichannel pipette, aliquot 22.5 µl of Pool A and Pool B into a clean 96-well plate(s) as follows:**

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

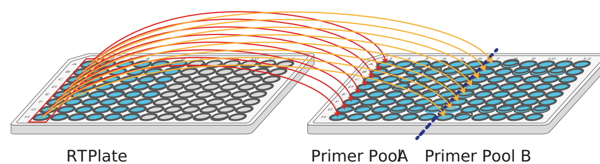
Note: For x96 samples, Pool A is a separate plate to Pool B.



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.

Example for X48 samples:



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 briefly.**

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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	35
Annealing and extension	65°C	5 min	
Hold	4°C	∞	

END OF STEP

When PCR reaches 30-35 cycles, assemble the Rapid Barcode reaction plate as described in the next section.

Addition of rapid barcodes

~10 minutes

Materials
- Rapid Barcode Plate (RB96)

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.**

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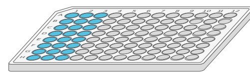
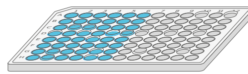
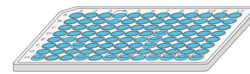
2 **Using a stepper pipette or a multichannel pipette, add 2.5 µl of nuclease-free water to the wells in a clean 96-well plate (Barcode Attachment Plate).**

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

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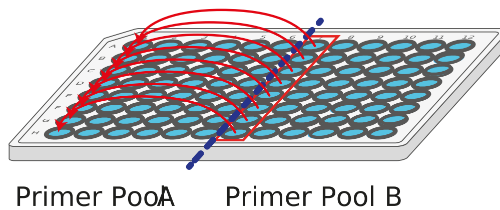
3 **Using a multichannel pipette, transfer 25 µl of each well of PCR Pool B to the corresponding well of PCR Pool A and mix by pipetting.**

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 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:



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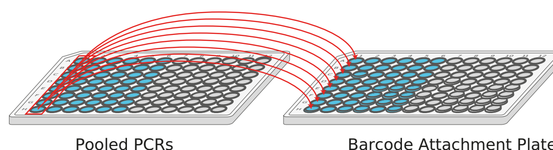
Addition of rapid barcodes

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:

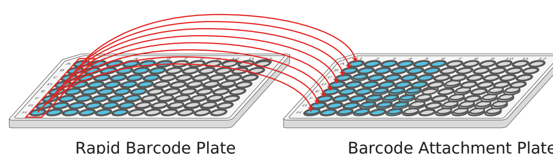


5 **Using a multichannel pipette, transfer 2.5 µl from the Rapid Barcode Plate to the corresponding well of the Barcode Attachment Plate, taking care not to cross-contaminate different wells. Mix by pipetting.**

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:



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.**

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Pooling samples and clean-up

~30 minutes

Materials
- SPRI beads (SPRI)
- Elution Buffer from the Oxford Nanopore kit (EB)
- Rapid Adapter F (RAP F)

Consumables
- Freshly-prepared 80% ethanol in nuclease-free water
- 1.5 ml Eppendorf DNA LoBind tubes
- 2 ml Eppendorf DNA LoBind tubes
- Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)

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

Optional Equipment
- 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 **Resuspend the SPRI beads by vortexing.**

4 **To the entire pooled barcoded sample, add an equal volume of resuspended SPRI beads and mix by flicking the tube.**

	x24 samples	x48 samples	x96 samples
Volume of SPRI to add	240 µl	480 µl	960 µl

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

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

7 **Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.**

8 **Keep the tube on the magnet and wash the beads with 1.5 ml of freshly-prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

9 **Repeat the previous step.**

10 **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.**

11 **Remove the tube from the magnetic rack and resuspend the pellet by pipetting in 30 µl Elution Buffer (EB). Incubate for 10 minutes at room temperature.**

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

13 **Remove and retain 30 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Dispose of the pelleted beads

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

14 **Take forward 600–800 ng of library and make up the volume to 11 µl with EB.**

15 **Add 1 µl of Rapid Adapter F (RAP F) to 11 µl of barcoded DNA.**

16 **Incubate at room temperature for 5 minutes.**

END OF STEP

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

Priming and loading the SpotON Flow Cell

~10 minutes

Materials

- Flush Buffer (FB)
- Flush Tether (FLT)
- Loading Beads II (LBII)
- Sequencing Buffer II (SBII)
- Loading Solution (LS)

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Consumables	• 1.5 ml Eppendorf DNA LoBind tubes
Equipment	• MinION Mk1B • SpotON Flow Cell • P1000 pipette and tips • P100 pipette and tips • P20 pipette and tips • P10 pipette and tips

Using the Loading Solution

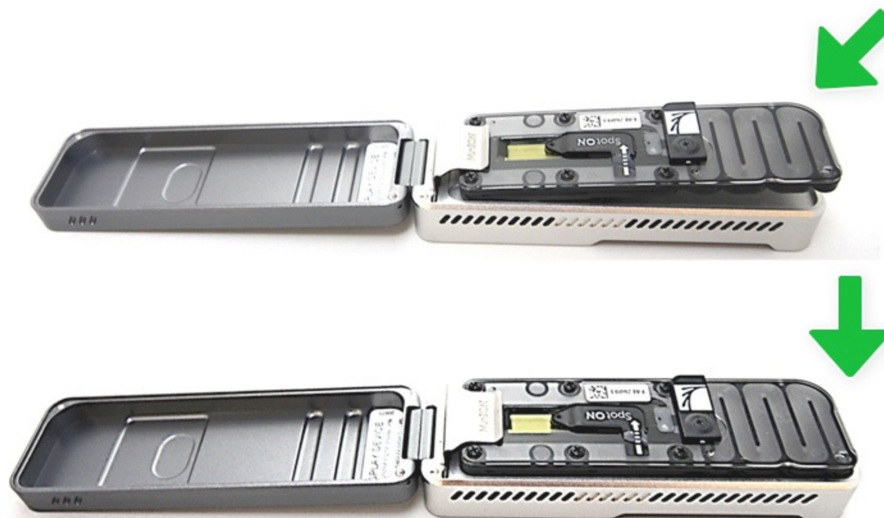
We recommend using the Loading Beads II (LBII) for loading your library onto the flow cell for most sequencing experiments. However, if you have previously used water to load your library, you must use Loading Solution (LS) instead of water. Note: some customers have noticed that viscous libraries can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

1 **Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) or Loading Solution (LS, if using), Flush Tether (FLT) and Flush Buffer (FB) at room temperature.**

2 **Mix the Sequencing Buffer II (SBII), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing. Spin down the SBII and FLT 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.



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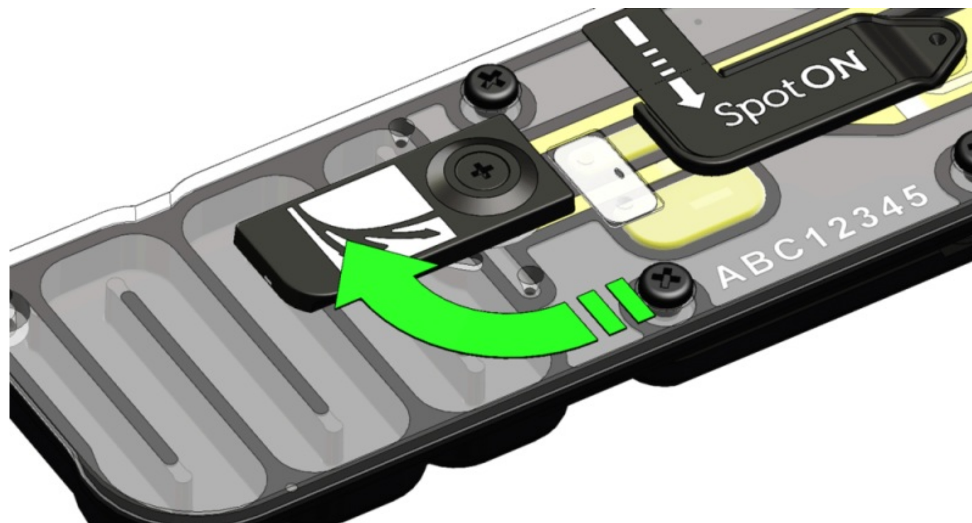
4 Slide the priming port cover clockwise to open the priming port.



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 To prepare the flow cell priming mix, add 30 µl of thawed and mixed Flush Tether (FLT) to 1.17 ml of thawed and mixed Flush Buffer (FB), and mix by vortexing at room temperature.

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 II (LBII) by pipetting.

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IMPORTANT

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

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

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

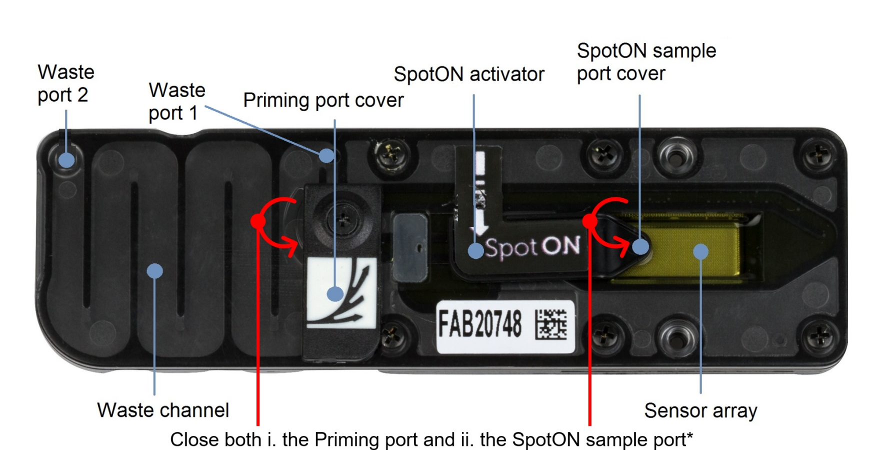
10 Complete the flow cell priming:

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.

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.



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Data acquisition and basecalling

Overview of nanopore data analysis

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

How to start sequencing

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

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

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

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

Follow the instructions in the GridION user manual.

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

Follow the instructions in the MinION Mk1C user manual.

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

Follow the instructions in the MinIT protocol.

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

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

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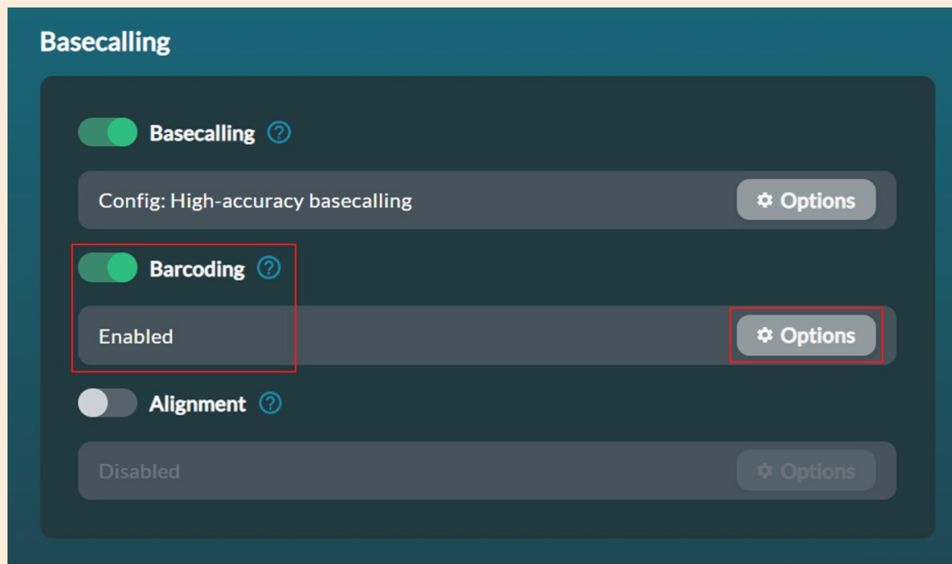
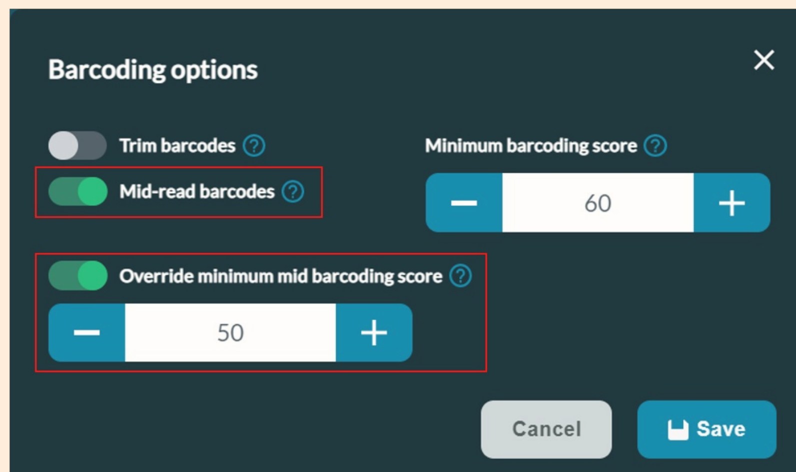
IMPORTANT

Required settings in MinKNOW

When setting the sequencing parameters in MinKNOW, in the **Basecalling** set barcoding as **Enabled**, and in the barcoding options, toggle ON the **Mid-read barcodes** and **Override minimum mid barcoding score** set to 50.

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





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

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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

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 - target coverage

The graphs below show how many hours of sequencing was required to cover the SARS-CoV-2 genome to different depths of coverage using the PCR tiling of SARS-CoV-2 with SQK-RBK110.96 protocol. Higher depths of coverage and higher numbers of multiplexed samples require a longer sequencing time. However, in most cases, 8-12 hours are sufficient to achieve full coverage of the genome.

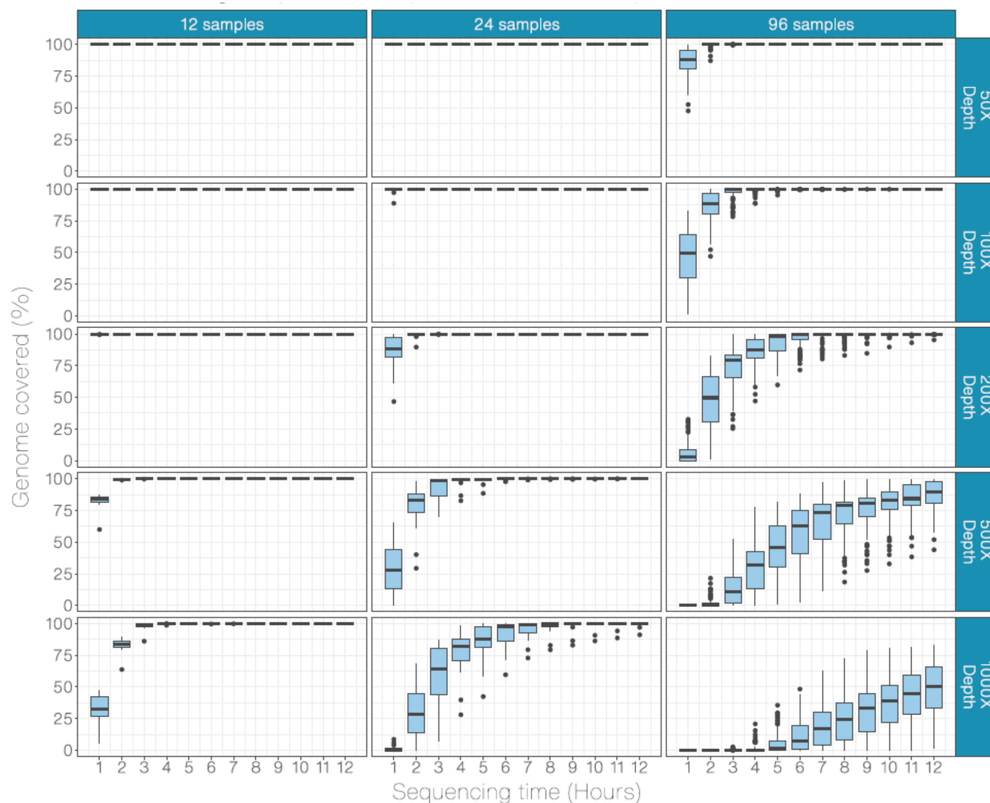

Figure 1. Sequencing time required to cover the SARS-CoV-2 genome to different depths with increasing sample numbers per flow cell. This experiment used 1.2 kb amplicons barcoded using the Rapid Barcoding Kit 96 (SQK-RBK110.96) that were sequenced on the GridION.

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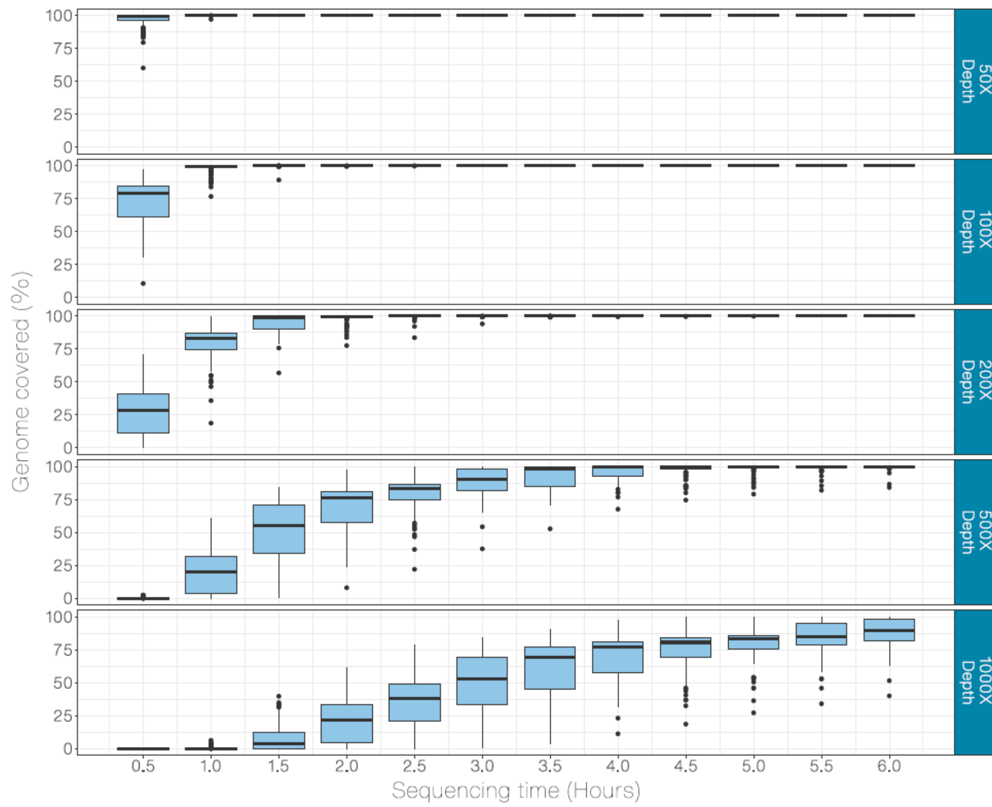

Figure 2. Sequencing time required to cover the SARS-CoV-2 genome to different depths with 96 samples. This experiment used 1.2 kb amplicons barcoded using the Rapid Barcoding Kit 96 (SQK-RBK110.96) that were sequenced on the PromethION.

When assessing coverage of the genome with varying numbers of viral copies as input, 1000 copies of the Twist synthetic SARS-CoV-2 RNA control were sufficient for nearly full coverage.

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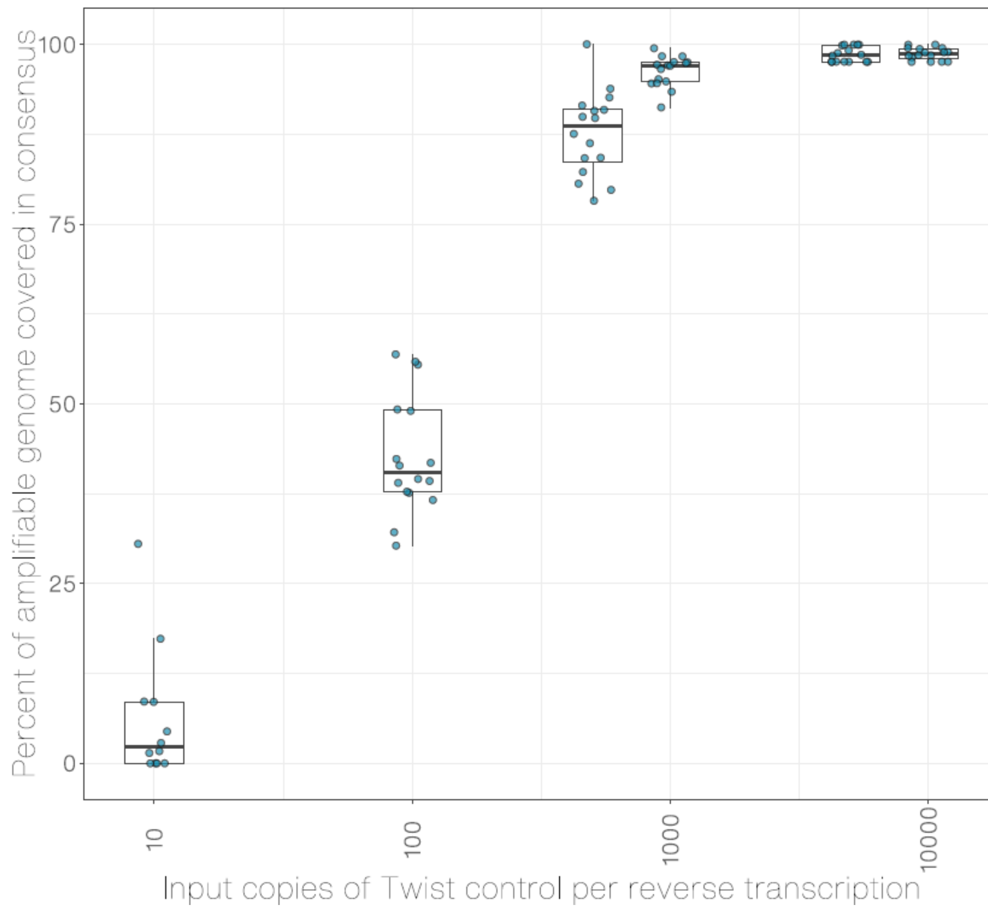

Figure 3. Coverage of the genome in a final consensus sequence prepared using the PCR tiling of SARS-CoV-2 using the Rapid Barcoding 96 protocol. 96 samples were sequenced on a single flow cell across an input titration gradient.

Ending the experiment

Materials

- Flow Cell Wash Kit (EXP-WSH004)

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

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

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.

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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

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.

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Observation	Possible cause	Comments and actions
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.

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

Issues during the sequencing run

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

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

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Issues during the sequencing run

Version: PCTR_9125_v110_revE_24Mar2021

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

Pore occupancy below 40%

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

Shorter than expected read length

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

Large proportion of recovering pores

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

Large proportion of inactive pores

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

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

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

Temperature fluctuation

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Issues during the sequencing run

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

Guppy – no Pass or Fail folders were generated after basecalling

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

Guppy – unusually slow processing on a GPU computer

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

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

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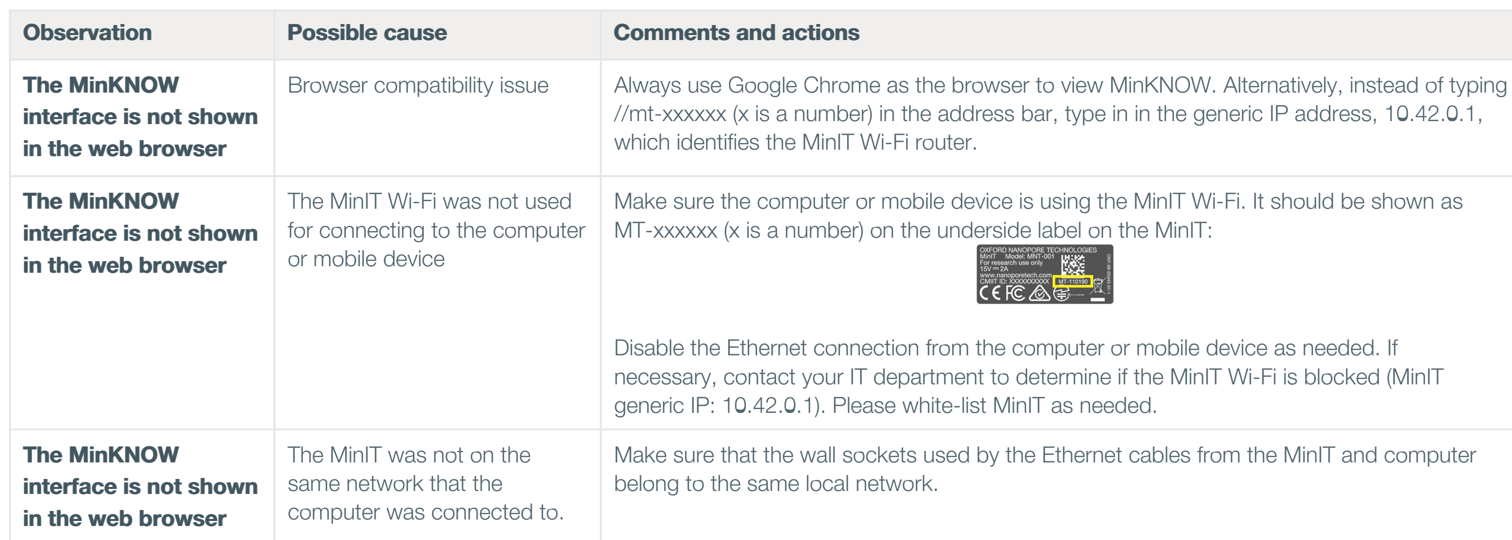
Observation	Possible cause	Comments and actions
The MinKNOW interface is not shown in the web browser	Browser compatibility issue	Always use Google Chrome as the browser to view MinKNOW. Alternatively, instead of typing //mt-xxxxxx (x is a number) in the address bar, type in in the generic IP address,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

MinIT – the MinIT software cannot be updated

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

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