Version 3

Aug 25, 2020

# nCoV-2019 sequencing protocol v3 (LoCost) V.3

Version 1 is forked from Ebola virus sequencing protocol

In 1 collection

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ARTIC Coronavirus Method Development Community 1 more workspace

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ABSTRACT

Amplicon sequencing protocol for SARS-CoV-2 v3 (LoCost)

We thank the ARTIC network, Oxford Nanopore Technologies, New England Biolabs, BCCDC, COG-UK, CanCOGen and protocols.io commenters for their assistance developing this protocol.

Changes in this version:

-Up to 95 samples per run with EXP-NBD196 native barcode kit
-Substitution of SuperScript IV for LunaScript RT SuperMix and reaction volume reduced to 10 uL.
-Substitution of Ultra II Ligation Module for Blunt/TA Ligase Master Mix and reaction volume reduced to 10 µL.
-Native barcode ligation reaction volume reduced to 10 uL.
-SFB wash volume reduced.

PROTOCOL CITATION

Josh Quick 2020. nCoV-2019 sequencing protocol v3 (LoCost). **protocols.io** https://protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-bh42j8ye Version created by Josh Quick

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Improvements to the ARTIC multiplex PCR method for SARS-CoV-2 genome sequencing using nanopore. John R Tyson, et. al. bioRxiv 2020.09.04.283077; doi:https://doi.org/10.1101/2020.09.04.283077

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MATERIALS TEXT

| Component | Supplier | Part number |
|---|---|---|
| ARTIC nCoV-2019 V3 panel (100uM) | IDT | See links below |
| LunaScript RT SuperMix Kit | NEB | E3010 |
| Q5 Hot Start HF Polymerase or | NEB | M0493 |
| Q5 Hot Start High-Fidelity 2X Master Mix | NEB | M0494 |
| dNTP Solution Mix (10 mM ea.) | NEB | N0447 |
| Nuclease-free water (100 mL) | NEB | B1500 |
| NEBNext Ultra II End Repair/dA-tailing module | NEB | E7546 |
| Blunt/TA Ligase Master Mix | NEB | M0367 |
| Native Barcoding Expansion Kit 1-12 and/or | ONT | EXP-NBD104 |
| Native Barcoding Expansion Kit 13-24 or | ONT | EXP-NBD114 |
| Native Barcoding Expansion Kit 96 | ONT | EXP-NBD196 |
| AMPure XP beads | Beckman | A63881 |
| NEBNext Quick Ligation Module | NEB | E6056S |
| Sequencing Auxiliary Vials | ONT | EXP-AUX001 |
| Short Fragment Buffer Expansion Kit | ONT | EXP-SFB001 |
| Qubit dsDNA HS Assay Kit | Thermo | Q32854 |
| Flow Cell Priming Kit | ONT | EXP-FLP002 |
| Flow Cell Wash Kit (optional) | ONT | EXP-WSH003 |
| R9.4.1 flow cells | ONT | FLO-MIN106 |

IDT premixed ARTIC nCoV-2019 V3 panel or order oligos individually.

BEFORE STARTING

Prepare between 11 and 95 RNA samples plus 1 negative control using this protocol.

## cDNA preparation 30m

1 Prepare between 11 and 95 RNA samples plus 1 negative control of nuclease-free water per library. If previously frozen, mix by briefly vortexing and pulse spin to collect liquid. Keep samples on ice at all times.

At least 11 samples are required to have sufficient material to load on the sequencer at the end. If you process >23 you will need the EXP-NBD196 expansion kit.

A positive control can also be included which may be a synthetic RNA constructs or high-titre clinical sample which can be diluted. This can help monitor run performance.

2 Mix the following components in a PCR strip-tubes/plate. Gently mix by pipetting and pulse spin the tube to collect liquid.

| Component | Volume |
|---|---|
| LunaScript RT SuperMix (5X) | 2 µL |
| Template RNA | 8 µL |
| **Total** | **10 µL** |

Viral RNA input from a clinical sample should be between Ct 18-35. If Ct is between 12-15, then dilute the sample 100-fold in water, if between 15-18 then dilute 10-fold in water. This will reduce the likelihood of PCR-inhibition.

To prevent pre-PCR contamination the mastermix should be added to the PCR strip-tubes/plate in the **mastermix** cabinet which should should be cleaned with decontamination wipes and UV sterilised before and after use.

RNA samples should be added in the **extraction/sample addition** cabinet which should should be cleaned with decontamination wipes and UV sterilised before and after use.

3 Incubate the reaction as follows:

**25 °C** for **00:02:00**

**55 °C** for **00:10:00**

**95 °C** for **00:01:00**

Hold at **4 °C**

Primer pool preparation (optional) 2h

4 If making up primer pools from individual oligos fully resuspend lyophilised oligos in 1xTE to a concentration of **100 Micromolar (µM)** , vortex thoroughly and spin down.

If using IDT ARTIC nCoV-2019 V3 Panel ( **100 Micromolar (µM)** ) skip to step 6.

5 Sort all odd regions primers into one or more tube racks. Add **5 µl** of each odd region primer to a **1.5 mL** Eppendorf tube labelled "Pool 1 ( **100 Micromolar (µM)** )". Repeat the process for all even region primers for Pool 2. These are your **100 Micromolar (µM)** stocks of each primer pool.

Primers should be diluted and pooled in the **mastermix** cabinet which should be cleaned with decontamination wipes and UV sterilised before and after use.

For more information see Figure 2 in;

Quick, J. et al. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. Nat Protoc 12, 1261–1276 (2017). https://doi.org/10.1038/nprot.2017.066

6 Dilute [M]**100 Micromolar (µM)** pools 1:10 in molecular grade water, to generate [M]**10 Micromolar (µM)** primer stocks.

Primers are used at a final concentration of [M]**15 Nanomolar (nM)** per primer. In this case V3 pools have 110 primers in pool 1 and 108 primers in pool 2. so the requirement is ~ **4 µl** primer pool ( [M]**10 Micromolar (µM)** ) per **25 µl** reaction.

Make up multiple **100 µl** aliquots of [M]**10 Micromolar (µM)** primer dilutions and freeze them in case of degradation or contamination.

Multiplex PCR 4h

7 Set up the two PCR reactions per sample as follows in strip-tubes or plates. Gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

| Component | Reaction 1 | Reaction 2 |
|---|---|---|
| 5X Q5 Reaction Buffer | 5 µL | 5 µL |
| 10 mM dNTPs | 0.5 µL | 0.5 µL |
| Q5 Hot Start DNA Polymerase | 0.25 µL | 0.25 µL |
| V3 Pool 1 (10µM) | 4 µL | 0 µL |
| V3 Pool 2 (10µM) | 0 µL | 4 µL |
| Nuclease-free water | 12.75 µL | 12.75 µL |
| **Total** | **22.5 µL** | **22.5 µL** |

For M0493

or

| Component | Reaction 1 | Reaction 2 |
|---|---|---|
| Q5 Hot Start High-Fidelity 2X Master Mix | 12.5 µL | 12.5 µL |
| V3 Pool 1 (10µM) | 4 µL | 0 µL |
| V3 Pool 2 (10µM) | 0 µL | 4 µL |
| Nuclease-free water | 6 µL | 6 µL |
| **Total** | **22.5 µL** | **22.5 µL** |

For M0494

Q5 Hot Start High-Fidelity 2X Master Mix can also be used instead of the component kit. Half-scale PCR reactions can also be used to save costs as you will only require **2.5 µl** for downstream steps.

To prevent pre-PCR contamination the mastermix for each pool should be made up in the **mastermix** cabinet which should should be cleaned with decontamination wipes and UV sterilised before and after use and aliquoted into PCR strip-tubes/plate

8 Add **2.5 µl** cDNA to each of the PCR reactions, gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

Up to **5 µl** cDNA can be added to each PCR reaction (in place of nuclease-free water) to improve amplification of low titre samples. Using **5 µl** cDNA will require a **20 µl** cDNA reaction and may be more likely to cause inhibition so use cautiously.

cDNA should be added in the **extraction and sample addition** cabinet which should should be cleaned with decontamination wipes and UV sterilised before and after use.

9 **0 µl** Set-up the following program on the thermal cycler:

| Step | Temperature | Time | Cycles |
|---|---|---|---|
| Heat Activation | **98 °C** | **00:00:30** | 1 |
| Denaturation | **98 °C** | **00:00:15** | 25-35 |
| Annealing | **65 °C** | **00:05:00** | 25-35 |
| Hold | **4 °C** | Indefinite | 1 |

Cycle number should be 25 for Ct 18-21 up to a maximum of 35 cycles for Ct 35.

Thermocycler calibration can vary instrument to instrument. If you see amplicon 64 dropout then decrease the annealing/extension temperature to **63 °C** . Denaturation temperature of **95 °C** can also be used and may slightly increase PCR yields.

PCR dilution 10m

10 Label strip-tubes/plate and combine the following volumes of each PCR reaction for **10 µl** each sample:

| Component | Volume |
|---|---|
| Pool 1 PCR reaction | 2.5 µL |
| Pool 2 PCR reaction | 2.5 µL |
| Nuclease-free water | 45 µL |
| **Total** | **50 µL** |

The PCR post-clean up concentration is typically around **100 Mass Percent** . This means we can pool them without quantification/normalisation to make a significant time saving. If you require very even barcode representation perform clean-up and normalise to **10 Mass Percent** then continue.

Amplicons should be added in the **post-PCR** cabinet which should should be cleaned with decontamination wipes and UV sterilised before and after use.

Native barocoding 2h

11 Barcode the amplicon pools using the one-pot native barcoding approach.

One-pot native barcoding of amplicons v3 (LoCost)
**by Josh Quick**

PREVIEW RUN

11.1 In a new PCR strip-tube/plate set up the following reaction for each sample:

| Component | Volume |
|---|---|
| PCR dilution from previous step | 3.3 µL |
| Ultra II End Prep Reaction Buffer | 1.2 µL |
| Ultra II End Prep Enzyme Mix | 0.5 µL |
| Nuclease-free water | 5 µL |
| **Total** | **10 µL** |

Make a master mix of end-preparation reagents and nuclease-free water and aliquot into strip-tube/plate to improve reproducability.

11.2 Incubate at room temperature for **00:15:00**

Incubate at **65 °C** for **00:15:00**

Incubate on ice for **00:01:00**

11.3 In a new PCR strip-tube/plate set up the following reaction for each sample:

| Component | Volume |
|---|---|
| End-preparation reaction mixture | 0.75 µL |
| NBXX barcode | 1.25 µL |
| Blunt/TA Ligase Master Mix | 5 µL |
| Nuclease-free water | 3 µL |
| **Total** | **10 µL** |

Use one native barcode from the EXP-NBD104 (1-12), EXP-NBD114 (13-24) or EXP-NBD196 per sample. Use 12 or more barcodes per library or there will be insufficient total material to achieve good yields.

11.4 Incubate at room temperature for **00:20:00**

Incubate at **65 °C** for **00:10:00**

Incubate on ice for **00:01:00**

The 65°C incubation is to inactivate the DNA ligase to prevent barcode cross-ligation when reactions are pooled in the next step.

11.5 In a new **1.5 mL** Eppendorf tube pool all one-pot barcoding reactions together.

If processing 12-24 samples pool all **10 µl** from each native barcoding reaction.

if processing 48 samples pool **5 µl** from each native barcoding reaction.

If processing 96 samples pool **2.5 µl** from each native barcoding reaction so as not to exceed a pool volume of **240 µl** which would make the clean-up volume too large.

11.6 Add 0.4x volume of SPRI beads to the sample tube and mix gently by either flicking or pipetting. For example add **96 µl** SPRI beads to **240 µl** pooled one-pot barcoding reactions.

0.4x volume of SPRI is sufficient to bind 400 bp amplicons in the presence of ligation buffer, do not use 1x as this will result in an excessive large bead pellet.

11.7 Mix by vortexing and pulse centrifuge to collect all liquid at the bottom of the tube. Incubate for **00:05:00** at room temperature.

11.8 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear. Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

11.9 Add **250 µl** SFB and resuspend beads completely by pipette mixing. Pulse centrifuge to collect all liquid at the bottom of the tube and place on the magnet. Remove supernatant and discard.

> SFB will remove excess adapter without damaging the adapter-protein complexes. Do not use 70% ethanol as in early clean-ups.

1.10 Repeat steps 11.9 to perform a second SFB wash. Pulse centrifuge and remove any residual SFB.

> You do not need to allow to air dry with SFB washes.

1.11 Add **200 µl** of room-temperature [M]**70 % volume** ethanol to bathe the pellet. Carefully remove and discard ethanol, being careful not to touch the bead pellet.

> Only perform 1x 70% ethanol wash

1.12 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette.

1.13 With the tube lid open incubate for **00:01:00** or until the pellet loses it's shine (if the pellet dries completely it will crack and become difficult to resuspend).

1.14 Resuspend pellet in **30 µl** [M]**10 Milimolar (mM)** Tris pH 8.0, mix gently by either flicking or pipetting and incubate for **00:02:00** .

1.15 Place on magnet and transfer sample to a clean **1.5 mL** Eppendorf tube ensuring no beads are transferred into this tube.

12 Quantify **1 µl** of the barcoded amplicons using the Quantus Fluorometer using the ONE dsDNA assay.

Concentration will vary depending on number and Ct of samples and but you need about **30 ng** total at this stage to acheive maximum run yield.

DNA quantification using the Quantus fluorometer
**by Josh Quick**

PREVIEW RUN

12.1 Remove Lambda DNA 400 ng/µL standard from the freezer and leave on ice to thaw. Remove ONE dsDNA dye solution from the fridge and allow to come to room temperature.

QuantiFluor(R) ONE dsDNA System, 500rxn **Promega Catalog #E4870**

12.2 Set up two **0.5 mL** tubes for the calibration and label them 'Blank' and 'Standard'

12.3 Add **200 µl** ONE dsDNA Dye solution to each tube.

12.4 Mix the Lambda DNA standard 400 ng/µL standard by pipetting then add **1 µl** to one of the standard tube.

12.5 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

12.6 Allow both tubes to incubate at room temperature for **00:02:00** before proceeding.

12.7 Selection 'Calibrate' then 'ONE DNA' then place the blank sample in the reader then select 'Read Blank'. Now place the standard in the reader and select 'Read Std'.

12.8 Set up the required number of **0.5 mL** tubes for the number of DNA samples to be quantified.

Use only thin-wall, clear, 0.5mL PCR tubes such as Axygen #PCR-05-C

12.9 Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading.

2.10 Add **199 µl** ONE dsDNA dye solution to each tube.

2.11 Add **1 µl** of each user sample to the appropriate tube.

> Use a P2 pipette for highest accuracy.

2.12 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

2.13 Allow all tubes to incubate at room temperature for **00:02:00** before proceeding.

2.14 On the Home screen of the Quantus Fluorometer, select `Protocol`, then select `ONE DNA` as the assay type.

> If you have already performed a calibration for the selected assay you can continue, there is no need to perform repeat calibrations when using ONE DNA pre diluted dye solution. If you want to use the previous calibration, skip to step 11. Otherwise, continue with step 9.

2.15 On the home screen navigate to 'Sample Volume' and set it to **1 µl** then 'Units' and set it to ng/µL.

2.16 Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.

2.17 Repeat step 16 until all samples have been read.

2.18 The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory notebook.

13 Set up the following AMII adapter ligation and clean-up with SFB.

Adapter ligation with AMII v2
**by Josh Quick**

PREVIEW RUN

13.1 In a new **1.5 µl** Eppendorf tube set up the following AMII adapter ligation reaction.

| Component | Volume |
|---|---|
| Barcoded amplicon pool | 30 µL |
| NEBNext Quick Ligation Reaction Buffer (5X) | 10 µL |
| Adapter Mix (AMII) | 5 µL |
| Quick T4 DNA Ligase | 5 µL |
| **Total** | **50 µL** |

13.2 Incubate at room temperature for **00:20:00**

13.3 Add **50 µl** (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. Pulse centrifuge to collect all liquid at the bottom of the tube.

Vortex SPRI beads thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown colour.

There will be some variation in clean-up efficiencies but expect to carry around 50% through this clean-up

13.4 Incubate for **00:05:00** at room temperature.

13.5 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear. Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

13.6 Add **250 µl** SFB and resuspend beads completely by pipette mixing. Pulse centrifuge to collect all liquid at the bottom of the tube. Remove supernatant and discard.

SFB will remove excess adapter without damaging the adapter-protein complexes. Do not use 70% ethanol as in early clean-ups.

13.7 Repeat steps 13.6 to perform a second SFB wash.

13.8 Pulse centrifuge and remove any residual SFB. Add **15 µl** EB (ONT) and resuspend beads by pipette mixing.

You do not need to allow to air dry with SFB washes.

13.9 Incubate at room temperature for **00:02:00** .

3.10 Place on magnetic rack until clear. Transfer final library to a new 1.5mL Eppendorf tube.

14 Quantify **1 µl** of the final library using the Quantus Fluorometer using the ONE dsDNA assay. Concentration will vary depending on number and Ct of samples but **15 ng** final library is usually required to acheive maximum run yield.

DNA quantification using the Quantus fluorometer
**by Josh Quick**

PREVIEW RUN

Final library can be now be stored in **10 Milimolar (mM)** Tris **pH 8** at **4 °C** for up to a week if needed otherwise proceed directly to MinION sequencing.

14.1 Remove Lambda DNA 400 ng/µL standard from the freezer and leave on ice to thaw. Remove ONE dsDNA dye solution from the fridge and allow to come to room temperature.

QuantiFluor(R) ONE dsDNA System, 500rxn **Promega Catalog #E4870**

14.2 Set up two **0.5 mL** tubes for the calibration and label them 'Blank' and 'Standard'

14.3 Add **200 µl** ONE dsDNA Dye solution to each tube.

14.4 Mix the Lambda DNA standard 400 ng/µL standard by pipetting then add **1 µl** to one of the standard tube.

14.5 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

14.6 Allow both tubes to incubate at room temperature for **00:02:00** before proceeding.

14.7 Selection 'Calibrate' then 'ONE DNA' then place the blank sample in the reader then select 'Read Blank'. Now place the standard in the reader and select 'Read Std'.

14.8 Set up the required number of **0.5 mL** tubes for the number of DNA samples to be quantified.

Use only thin-wall, clear, 0.5mL PCR tubes such as Axygen #PCR-05-C

14.9 Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading.

4.10 Add **199 µl** ONE dsDNA dye solution to each tube.

4.11 Add **1 µl** of each user sample to the appropriate tube.

Use a P2 pipette for highest accuracy.

4.12 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

4.13 Allow all tubes to incubate at room temperature for **00:02:00** before proceeding.

4.14 On the Home screen of the Quantus Fluorometer, select `Protocol`, then select `ONE DNA` as the assay type.

If you have already performed a calibration for the selected assay you can continue, there is no need to perform repeat calibrations when using ONE DNA pre diluted dye solution. If you want to use the previous calibration, skip to step 11. Otherwise, continue with step 9.

4.15 On the home screen navigate to 'Sample Volume' and set it to **1 µl** then 'Units' and set it to ng/µL.

4.16 Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.

4.17 Repeat step 16 until all samples have been read.

4.18 The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory notebook.

## MinION sequencing 1d

15 Prime the flowcell and load **15 ng** sequencing library onto the flowcell.

Priming and loading a MinION flowcell v2
**by Josh Quick**
PREVIEW RUN

From experience we know **15 ng** is optimum loading input for short amplicons. Speed drop during the run indicates excessive library was loaded. Low run yield <20M reads indicates insufficient library.

15.1 Thaw the following reagents at room temperature before placing on ice:

Sequencing buffer (SQB)
Loading beads (LB)
Flush buffer (FLB)
Flush tether (FLT)

15.2 Add **30 µl** FLT to the FLB tube and mix well by vortexing.

15.3 If required place a new MinION flowcell onto the MinION by flipping open the lip and pushing one end of the flowcell under the clip and pushing down gently.

15.4 Rotate the inlet port cover clockwise by 90° so that the priming port is visible.

15.5 Take a P1000 pipette and tip and set the volume to **800 µl** . Place the tip in the inlet port and holding perpendicularly to the plane of the flowell remove any air from the inlet port by turning the volume dial anti-clockwise.

Be careful not to remove so much volume that air is introduced onto the rectangular array via the outlet.

15.6 Load **800 µl** of FLB (plus FLT) into the flow cell via the inlet port, dispense slowly and smoothly trying to avoid the

introduction of any air bubbles.

15.7 Wait for **00:05:00** .

15.8 Gently lift the SpotON cover to open the SpotON port.

15.9 Load another **200 µl** of FLB (plus FLT) into the flow cell via the inlet port, this will initiate a siphon at the SpotON port to allow you to load the library dilution.

5.10 In a new tube prepare the library dilution for sequencing:

| Component | Volume |
|---|---|
| SQB | 37.5 µL |
| LB | 25.5 µL |
| Library | 12 µL |
| **Total** | **75 µL** |

Mix LB immediately before use as they settle quickly.

Make up with EB if less than 12 µL library is required.

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

5.12 Add the **75 µl** library dilution to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop siphons into the port before adding the next.

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

16 Start the sequencing run using MinKNOW.

Starting a MinION sequencing run using MinKNOW
**by Josh Quick**

PREVIEW RUN

If using Live basecalling ensure to turn on double-ended barcoding in the basecalling settings.

16.1 If required plug the MinION into the computer and wait for the MinION and flowcell to ben detected.

16.2 Choose flow cell 'FLO-MIN106' from the drop-down menu.

16.3 Then select the flowcell so a tick appears.

16.4 Click the 'New Experiment' button in the bottom left of the screen.

16.5 On the New experiment popup screen, select the running parameters for your experiment from the individual tabs:

**Experiment:** Name the run in the experiment field, leave the sample field blank.

**Kit: Selection:** Select LSK109 as there is no option for native barcoding (NBD104).

**Run Options:** Set the run length to 6 hours (you can stop the run once sufficient data has been collected as determined using RAMPART).

**Basecalling:** Leave basecalling turned but select 'fast basecalling'.

**Output:** The number of files that MinKNOW will write to a single folder. By default this is set to 4000 but can be reduced to make RAMPART update more frequently.

Click 'Start run'.

16.6 Monitor the progress of the run using the MinKNOW interface.