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nCoV-2019 sequencing protocol v2

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1 Works for me dx.doi.org/10.17504/protocols.io.bdp7i5rn

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Josh Quick

ABSTRACT

ARTIC amplicon sequencing protocol for MinION for nCoV-2019

This one-pot native barcoding protocol was developed in conjunction with Oxford Nanopore Technologies, New England Biolabs and BCCDC.

MATERIALS TEXT

Primers 25nm, desalted, ideally LabReady formulation from IDT nCoV-2019/V2

Extraction kits; Zymo Quick-RNA Viral Kit	Zymo	R1034 **or**
QIAamp Viral RNA Mini	Qiagen	52904
SuperScript IV (50 rxn)	Thermo	18090050
dNTP mix (10 mM each)	Thermo	R0192
Random Hexamers (50 µM)	Thermo	N8080127
RNase OUT (125 rxn)	Thermo	10777019
Q5 Hot Start HF Polymerase	NEB	M0493S
NEBNext Ultra II End-prep	NEB	E7546S
NEBNext Quick Ligation Module	NEB	E6056S
Native Barcoding Expansion Kit 1-12	Nanopore	EXP-NBD104
Native Barcoding Expansion Kit 13-24	Nanopore	EXP-NBD114
Sequencing Auxiliary Vials	Nanopore	EXP-AUX001
Short Fragment Buffer Expansion kit	Nanopore	EXP-SFB001
Flow Cell Priming Kit	Nanopore	EXP-FLP002
R9.4.1 flow cells	Nanopore	FLO-MIN106

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cDNA preparation

1 Mix the following components in an 0.2mL 8-strip tube;

Component	Volume
50µM random hexamers	1 µl
10mM dNTPs mix (10mM each)	1 µl
Template RNA	11 µl
Total	13 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.

A mastermix should be made up in the **mastermix cabinet** and aliquoted into PCR strip tubes. Tubes should be wiped down when entering and leaving the mastermix cabinet.

2 Gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

3 Incubate the reaction as follows:

65 °C for 00:05:00

Place on ice for 00:01:00

4 Add the following to the annealed template RNA:

Component	Volume
SSIV Buffer	4 µl
100mM DTT	1 µl
RNaseOUT RNase Inhibitor	1 µl
SSIV Reverse Transcriptase	1 µl
Total	20 µl

A mastermix should be made up in the **mastermix cabinet** and added to the denatured RNA in the **extraction and sample addition cabinet**. Tubes should be wiped down when entering and leaving the mastermix cabinet.

5 Gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

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6 Incubate the reaction as follows:

42 °C **00:50:00**

70 °C **00:10:00**

Hold at **5 °C**

Primer pool preparation

7 If required resuspend lyophilised primers at a concentration of 100µM each

nCov-2019/V3 primers for this protocol were designed using Primal Scheme and generate overlapping 400nt amplicons. Primer names and dilutions are listed in the table below.

8 Generate primer pool stocks by adding **5 µl** of each odd region primer to a **1.5 ml** Eppendorf labelled "Pool 1 (100µM)" and each even region primer to a **1.5 ml** Eppendorf labelled "Pool 2 (100µM)". The pool is also given in the .TSV files in the primalscheme output. These are your 100µM 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.

9 Dilute this primer pool 1:10 in molecular grade water, to generate 10µM primer stocks. It is recommend that multiple aliquots of each primer pool are made to in case of degradation or contamination.

Primers need to be used at a final concentration of 0.015µM per primer. In this case V3 pools have 110 primers in pool 1 and 108 primers in pool 2. so the requirement is ~4µL primer pool (10uM) per 25µL reaction. For other schemes, adjust the volume added appropriately.

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Multiplex PCR

10 In the mastermix hood set up the multiplex PCR reactions as follows in 0.2mL 8-strip PCR tubes:

Component	Pool 1	Pool 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 Primer Pool 1 or 2 (10µM)	**4 µl**	**4 µl**
Nuclease-free water	**12.75 µl**	**12.75 µl**
Total	**22.5 µl**	**22.5 µl**

A PCR mastermix for each pool should be made up in the **mastermix cabinet** and aliquoted into PCR strip tubes. Tubes should be wiped down when entering and leaving the mastermix cabinet.

11 In the **extraction and sample addition cabinet** add **2.5 µl** cDNA to each tube and mix well by pipetting.

The **extraction and sample addition cabinet** should should be cleaned with decontamination wipes and UV sterilised before and after use.

12 Pulse centrifuge the tubes to collect the contents at the bottom of the tube.

13 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

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PCR clean-up

14 Label a **1.5 ml** Eppendorf tube for each sample and assemble the following PCR dilution for each sample:

Component	Volume
Pool 1 PCR reaction	**2.5 µl**
Pool 2 PCR reaction	**2.5 µl**
Nuclease-free water	**45 µl**

The PCR post-clean up concentration is typically around **[M]100 ng/µL** . This assumption means we can make a significant saving in terms of protocol time for only a slight loss in barcode representation.

Quantification and normalisation

15 Label another **1.5 ml** Eppendorf tube for each sample.

Input to the one-pot native barcoding reaction is 50ng per sample. Process at least 6 samples plus one negative control per library in order to have sufficient material to load on the sequencer at the end.

Native barocoding

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

One-pot native barcoding of amplicons v2
by Josh Quick

PREVIEW RUN

16.1 Set up the following reaction for each sample:

Component	Volume
PCR dilution from previous step	**5 µl**
Nuclease-free water	**7.5 µl**
Ultra II End Prep Reaction Buffer	**1.75 µl**
Ultra II End Prep Enzyme Mix	**0.75 µl**
Total	**15 µl**

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16.2 Incubate at room temperature for **00:10:00**

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

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

16.3 In a new 1.5mL Eppendorf tube set up the following reaction:

Component	Volume
Previous reaction mixture	**1.5 µl**
Nuclease-free water	**5.7 µl**
NBXX barcode	**2.5 µl**
Ultra II Ligation Master Mix	**10 µl**
Ligation Enhancer	**0.3 µl**
Total	**20 µl**

Use one native barcode from the EXP-NBD104 (1-12) or EXP-NBD114 (13-24) per sample. Use from 6 to 24 barcodes in a library, any fewer and there will be insufficient total material to achieve good yields.

16.4 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.

16.5 In a new 1.5 ml Eppendorf tube pool all **20 µl** one-pot barcoding reactions together.

16.6 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** 12-plex pooled one-pot native barcoding reactions.

0.4x volume of SPRI will only bind 400 bp amplicons in the presence of ligation buffer as in a one-pot reaction, do not use 1x as this will result in excessive native barcode carryover.

16.7 Pulse centrifuge to collect all liquid at the bottom of the tube.

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

16.9 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear.

16.10 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

16.11 Add 700 µl SFB and resuspend beads completely by pipette mixing.

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

16.12 Pulse centrifuge to collect all liquid at the bottom of the tube.

16.13 Remove supernatant and discard.

16.14 Repeat steps 11-13 to perform a second SFB wash.

16.15 Pulse centrifuge and remove any residual SFB.

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

16.16 Add 200 µl of room-temperature 70 % volume ethanol to bathe the pellet.

16.17 Carefully remove and discard ethanol, being careful not to touch the bead pellet.

Only perform 1x 70% ethanol wash

16.18 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.

16.19 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).

16.20 Resuspend pellet in **30 µl** Elution Buffer (EB), mix gently by either flicking or pipetting and incubate for **00:02:00** .

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

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17 Quantify **1 µl** of the barcoded amplicon pool using the Quantus Fluorometer using the ONE dsDNA assay.

DNA quantification using the Quantus fluorometer
by Josh Quick
PREVIEW RUN

17.1 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
by Promega
Catalog #: E4870

17.2 Set up two **0.5 ml** tubes for the calibration and label them 'Blank' and 'Standard'

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

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

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

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

17.7 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'.

17.8 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

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

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

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

Use a P2 pipette for highest accuracy.

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

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

17.14 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.

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

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

17.17 Repeat step 16 until all samples have been read.

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

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

Adapter ligation with AMII
by Josh Quick

PREVIEW RUN

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18.1 Set up the following AMII adapter ligation reaction:

Component	Volume
End-repaired amplicon pools	**30 µl**
NEBNext Quick Ligation Reaction Buffer (5X)	**10 µl**
Adapter Mix (AMII)	**5 µl**
Quick T4 DNA Ligase	**5 µl**
Total	**50 µl**

There will be some variation in clean-up efficiencies but expect to carry around 80% through a clean-up.

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

18.3 Add **50 µl** (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting.

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

18.4 Pulse centrifuge to collect all liquid at the bottom of the tube.

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

18.6 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear.

18.7 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

18.8 Add **250 µl** SFB and resuspend beads completely by pipette mixing.

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

18.9 Pulse centrifuge to collect all liquid at the bottom of the tube.

18.10 Remove supernatant and discard.

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18.11 Repeat steps 14-16 to perform a second SFB wash.

18.12 Pulse centrifuge and remove any residual SFB.

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

18.13 Add **15 µl** EB and resuspend beads by pipette mixing.

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

18.15 Place on magnetic rack.

18.16 Transfer final library to a new 1.5mL Eppendorf tube.

19 Quantify **1 µl** of the final library using the Quantus Fluorometer using the ONE dsDNA assay.

DNA quantification using the Quantus fluorometer
by Josh Quick
PREVIEW RUN

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

19.1 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
by Promega
Catalog #: E4870

19.2 Set up two **0.5 ml** tubes for the calibration and label them 'Blank' and 'Standard'

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

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

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

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

19.7 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'.

19.8 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

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

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

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

Use a P2 pipette for highest accuracy.

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

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

19.14 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.

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

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

19.17 Repeat step 16 until all samples have been read.

19.18 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

20 Prime the flowcell and load **20 ng** sequencing library onto the flowcell.

Priming and loading a MinION flowcell
by Josh Quick
PREVIEW RUN

From experience we know 20 ng is optimum loading input for short amplicons.

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

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

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

20.3 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.

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

20.5 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.

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20.6 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.

20.7 Wait for **00:05:00** .

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

20.9 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.

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

Component	Volume
SQB	**37.5 µl**
LB	**25.5 µl**
Final library	**12 µl**
Total	**75 µl**

Mix LB immediately before use as they settle quickly.

Dilute library in EB if required.

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

20.12 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.

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

21 Start the sequencing run using MinKNOW.

Starting a MinION sequencing run using MinKNOW
by Josh Quick

PREVIEW RUN

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

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

21.3 Then select the flowcell so a tick appears.

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

21.5 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'.

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

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