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.

Introduction to the protocol

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

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

This protocol is based on the ARTIC amplicon sequencing protocol for MinION for COVID-19 by Josh Quick. The protocol generates 400 bp amplicons in a tiled fashion across the whole COVID-19 genome. Some example data is shown in the Downstream analysis and expected results section, this is generated using human coronavirus 229E to show what would be expected when running this protocol with COVID-19 samples.

This version of the protocol does not incorporate the one-step barcode/adapter ligation suggested by the ARTIC group, as the Oxford Nanopore team have not yet run this extensively enough to offer the support being requested. We will be working with the ARTIC team as we establish its performance internally.

Before starting

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

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

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

qPCR ct	Dilution factor
18–35	none
15–18	1:10
12–25	1:100

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

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

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Ligation Sequencing Kit (SQK-LSK109)
- Native Barcoding Expansions 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114)
- FLO-MIN106D, FLO-MINSP6 or FLO-MIN111 flow cells
- Wash Kit (EXP-WSH003)

Equipment and consumables

Materials

- Input RNA
- Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) if multiplexing more than 12 samples
- Ligation Sequencing Kit (SQK-LSK109)
- Flow Cell Priming Kit (EXP-FLP002)

Consumables

- Q5® Hot Start High-Fidelity 2X Master Mix (NEB, M0494)
- Random Primer Mix (NEB, S1330S)
- 10 mM dNTP solution (e.g. NEB N0447)
- SuperScript IV reverse transcriptase, 5X RT buffer and 100 mM DTT (ThermoFisher Scientific, 18090010)
- RNaseCUT™, 40 U/µl (Life Technologies, 10777019)
- COVID-19 primers (lab-ready at 100 µM, IDT)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Agencourt AMPure XP beads
- Freshly prepared 80% ethanol in nuclease-free water
- Qubit dsDNA HS Assay Kit (ThermoFisher Q32851)
- NEBNext Ultra II End repair / dA-tailing Module (E7546)
- NEBNext Ultra II Ligation Module (E7595)
- NEBNext Quick Ligation Module (E6056)
- DNA 12000 Kit & Reagents - optional (Agilent Technologies)
- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes

Equipment

- Hula mixer (gentle rotator mixer)
- Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- Microfuge
- Vortex mixer
- Thermal cycler
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips

- P10 pipette and tips
- P2 pipette and tips
- Ice bucket with ice
- Timer

Optional Equipment

- Agilent Bioanalyzer (or equivalent)
- Qubit fluorometer (or equivalent for QC check)
- Eppendorf 5424 centrifuge (or equivalent)
- PCR hood with UV steriliser (optional but recommended to reduce cross-contamination)
- PCR-Cooler (Eppendorf)

Input RNA guidelines

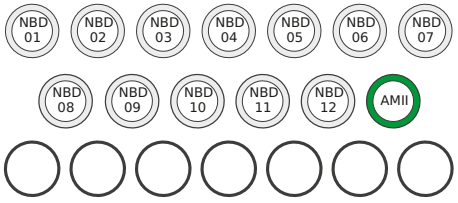
Where sample RNA is added to the below reaction, it is likely advantageous to follow the dilution guidelines proposed by Josh Quick:

qPCR ct	Dilution factor
18–35	none
15–18	1:10
12–25	1:100

If the sample has a low copy number (ct 18–35) use 11 µl of undiluted sample, but be aware that co-extracted compounds may inhibit reverse transcription and PCR.

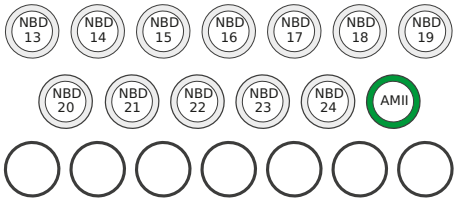
Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) contents

EXP-NBD104 kit contents



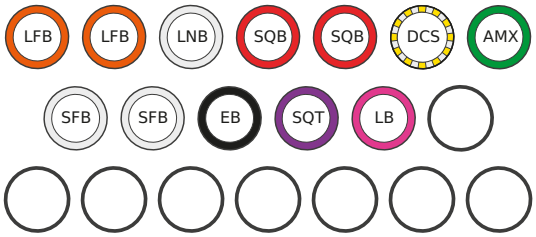
NBD01: Native barcode 1
NBD02: Native barcode 2
NBD03: Native barcode 3
NBD04: Native barcode 4
NBD05: Native barcode 5
NBD06: Native barcode 6
NBD07: Native barcode 7
NBD08: Native barcode 8
NBD09: Native barcode 9
NBD10: Native barcode 10
NBD11: Native barcode 11
NBD12: Native barcode 12
AMII: Adapter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

EXP-NBD114 kit contents



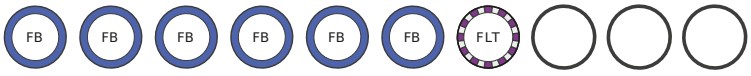
NBD13: Native barcode 13
NBD14: Native barcode 14
NBD15: Native barcode 15
NBD16: Native barcode 16
NBD17: Native barcode 17
NBD18: Native barcode 18
NBD19: Native barcode 19
NBD20: Native barcode 20
NBD21: Native barcode 21
NBD22: Native barcode 22
NBD23: Native barcode 23
NBD24: Native barcode 24
AMII: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

Ligation Sequencing Kit contents



LFB: L fragment buffer
LNB: Ligation buffer
SQB: Sequencing buffer
DCS: DNA control strand
AMX: Adapter mix
SFB: S fragment buffer
EB: Elution buffer
SQT: Sequencing tether
LB: Loading beads

Flow Cell Priming Kit contents (EXP-FLP002)



FB: Flush buffer
FLT: Flush tether

Native barcode sequences

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

Native Barcoding Expansion 1-12

Component	Sequence
NB01	CACAAAGACACCGACAACTTTCTT

Component	Sequence
NB02	ACAGACGACTACAAACGGAATCGA
NB03	CCTGGTAACTGGGACACAAGACTC
NB04	TAGGGAAACACGATAGAATCCGAA
NB05	AAGGTTACACAAACCCTGGACAAG
NB06	GACTACTTTCTGCCTTTGCGAGAA
NB07	AAGGATTCATTCCCACGGTAACAC
NB08	ACGTAACTTGGTTTGTTCCCTGAA
NB09	AACCAAGACTCGCTGTGCCTAGTT
NB10	GAGAGGACAAAGGTTTCAACGCTT
NB11	TCCATTCCCTCCGATAGATGAAAC
NB12	TCCGATTCTGCTTCTTTCTACCTG

Native Barcoding Expansion 13-24

Component	Sequence
NB13	TCACACGAGTATGGAAGTCGTTCT
NB14	TCTATGGGTCCCAAGAGACTCGTT
NB15	CAGTGGTGTTAGCGAGGTAGACCT
NB16	AGTACGAACCACTGTCAGTTGACG
NB17	ATCAGAGGTACTTTCCTGGAGGGT
NB18	GCCTATCTAGGTTGTTGGGTTTGG
NB19	ATCTCTTGACACTGCACGAGGAAC
NB20	ATGAGTTCTCGTAACAGGACGCAA
NB21	TAGAGAACGGACAATGAGAGGCTC
NB22	CGTACTTTGATACATGGCAGTGGT
NB23	CGAGGAGGTTCACTGGGTAGTAAG
NB24	CTAACCCATCATGCAGAACTATGC

Computer requirements and software

MinION Mk 1B IT requirements

Unless you are using a MinIT device, sequencing on a MinION Mk 1B 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 MinKNOW for every sequencing experiment. MinKNOW can also demultiplex reads by barcode, and basecall/demultiplex data after a sequencing run has completed.

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.

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.

MinKNOW installation and use

For instructions on how to install the MinKNOW software, please refer to the relevant "Download and install MinKNOW" section in the MinKNOW protocol.

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 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:

Flow cell	Minimum number of active pores covered by warranty
Flongle Flow Cell	60
MinION/GridION Flow Cell	800
PromethION Flow Cell	5000

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.

Reverse transcription

~75 minutes

Materials	• Input RNA
Consumables	• Random Primer Mix (NEB, S1330S) • 10 mM dNTP solution (e.g. NEB N0447) • SuperScript IV reverse transcriptase, 5X RT buffer and 100 mM DTT (ThermoFisher Scientific, 18090010) • RNaseCUT™, 40 U/µl (Life Technologies, 10777019) • 0.2 ml thin-walled PCR tubes • 1.5 ml Eppendorf DNA LoBind tubes
Equipment	• P200 pipette and tips • P2 pipette and tips • Thermal cycler • Microfuge • Ice bucket with ice
Optional Equipment	• PCR-Cooler (Eppendorf) • PCR hood with UV steriliser (optional but recommended to reduce cross-contamination)

Input RNA guidelines

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

qPCR ct	Dilution factor
18–35	none
15–18	1:10
12–25	1:100

If the sample has a low copy number (ct 18–35) use 11 µl of undiluted sample, but be aware that co-extracted compounds may inhibit reverse transcription and PCR.

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, mix together the following components in a 0.2 ml PCR tube on ice or in a PCR cool rack such as the Eppendorf PCR-Cooler:

Reagent	Volume
60 µM random hexamers and anchored polyT(23)	1 µl
10 mM dNTPs	1 µl
RNA sample	11 µl
Total	**13 µl**

2 **Mix gently by flicking the tube, and spin down. Return the tube to ice.**

3 **Preheat the thermal cycler to 65°C, with a heated lid at 105°C.**

4 **Incubate the reaction in the thermal cycler at 65°C for 5 mins.**

5 **Immediately snap-cool the sample by placing on ice for >1 min. More consistent cooling may be achieved using a PCR tube cool block such as the Eppendorf PCR-Cooler.**

6 **In a clean 1.5 ml Eppendorf DNA LoBind tube, mix together the following reagents in a clean pre-PCR hood:**

Reagent	Volume
5X SuperScript IV buffer	4 µl
100 mM DTT	1 µl
RNaseOUT RNase Inhibitor	1 µl
Superscript IV Reverse Transcriptase	1 µl
Total	**7 µl**

A master mix can be made if processing multiple samples simultaneously.

7 **After the RNA sample has cooled for >1 min, add 7 µl of the above master mix to the sample in the pre-PCR hood.**

8 **Mix gently by flicking the tube, and spin down. Return the tube to ice.**

9 **Preheat the thermal cycler to 42°C, with a heated lid at 105°C.**

10 **Incubate the sample in the thermal cycler using the following program:**

Temperature	Time
42°C	50 min
70°C	10 min
4°C	Hold

END OF STEP

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

PCR and clean-up

~210 minutes

Consumables

- COVID-19 primers (lab-ready at 100 µM, IDT)
- Q5® Hot Start High-Fidelity 2X Master Mix (NEB, M0494)
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Agencourt AMPure XP beads
- Freshly prepared 80% ethanol in nuclease-free water
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes

Equipment

- Microfuge
- Thermal cycler
- Hula mixer (gentle rotator mixer)
- Magnetic rack
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips

Optional Equipment

- Qubit fluorometer (or equivalent for QC check)
- Agilent Bioanalyzer (or equivalent)
- PCR hood with UV steriliser (optional but recommended to reduce cross-contamination)

Primer design

To generate tiled PCR amplicons from the COVID-19 viral cDNA, primers were designed by Josh Quick using Primal Scheme. These primers are designed to generate 400 bp amplicons that overlap by approximately 20 bp. These primer sequences can be found here. Where we show example data outputs in this protocol, the same parameters were used to design primers to the human coronavirus 229E to provide guideline statistics.

IMPORTANT

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

IMPORTANT

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

1 **Add 5 µl of each primer from pool A to a 1.5 ml Eppendorf DNA LoBind tube to give a 100 µM stock primer pool.**

2 **Add 5 µl of each primer from pool B to a 1.5 ml Eppendorf DNA LoBind tube to give a 100 µM stock primer pool.**

3 Dilute each 100 µM stock 1 in 10 with nuclease-free water to form a working stock of each pool at 10 µM.

Note: To achieve the desired final concentration of each primer in the pool at 0.015 µM in the PCR reaction, 3.7 µl of the 10 µM working stock is needed for each PCR reaction. Two separate PCR reactions will be performed per sample, one for pool A primers and one for pool B. This results in tiled amplicons that have approximately 20 bp overlap.

4 In a clean pre-PCR hood, set up two individual reactions using primer pool A and primer pool B in clean 0.2 ml PCR tubes:

Reagent	Volume (pool A)	Volume (pool B)
Reverse-transcribed sample from above	2.5 µl	2.5 µl
Q5® Hot Start High-Fidelity 2X Master Mix	12.5 µl	12.5 µl
Primer pool at 10 µM (A or B)	3.7 µl	3.7 µl
Nuclease-free water	6.3 µl	6.3 µl
Total	**25 µl**	**25 µl**

Note: If running multiple samples, make up individual master mixes for each primer pool and aliquot 22.5 µl to the corresponding 0.2 ml PCR tubes prior to adding the sample.

IMPORTANT

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

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

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	98°C	15 sec	25–35
Annealing and extension	65°C	5 min	25–35
Hold	4°C	∞	

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

IMPORTANT

If available, a clean post-PCR hood should be used for all steps that involve handling amplified material. Decontamination with UV and or DNAzap between sample batches is recommended.

7 Combine the 25 µl reaction from pool A and the 25 µl reaction from pool B into a new 1.5 ml Eppendorf DNA LoBind tube; one tube per sample.

8 **Resuspend the AMPure XP beads by vortexing.**

9 **Add 50 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube.**

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

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

12 **Spin down the sample and pellet the beads on a magnet for 5 mins. Keep the tube on the magnet, and pipette off the supernatant.**

13 **Keep the tube on the magnet and wash the beads with 200 µl of freshly-prepared 80% ethanol without disturbing the pellet. Keeping the magnetic rack on the benchtop, rotate the bead-containing tube by 180°. Wait for the beads to migrate towards the magnet and form a pellet. Remove the ethanol using a pipette and discard.**

14 **Repeat the previous step.**

15 **Spin down and place the tubes 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.**

16 **Remove the tube from the magnetic rack and resuspend pellet in 15 µl nuclease-free water. Incubate for 2 minutes at room temperature.**

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

18 **Remove and retain 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

- Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube
- Dispose of the pelleted beads

19 **Quantify 1 µl of eluted sample using a Qubit fluorometer.**

20 **Store any unused amplified material at -20°C for use in later experiments.**

Expected results

During initial method development, it is useful to analyse 1 µl on an Agilent Bioanalyzer chip or an appropriate amount on an agarose gel. The traces below show expected results where a dilution series of coronavirus 229E was spiked into 100 ng of human RNA extracted from GM12878 (primers were designed against the human coronavirus 229E reference genome using Primal Scheme). Here, Qubit hsDNA results and Agilent Bioanalyser (DNA 12000 assay) traces are shown for 30 and 35 cycles of PCR with input concentrations ranging from 10 pg to 0.001 pg in 100 ng human RNA. While not directly comparable to Ct values of a real biological sample, these give a rough approximation of high to low viral titres. A human-only and reverse transcription negative control were also included.

Note: The viral RNA that was used for this spike-in experiment was obtained from ATCC and is total RNA extracted from human cell lines infected with coronavirus 229E. So, 10 pg of spike-in represents a mix of human and viral RNA, spiked into 100 ng of human RNA extracted from GM12878 cells.

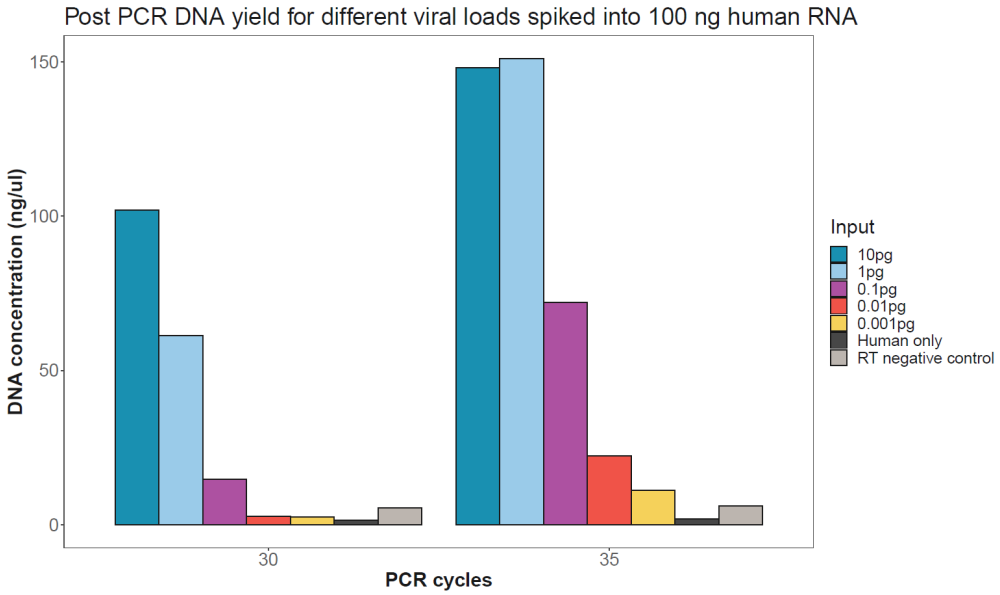


Figure 1. DNA yield after PCR and AMPure XP clean-up for decreasing viral input and different PCR cycle numbers in a background of 100 ng human RNA.

For a 400 bp amplicon, approximately 50 ng (~0.2 pmol) is required for the end-prep step. PCR cycles can be adjusted based on initial results to minimise the number of cycles. For samples that provide less than 50 ng total yield, further PCRs may be carried out on the remaining reverse transcription reaction.

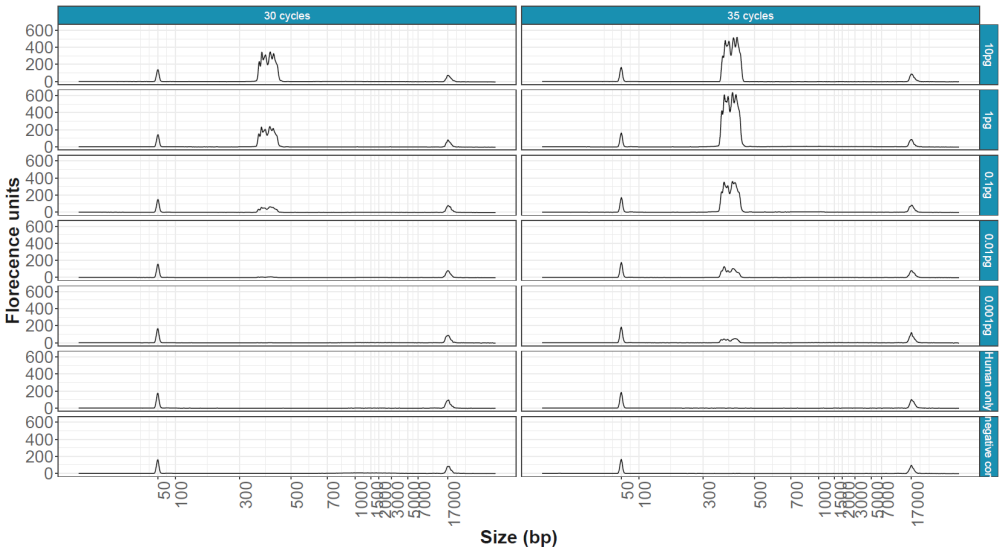


Figure 2. Bioanalyser traces of 1 µl of post-PCR cleaned up samples with decreasing input quantities, spiked into 100 ng human RNA amplified with 30 and 35 cycles. RT negative controls and human-only negative controls show no product in the 300–400 bp range.

End-prep

~20 minutes

Consumables

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

- NEBNext Ultra II End repair / dA-tailing Module (E7546)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes

Equipment

- P1000 pipette and tips
- P100 pipette and tips
- P10 pipette and tips
- Thermal cycler at 20° C and 65° C
- Microfuge
- Ice bucket with ice

IMPORTANT

For optimal efficiency of the end-prep reaction, use ~200 fmol (50 ng for 400 bp amplicons) of cDNA from the previous step.

The end_prep_molarity Excel file allows for easy calculation of this.

IMPORTANT

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

1 **Determine the volume of the cleaned-up PCR reaction that yields 200 fmol (50 ng) of DNA.**

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

3 **In a 0.2 ml thin-walled PCR tube, mix in the following order:**

Reagent	Volume
cDNA	x µl (50 ng per sample)
Nuclease-free water	12.5-x µl
Ultra II End-prep reaction buffer	1.75 µl
Ultra II End-prep enzyme mix	0.75 µl
Total	**15 µl**

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

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

END OF STEP

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

Native barcode ligation

~60 minutes

Materials	• Native Barcoding Expansion 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) if multiplexing more than 12 samples • Short Fragment Buffer (SFB)
Consumables	• Freshly prepared 80% ethanol in nuclease-free water • 1.5 ml Eppendorf DNA LoBind tubes • Nuclease-free water (e.g. ThermoFisher, cat # AM9937) • Agencourt AMPure XP beads • NEBNext Ultra II Ligation Module (E7595)
Equipment	• Magnetic separator, suitable for 1.5 ml Eppendorf tubes • Thermal cycler • Hula mixer (gentle rotator mixer) • Vortex mixer • Ice bucket with ice • Microfuge • P1000 pipette and tips • P100 pipette and tips • P10 pipette and tips
Optional Equipment	• Qubit fluorometer (or equivalent for QC check)

IMPORTANT

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

1 **Thaw the Native Barcodes at room temperature, enough for one barcode per sample. Individually mix the barcodes by pipetting, and place them on ice.**

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

3 **Select a unique barcode for every sample to be run together on the same flow cell, from the provided 24 barcodes. Up to 24 samples can be barcoded and combined in one experiment.**

4 **Add the reagents in the order given below, mixing by flicking the tube between each sequential addition:**

Reagent	Volume
Nuclease-free water	5.5 µl
End-prepped DNA	1.5 µl
Native Barcode	2.5 µl
NEBNext Ultra II Ligation Master Mix	10 µl
NEBNext Ligation Enhancer	0.5 µl
Total	**20 µl**

5 **Mix contents thoroughly by pipetting and spin down briefly.**

6 **Using a thermal cycler, incubate at 20°C for 20 mins and at 65°C for 10 mins.**

7 **Pool all barcoded samples together into a 1.5 ml Eppendorf DNA LoBind tube.**

8 **Resuspend the AMPure XP beads by vortexing.**

9 **Add 0.4x volumes of resuspended AMPure XP beads to the reaction and mix by pipetting. For example, 24 reaction pooled to a total of 480 µl would require 192 µl AMPure XP beads.**

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

11 **Prepare sufficient fresh 80% ethanol in nuclease-free water.**

12 **Spin down the sample and pellet the beads on a magnet for 5 mins. Keep the tube on the magnet, and pipette off the supernatant.**

13 **Wash the beads by adding 700 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.**

14 **Repeat the previous step.**

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

16 **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.**

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

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

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

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

Adapter ligation and clean-up

~70 minutes

Materials	• Short Fragment Buffer (SFB) • Elution Buffer (EB) • Adapter Mix II (AMII)
Consumables	• NEBNext Quick Ligation Module (E6056) • Agencourt AMPure XP beads • 1.5 ml Eppendorf DNA LoBind tubes
Equipment	• Microfuge • Magnetic rack • Vortex mixer • Hula mixer (gentle rotator mixer)
Optional Equipment	• Qubit fluorometer (or equivalent for QC check)

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

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

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

Reagent	Volume
Pooled barcoded sample	x µl (~30-50 ng of pooled barcoded material)
Nuclease-free water	30-x µl
Adapter Mix II (AMII)	5 µl
NEBNext Quick Ligation Reaction Buffer (5X)	10 µl
Quick T4 DNA Ligase	5 µl
Total	**50 µl**

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

5 Incubate the reaction for 20 minutes at room temperature.

IMPORTANT

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

6 Resuspend the AMPure XP beads by vortexing.

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

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

9 Spin down the sample and pellet the beads on a magnet for 5 mins. Keep the tube on the magnet, and pipette off the supernatant.

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

11 Repeat the previous step.

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

13 Remove the tube from the magnetic rack and resuspend pellet in 15 µl Elution Buffer (EB).

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

15 Remove and retain 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

- Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube
- Dispose of the pelleted beads

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

IMPORTANT

We recommend loading ~15 ng of this final prepared library onto the flow cell. Loading more than 15 ng can have a detrimental effect on throughput. Dilute the library in EB if required.

END OF STEP

The prepared library is used for loading into the MinION Mk 1B flow cell. Store the library on ice until ready to load.

TIP

Library storage recommendations

We recommend storing libraries in Eppendorf LoBind tubes at **4°C for short term** storage or repeated use, for example, re-loading flow cells between washes.
For single use and **long term storage** of more than 3 months, we recommend storing libraries at **-80°C** in Eppendorf LoBind tubes.
For further information, please refer to the Library Stability Know-How document.

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

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

Priming and loading the SpotON flow cell

~10 minutes

Materials	• Flush Tether (FLT) • Flush Buffer (FB) • Loading Beads (LB) • Sequencing Buffer (SQB)
Consumables	• 1.5 ml Eppendorf DNA LoBind tubes • Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
Equipment	• MinICN Mk 1B • SpotCN Flow Cell • P1000 pipette and tips • P100 pipette and tips

- P20 pipette and tips
- P10 pipette and tips

IMPORTANT

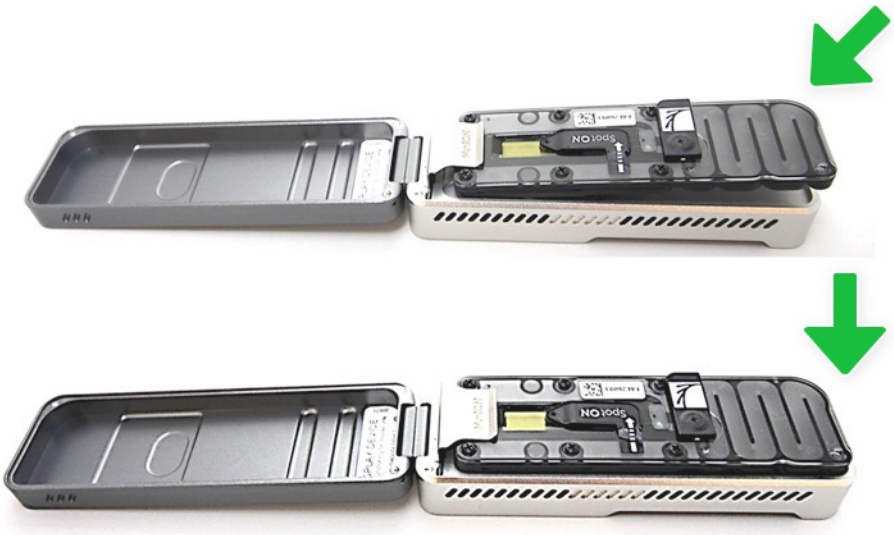
Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.

1 **Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature before placing the tubes on ice as soon as thawing is complete.**

2 **Mix the Sequencing Buffer (SQB), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing, spin down then return to ice.**

3 **Open the MinION Mk 1B lid and slide the flow cell under the clip.**

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



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 µls, 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 µls):**

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

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

6 **Prepare the flow cell priming mix: add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing.**

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

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

IMPORTANT

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

TIP

Using the Loading Beads

Demo of how to use the Loading Beads.

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

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

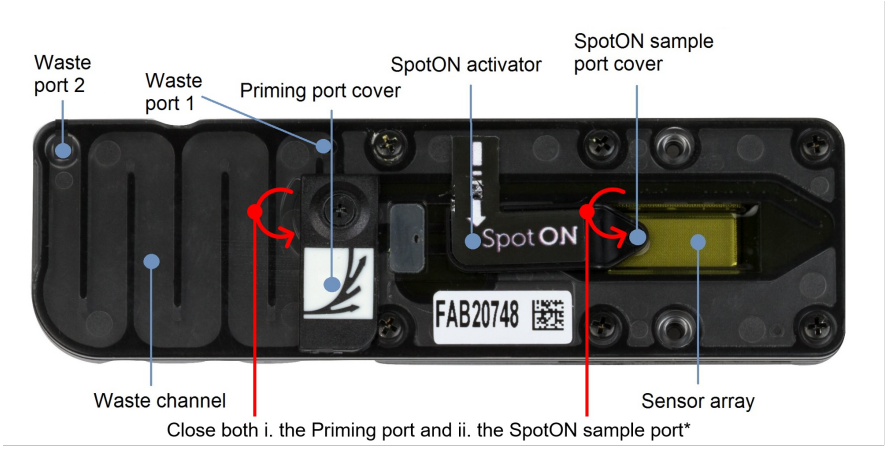
10 Complete the flow cell priming:

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

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

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

13 **Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION Mk 1B lid.**



*Both ports are shown in a closed position

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

Please 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 MinIT device

Please follow the instructions in the MinIT protocol.

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

Please 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, please follow the instructions in the Guppy protocol starting from the "Quick Start Guide for Guppy" section.

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 Rampart software (https://github.com/artic-network/rampart) provides a real-time analysis of sequencing results. The demultiplexed results from MinKNOW basecalling are mapped to the reference COVID-19 genome and graphical presentation of the data allows for a review of sequencing performance. The number of reads from each barcoded sample, their distribution over the reference genome and overall depth of coverage are presented. These real-time results can be used to ensure that sufficient reads have been sequenced from each of the barcoded samples prior to the genome assembly and variant calling steps.

The reference guided genome assembly and variant calling are also performed according to the bioinformatics protocol provided by the ARTIC-network. Their best practises guide uses the software contained within the FieldBioinformatics project on GitHub (https://github.com/artic-network/fieldbioinformatics).

This workflow uses only the basecalled FASTQ files to perform a high-quality reference-guided assembly of the COVID-19 genome. Sequenced reads are re-demultiplexed with the requirement that reads must contain a barcode at both ends of the sequence, 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.

The Rampart and genome analysis workflows can both be deployed on computers running either Linux or MacOS. The ARTIC authors provide instructions and environment files to build an ARTIC bioinformatics environment using the CONDA software. The Rampart and coronavirus bioinformatics protocols include the precise instructions to install and run the software.

To further simplify the installation of the Rampart and coronavirus bioinformatics protocols, the workflows have been packages as Docker containers.

Docker is a tool designed to make the deployment and sharing of workflows easier using "containers". A container packages the application along with (in our case) its bioinformatics dependencies and required linux libraries. Docker containers for both Rampart and the coronavirus bioinformatics protocol have been deposited on Dockerhub (https://hub.docker.com) - this is a platform for the sharing of open containers.

The Rampart container available at (https://hub.docker.com/r/ontresearch/artic_rampart) provides the real-time library checking workflow - installation pages are included on the DockerHub pages.

The coronavirus bioinformatics protocol is available at https://hub.docker.com/r/ontresearch/artic_bioinformatics.

The coronovirus bioinformatics protocol has been implemented as a Jupyter notebook. This is presented through a containerised Jupyter-labs installation. 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 containers have been tested on Linux, MacOS and Windows. The Rampart container has also been tested on MinIT.

Expected results

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

Note: The viral RNA from ATCC is generated from cell lines infected with human coronavirus 229E. The RNA supplied is total RNA extracted from the cell

lines and includes both human and viral RNA. Therefore, the levels of sensitivity are likely to be higher than those reported here.

Sample balancing

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

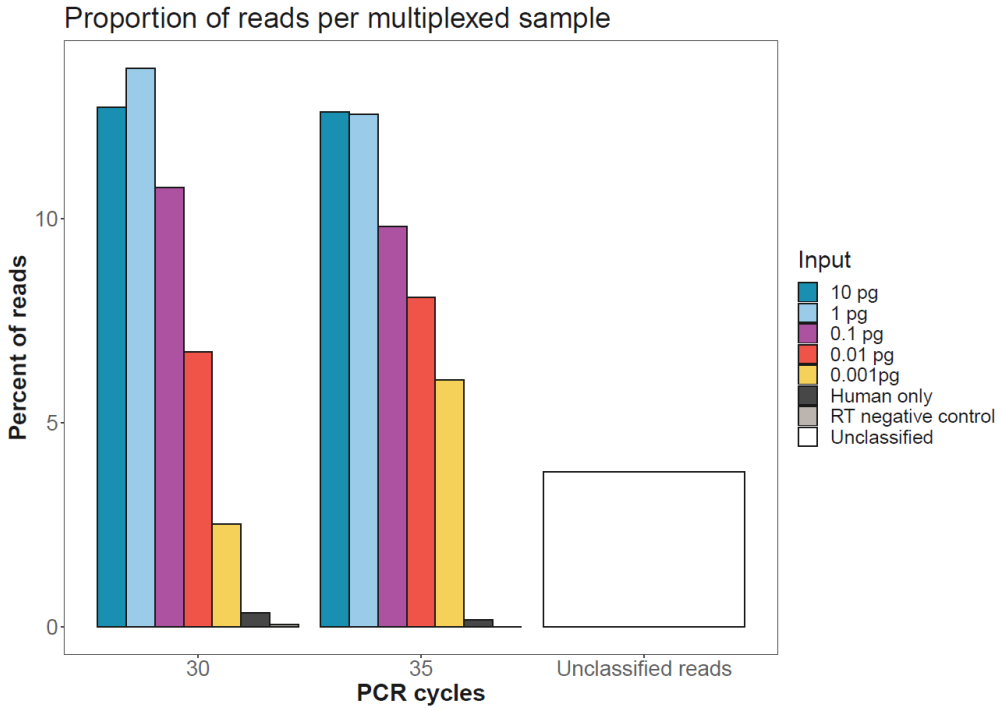


Figure 3. Number of reads per sample after native barcode demultiplexing in MinKNOW. All 14 samples were run on a single flow cell.

On-target rate

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

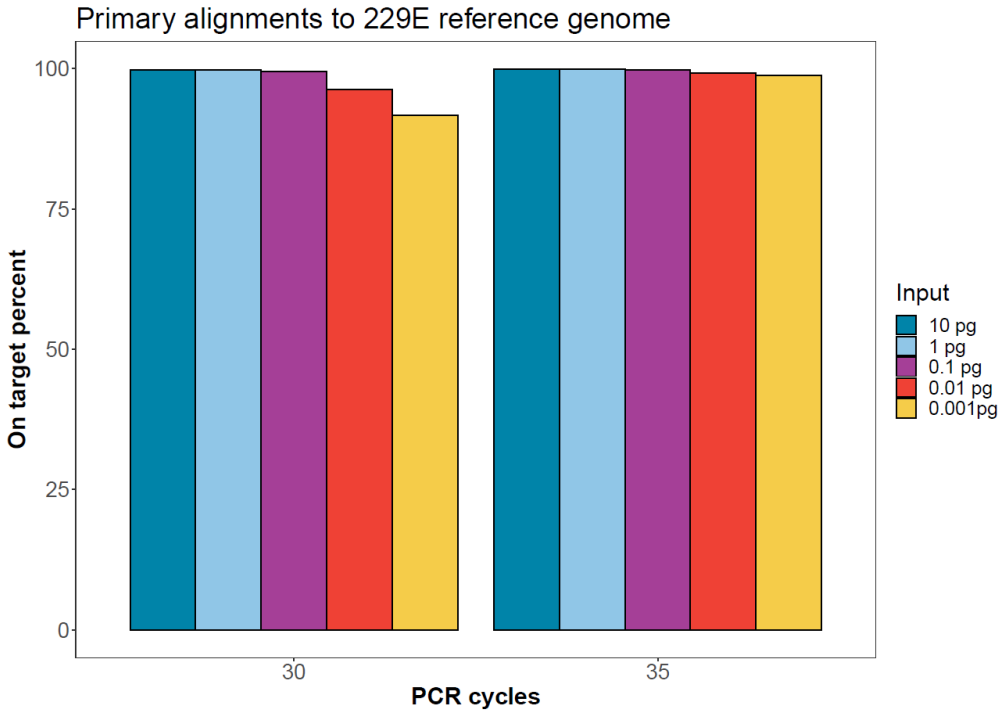


Figure 4. Proportion of reads for each sample aligning to the human coronavirus 229E reference genome.

Assessment of negative controls

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

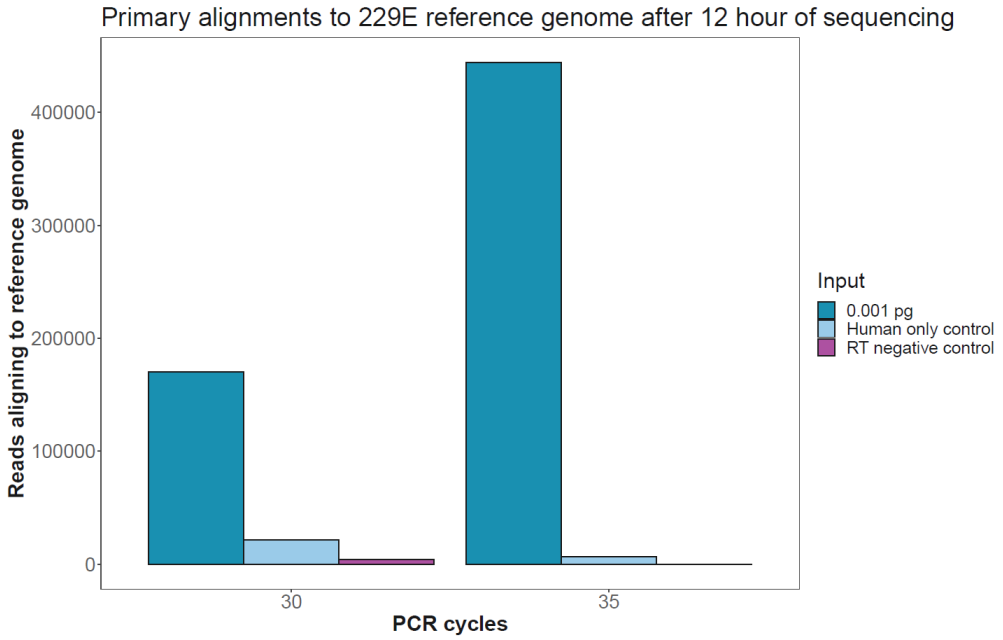


Figure 5. Absolute number of reads aligning to the human coronavirus 229E reference genome in the negative controls compared with the lowest input of viral RNA. Sequencing was carried out for 12 hours to pick up low levels of sequences assigned to barcodes representing these samples.

Target coverage for different PCR cycles and viral load

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

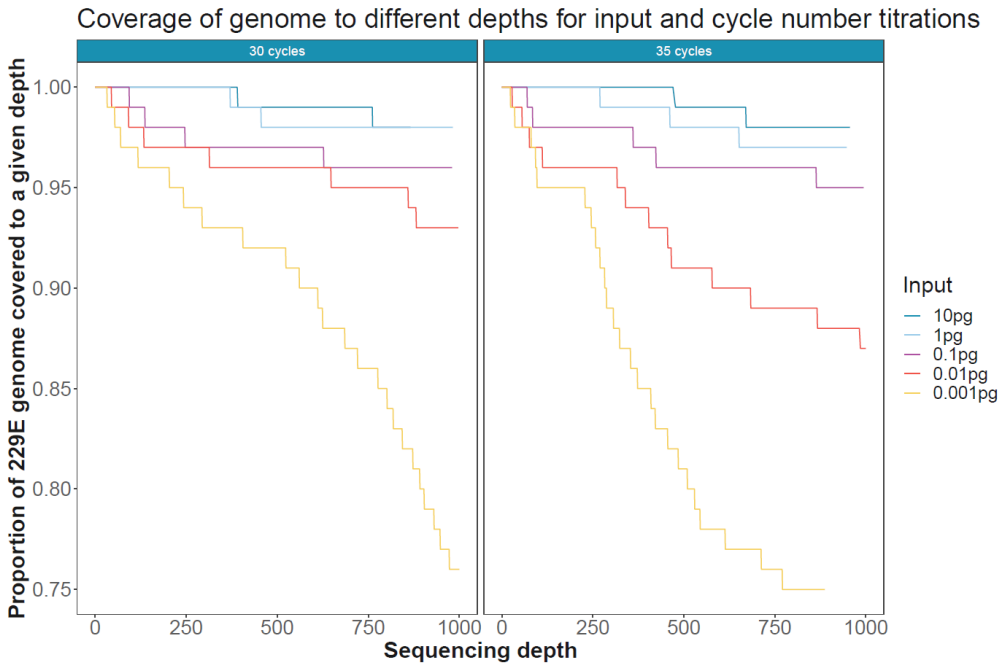


Figure 6. Coverage and depth of the human coronavirus 229E genome for different input quantities of viral RNA and different cycle numbers after 12 hours of sequencing on a single flow cell.

How much sequencing is required?

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

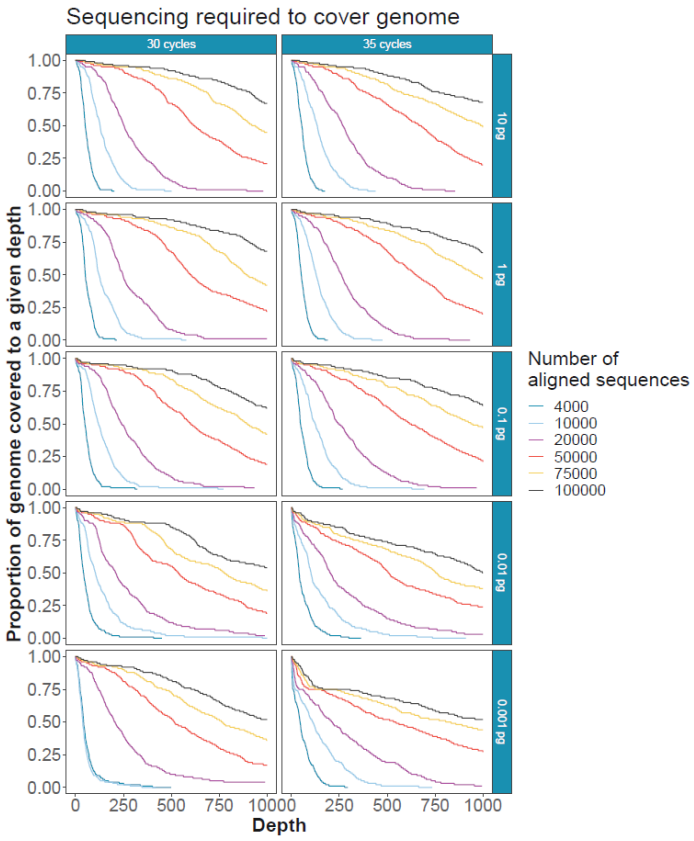


Figure 7. Subsampled sequences to give an indication of the depth of sequencing achievable covering different amounts of the human coronavirus 229E genome. Input quantities and cycle number titrations show that high cycle numbers should be avoided where possible to minimise amplicon drop out.

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

Ending the experiment

Materials

- Flow Cell Wash Kit (EXP-WSH003)

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

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

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

Instructions for returning flow cells can be found here.

IMPORTANT

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

Issues during DNA/RNA extraction and library preparation

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

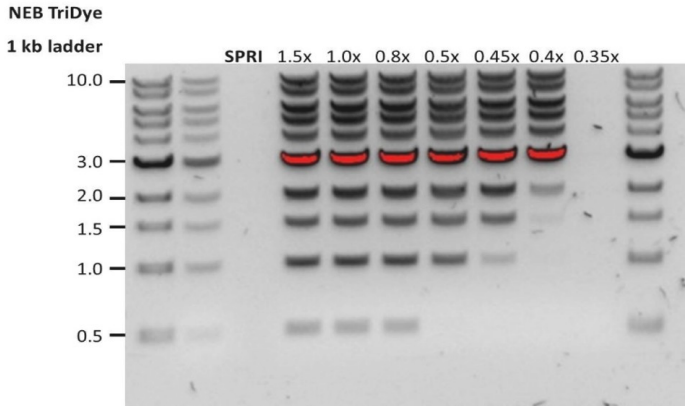
If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via LiveChat in the Nanopore Community.

Low sample quality

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

Low DNA recovery after AMPure bead clean-up

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.

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.

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.

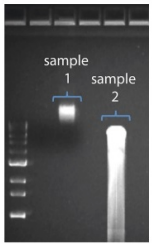
MinKNOW script failed

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.

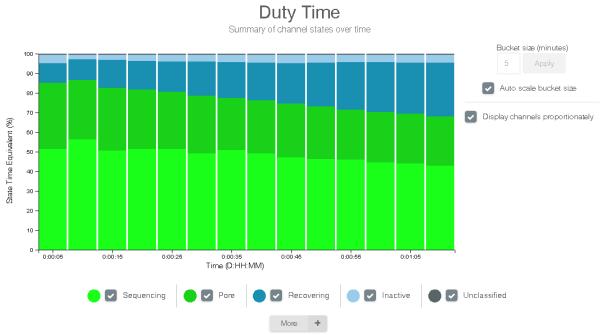
Pore occupancy below 40%

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 Mk 1B/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

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

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

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.