Mar 30, 2021

nCoV-2019 sequencing protocol v3 (LoCost) - InvitaeSF v2.0

Forked from nCoV-2019 sequencing protocol v3 (LoCost)

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Amanda Kahn-Kirby: minor modifications;
Lisa Cunden: minor modifications
Bianca Miani [Invitae: minor modifications

1 *Works for me* This protocol is published without a DOI.

Research Team, Invitae

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SUBMIT TO PLOS ONE

ABSTRACT

Amplicon sequencing protocol for SARS-CoV-2 v3 (LoCost), modified for automation and clean yield. Thank you Josh Quick and colleagues for publishing and revising the protocol on which this version is based.

We thank the ARTIC network, Oxford Nanopore Technologies, New England Biolabs, BCCDC, COG-UK, CanCOGen, other California SARS-CoV-2 sequencing labs, and protocols.io commenters for their assistance developing this protocol and sharing their work globally. We hope this work is useful to other investigators a) dealing with low yield or b) looking to streamline the protocol through automation and miniaturization.

Changes to the Quick lab v3 version:

-Batch size 24 or 48 wells per run with EXP-NBD196 native barcode kit
-Use of LunaScript RT SuperMix
-10uL ARTIC reactions
-Post-PCR SPRI to improve barcode ligation efficiency
-Post-adapter SPRI stringency increased to decrease adapter reads
- Calculations are for 3-col or 6-col preparation on an Agilent Bravo

PROTOCOL CITATION

Amanda Kahn-Kirby, Lisa Cunden, Bianca Miani [Invitae 2021. nCoV-2019 sequencing protocol v3 (LoCost) - InvitaeSF v2.0. **protocols.io**
https://protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-invitaesf-bts6nnhe

FORK NOTE

FORK FROM

Forked from nCoV-2019 sequencing protocol v3 (LoCost), Josh Quick

KEYWORDS

null, ARTIC, SARS-CoV-2, COVID-19, Nanopore, Bravo

CREATED

Mar 30, 2021

LAST MODIFIED

Mar 30, 2021

PROTOCOL INTEGER ID

48702

MATERIALS TEXT

A	B	C
Component	**Supplier**	**Part number**
ARTIC nCoV-2019 V3 panel (100uM)	IDT	See links below
LunaScript RT SuperMix Kit	NEB	E3010
Q5 Hot Start High-Fidelity 2X Master Mix	NEB	M0494
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 96	ONT	EXP-NBD196
AMPure XP beads (or equivalent)	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.

DISCLAIMER:

BEFORE STARTING

Prepare between 22 and 46 RNA samples plus 1 negative control and 1 positive control using this protocol.

cDNA preparation 30m

1 Input to the protocol is 22 or 46 RNA samples plus 1 positive control and one randomly-located negative control of nuclease-free water per library. (Use Cherrypicking program to prepare the input plate to this protocol). If previously frozen, mix by briefly vortexing and pulse spin to collect liquid. Keep samples on ice at all times.

The recommended positive control is Twist Control 2 diluted in pooled SARS-CoV-2 negative RNA extract from nasal swab testing, 10K copies per reaction. It is in a constant plate position for easy monitoring.

2 Stamp input plate to a 3-col or 6-col pre-aliquoted RT master mix plate using the Bravo protocol.

A	B
Component	**Volume**
LunaScript RT SuperMix (5X)	2 µL
Normalized 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. The cherrypicking protocol does this step automatically. RNA samples should be handled in the **Pre-PCR** cabinet which should should be cleaned with decontamination wipes and UV sterilised before and after use.

To prevent pre-PCR contamination the mastermix should be pre-aliquoted in a sterile cabinet and stored at -20.

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 (only for a fresh IDT box) 1h

4 Dilute IDT stock **100 Micromolar (µM)** pools 1:10 in molecular grade water, to generate **10 Micromolar (µM)** primer stocks. (**180 µl** ship volume + **1620 µl** molecular-grade water. Aliquot into convenient-sized labeled aliquots (**65 µl**) and store at -20.

Primers are used at a final concentration of **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.

Multiplex PCR 4h

5 Set up the master mixes for two half-scale PCR reaction pools as follows. Use 1 aliquot set of pre-diluted **10 uM** primers for a 3-col prep, and 2 sets for a 6-col prep.

Gently mix the MM tube by pipetting/flicking and pulse spin the tube to collect liquid at the bottom of the tube. Allocate to a single column of the Pre-PCR reagent plate, then distribute using the Bravo. There will be residual dead volume in the MM tube and in the distribution plate (15% overage).

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.

Pool 1 setup

A	B	C	D
Component	Reaction 1 (each)	Reaction 1 (3-col)	Reaction 1 (6-col)
Q5 Hot Start High-Fidelity 2X Master Mix	6.25 µL	172.5 µL	345 µL
V3 Pool 1 (10µM)	2 µL	55.2 µL	110.4 µL
Nuclease-free water	3 µL	82.8 µL	165.6 µL
Total	11.5 µL	310.5 µL	621 µL

For M0494, distribution volume for 8-strip is 72uL for 6-col or 37.5 uL for 3-col. Pool 1 goes to col 1.

and

Pool 2 setup

A	B	C	D
Component	Reaction 2 (each)	Reaction 2 (3-col)	Reaction 2 (6-col)
Q5 Hot Start High-Fidelity 2X Master Mix	6.25 µL	172.5 µL	345 µL
V3 Pool 2 (10µM)	2 µL	55.2 µL	110.4 µL
Nuclease-free water	3 µL	82.8 µL	165.6 µL
Total	11.5 µL	310.5 µL	621 µL

For M0494, distribution volume for 8-strip is 72uL for 6-col or 37.5 uL for 3-col. Pool 2 goes to col 4.

6 Add **1.25 µl** cDNA to each of the PCR reactions by Bravo stamp, followed by pipet-mix. Seal and pulse spin the

plate to collect liquid at the bottom of the tube.

7 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**	30
Annealing	**63 °C**	**00:05:00**	30
Hold	**4 °C**	Indefinite	1

Thermocycler calibration can vary instrument to instrument. We saw amplicon 64 dropout so decreased the annealing/extension temperature to **63 °C** .

Post-PCR cleanup 45m

8 Spin down ARTIC PCR products in the Post-PCR lab. Carefully remove seal. Run the Bravo protocol to merge **7.5 µl** of Reaction 1 and **7.5 µl** of Reaction 2 into a single plate for SPRI cleanup.

9 Perform a 1.0X bead cleanup protocol on Bravo with the following parameters per well

15 µl magnetic beads

350 µl 70% ethanol for two wash steps

15 µl Qiagen Buffer EB elution plate content

10 µl Qiagen Buffer EB elution input

9.0 µl Elution recovery

After SPRI, seal with PlateLoc and spin down before continuing.

Native barcoding 2h

10 Barcode the amplicon pools using the one-pot native barcoding approach. 3-col and 6-col plates are used as input for the protocol. [Not modifying the One-Pot embedded protocol, but see the substeps below for automation-specific calculations at each step.

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

PREVIEW RUN

10.1 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.

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

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

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

10.3 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.

10.4 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.

10.5 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.

10.6 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.

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

10.8 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.

10.9 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.

0.10 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.

0.11 Add **200 µl** of room-temperature **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

0.12 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.

0.13 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).

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

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

10.1 ER/AT Master Mix calculations. Make up in an Eppendorf tube, mix thorougly, then pipet to column 1 of the Post-PCR distribution plate.

A	B	C	D
Component	ER/AT(each)	ER/AT (3-col)	ER/AT(6-col)
Ultra II End Prep Buffer	1.2	33.12	66.24
Ultra II End Prep Enzyme	0.5	13.8	27.6
Nuclease-free water	5	138	276
Total Volume	6.7	184.92	369.84

Volumes account for 15% overage. Pipet 22.1 uL (3-col) or 42.0 (6-col) uL to distribution plate.

10.2 Bravo will stamp **3.3 µl** cleaned-up amplicons per ER/AT reaction.

10.3 For Native Barcoding, use the following quantities for 3-col or 6-col setup. Mix thoroughly, spin down, then pipet to column 4 of the Post-PCR reagent plate for Bravo distribution. Program will then add 0.75 uL ER/AT product and 1.25 barcode to each well. Mix, seal, spin, and continue with protocol.

A	B	C	D
Component	Ligation (each)	Ligation (3-col)	Ligation (6-col)
Blunt/TA Ligase Master Mix	5	138	276
nuclease-free water	3	82.8	165.6
Total Volume	8		441.6

Volumes account for 15% overage. Pipet 26.6 uL (3-col) or 52.0 uL (6-col) to distribution plate.

11 Quantify **1 µl** of the barcoded amplicons using the Qubit hsDNA assay. Concentration will vary depending on number and Ct of samples and but you need about **30 ng** total at this stage to achieve maximum run yield.

12 Set up the following AMII adapter ligation and clean-up with SFB, but **note that the SPRI ratio for the final clean should be 0.9X to minimize adapter reads**. Elution is with **Oxford Buffer EB**, not Qiagen. There is no Ethanol clean-up in the final SPRI.

Adapter ligation with AMII v2
by Josh Quick
PREVIEW RUN

12.1 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**

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

12.3 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

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

12.5 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.

12.6 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.

12.7 Repeat steps 13.6 to perform a second SFB wash.

12.8 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.

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

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

13 Quantify **1 µl** of the final library using the Qubit hsDNA assay. Concentration will vary depending on number and Ct of samples but **15 ng** final library is usually required to achieve maximum run yield.

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

MinION sequencing 1d

14 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.

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

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

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

14.3 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.

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

14.5 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.

14.6 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.

14.7 Wait for **00:05:00** .

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

14.9 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.

4.10 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.

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

4.12 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.

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

15 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.

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

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

15.3 Then select the flowcell so a tick appears.

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

15.5 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'.

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