Step-by-step MinION sequencing - nCoV-2019 sequencing protocol v3 (LoCost)

(with EXP-NBD196 and SQK-LSK109)

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Adapted from

https://www.protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-bh42j8ye/materials and Pater et. al, 2021.

This step must be performed in a pre-PCR area. The area must be clean with 1% sodium hypochlorite solution, RNAse and 70% EtOH.

IMPORTANT- you MUST have AT LEAST ONE negative control for every 30 samples tested and sequenced on EVERY library. This should ideally be an extraction/cDNA/PCR control, but if these are unexpectedly unavailable then you should include a water control instead at this stage. This is not ideal, but you MUST have a negative control.

1**. cDNA synthesis:**

Prepare 95 samples + 1 negative control (If previously frozen, mix by briefly vortexing and pulse spin to collect liquid. Keep samples on ice at all times) and map the 96 microplates with the positions of each sample. Indicate on the map the samples that need to be diluted. Viral RNA input from a clinical sample should have Ct < 36. If Ct is < 14, then dilute the sample 100-fold in water, if between 15-18 then dilute 10-fold in water and if > 18, don't dilute. This will reduce the likelihood of PCR-inhibition.

Mix the following components in an 0.2mL 8-strip tube or microplate of 200 µl;

Component	**Amount (µl)**
LunaScript RT SuperMix (5x)	2
Template RNA	8
Total Volume	10

Mix by pipetting and pulse spin the tube/plate to collect liquid at the bottom.

Seal the plate with a clear sealing film for RT-PCR.

Incubate the reaction as follows:

25 °C 00:02:00

55 °C 00:10:00

95 °C 00:01:00

Hold at 4 °C or keep on ice.

2. Multiplex PCR:

Generate primer pool stocks by adding 5 µl of each primer pair to a 1.5 ml Eppendorf labelled either “Pool 1 (100µM)” or “Pool 2 (100µM)”. Total volume should be 490 µl for Pool 1 (100µM) and 490 µl for Pool 2 (100µM). These are your 100µM stocks of each primer pool. Dilute this primer pool 1:10 in molecular grade water, to generate 10µM primer stocks. It is recommended that multiple aliquots of each primer pool are made in case of degradation or contamination.

NOTE: For the amplicons you see dropout then increase the primer concentration. Instead 5 µl of the follow primers for the pool preparation, add:

Pool 1 - 1 (15 µl), 13 (10 µl), 31 (10 µl), 45 (10 µl), 59 (15 µl), 73 (10 µl), 85 (10 µl), 91 (15 µl).

Pool 2 - 12 (15 µl), 26 (10 µl), 32 (10 µl), 58 (10 µl), 68 (15 µl), 72 (10 µl), 74 (10 µl), 76 (15 µl), 90 (10 µl), 98 (10 µl).

In the mastermix hood set up the multiplex PCR reactions as follows in 0.2mL 8-strip PCR tubes or microplate of 200 µl:

<u>Options 1 or 2 if using primer v3:</u>

Component	**Pool 1 (µl)**	**Pool 2 (µl)**
5X Q5 Reaction Buffer	5	5
10mM dNTPs	0.5	0.5
Q5 DNA polymerase	0.25	0.25
Primer V3 Pool 1 or 2 (10µM)	4.0	4.0
NFW	12.75	12.75
Total Volume	22.5	22.5

or

Component	Pool 1 (µl)	Pool 2 (µl)
Q5 Hot Start High-Fidelity 2x Master Mix	12.5	12.5
Primer V3 Pool 1 or 2 (10µM)	4.0	4.0
NFW	6.0	6.0
Total Volume	22.5	22.5

Options 1 or 2 if using primer v4:

Component	Pool 1 (µl)	Pool 2 (µl)
5X Q5 Reaction Buffer	5	5
10mM dNTPs	0.5	0.5
Q5 DNA polymerase	0.25	0.25
Primer V4 Pool 1 or 2 (10µM)	4.0	4.0
Primer V4.1 Pool 1 or 2 (10µM)	0.2	0.2
NFW	12.55	12.55
Total Volume	22.5	22.5

or

Component	Pool 1 (µl)	Pool 2 (µl)
Q5 Hot Start High-Fidelity 2x Master Mix	12.5	12.5
Primer V4 Pool 1 or 2 (10µM)	4.0	4.0
Primer V4.1 Pool 1 or 2 (10µM)	0.2	0.2
NFW	5.8	5.8
Total Volume	22.5	22.5

Add 2.5 µl cDNA to each tube and mix well by pipetting. **Seal the plate with an aluminum foil sealer.**

***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.**

Set-up the following program on the thermal cycler:

Step	Temperature	Time	Cycles
Heat Activation	**98 °C**	**00:00:30**	**1**
Denaturation	**98 °C**	**00:00:16**	**20**
Annealing	**65-63 °C**	**00:05:00**	**20**
	Touchdown (-0.1°C each cycle)		
Denaturation	**98 °C**	**00:00:16**	**15**
Annealing	**63 °C**	**00:05:00**	**15**
Hold	**4 °C**	**Indefinite**	**1**

This step must be performed in a post-PCR area. The area must be clean with 1% sodium hypochlorite solution, RNAse and 70% EtOH.

LIBRARY PREPARATION

IMPORTANT:

- You MUST have AT LEAST ONE negative control on EVERY library. This should ideally be an extraction/cDNA/PCR control, but if these are unexpectedly unavailable then you should include a water control instead at this stage. This is not ideal, but you MUST have a negative control.

- **After finishing each step of the library, store the plate in the refrigerator using the aluminum sealing film.**

Before starting:

- Label all the plates with the ID of the library, date and each step.

- Mix by inversion and spin down all reagent tubes before using.

- Prepare 70% EtOH in a falcon tube.
- Take AMPure beads out of the fridge and mix by vortexing. Use at room temperature.
- Make 1ml stocks of AMPure beads and nuclease-free water to avoid contamination.
- After using each plate, seal and store in the refrigerator.

Note: don't leave the reagents out of the freezer when not in use

3**. PCR dilution:**

Vortex each tube/plate and pulse spin to collect liquid at the bottom. Label the PCR plates to avoid changing them.

Quantify 1 µl of the amplicon pools A and B on samples with Ct >27 on the Qubit.

Indicate on the map the samples that don't need to be diluted (Qubit result < 30ng/µl).

Label strip-tubes/plate (Library_name+date+dilution) and combine the following volumes of each PCR reaction for 10 µl each sample:

Component	**Amount (µl)**
Pool 1 PCR reaction	2.5
Pool 2 PCR reaction	2.5
NFW	45
Total Volume	50

for samples with qubit result < 30ng/µl

Component	**Amount (µl)**
Pool 1 PCR reaction	24
Pool 2 PCR reaction	24
NFW	2
Total Volume	50

Vortex the tubes/plate and pulse spin to collect liquid at the bottom

4**. End-repair and dA-tailing:**

Label strip-tubes/plate (Library_name+date+EndPrep) and combine the following volumes of each PCR reaction for 10 µl each sample:

Component	Amount (µl)
PCR dilution (from Step 3)	3.3
Ultra II End Prep Reaction Buffer	1.2
Ultra II End Prep Enzyme Mix	0.5
NFW	5
Total Volume	10

Mix by inversion, seal the plate with a clear sealing film for RT-PCR, spin down and incubate for 15 min at RT, followed by 15 min at 65 °C. Incubate on ice for 1 min.

5**. Native barcode ligation.**

Before starting this step, organize and record the order of your samples with the order of the barcodes. Example: Sample 1 (A and B mixed) – Barcode 1

Sample 2 (A and B mixed) – Barcode 2

...

Vortex each barcode and pulse spin to collect liquid at the bottom

IMPORTANT: the negative control should be placed randomly within the library (e.g., do not always use BC12 or BC24 for the negative control). When we are running with the nuclease flush, it would be much harder to detect if there was any cross-library contamination if the negative control was always on the same barcode. Please make sure that the negative control is placed in a different barcode for each library.

Note: be careful in this step to not change the order of samples and barcodes. During the analysis, these notes will be necessary to know which samples correspond to each barcode.

Label strip-tubes/plate (Library_name+date+barcoding) and combine the following volumes of each PCR reaction for 10 µl each sample:

Component	Amount (µl)
dA-tailed amplicons (from Step 3)	1.0
Native barcode NB01-NB96	1.25
Blunt/TA Ligase Master Mix	5
NFW	2.75
Total volume	10

or

Component	Amount (µl)
dA-tailed amplicons (from Step 3)	1.0
Native barcode NB01-NB96	1.25
Ultra II ligation Master Mix	5
Ultra II Enhancer	1
NFW	1,75
Total volume	10

Mix by inversion, seal the plate with a clear sealing film for RT-PCR, spin down and incubate at room temperature (20 °C) for 20 min, followed by 65 °C for 10 min to denature the ligase. Incubate on ice for 1 min.

Spin down and combine all the barcode ligation reactions (all the tubes/wells) into a single 1.5-ml Eppendorf tube according to the list below:

- 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.

Perform SPRI cleanup by doing the **Step-by- step Clean-up**.

- add 0.4x AMPure beads previously homogenized;

- mix by inversion for 5 minutes to promote the binding of the library to the beads;

- spin down and place on a magnetic rack;

- once the beads have peeled and the liquid is completely clear, remove the supernatant with the 100 or 200 pipette by the opposite side of the pellet to avoid touching the pellet. Discard the supernatant;

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

- repeat the step above to perform a second SFB wash. Pulse centrifuge and remove any residual SFB.

- wash the pellet with 200 ul of 70% ethanol (or you may need more ethanol to get a good wash if there are lots of beads). Do not disturb the pellet; Pippet carefully;

- after discarding, close the tubes, spin down and place again on magnetic rack;

- remove the residual ethanol then leave open to air dry until the pellet loses its shine;

- when the pellet is already dry (no shining against the light), elute the pellet by adding **31 µl** of NFW and resuspend the beads by flicking or with the pipette, make sure all beads have been eluted from the wall tube;

- incubate for 10 minutes at RT;

- spin down, place on the magnetic rack, let the pellet beads on magnet, transfer the eluate to a new tube. (Note: be careful to not transfer the beads to the new tube).

Quantify 1 µl of the amplicon pools on the Qubit to confirm that not much DNA was lost during the purifications.

6. Adapter ligation and clean-up.

Set up the following ligation reaction, mixing between each addition

Component	Amount (µl)
Pooled barcoded sample (from Step 5)	30
Adapter Mix (AMII)	5
NEBNext Quick Ligation Reaction Buffer (5X)	10
Quick T4 DNA Ligase	5
Total volume	50

or

Component	Amount (µl)
Pooled barcoded sample (from Step 5)	30
Adapter Mix (AMII)	5
Ultra II ligation Master Mix	36
Ultra II Enhancer	1
Total volume	72

Mix by inversion, spin down and incubate at RT for 20 minutes.

- add 1x of AMPure beads previously homogenised;

- mix by inversion for 5 minutes to promote the binding of the library to the beads;

- spin down and place on the magnetic rack;

- once the beads have pelleted and the liquid is completely clear, remove the supernatant with the 100 or 200 pipette by the opposite side of the pellet to avoid touching the pellet. Discard the supernatant;

- with the tube still on the magnetic rack, wash the pellet with 250 ul of **SFB**. Close tube lid, resuspend beads by flicking.

- spin down, beads on magnet and remove supernatant- discard the supernatant and wash again with 250 uL of **SFB**. After discard the second time, close the tubes, spin down and place again on magnetic rack;

- remove the residual SFB then elute the pellet by adding **13 ul** of **EB** (Elution Buffer) and resuspend the beads by flicking or pipetting, make sure all beads have been eluted from the wall tube;

- incubate for 10 minutes at RT;

- spin down, place on the magnetic rack, let the pellet beads on magnet, transfer the eluate to a new tube. (Note: be careful to not transfer the beads to the new tube.

Quantify 1 ul of your eluted library using the Qubit. With the new R9.4.1 you only need 15 ng to sequence. So after quantifying, dilute the library to 15 ng in 12 ul, using **EB** to dilute.

7. Priming and loading the SpotON flow cell

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

Mix the Sequencing Buffer (**SQB**) and Flush Buffer (**FLB**) tubes by vortexing, spin down and return to ice.

Spin down the Flush Tether (**FLT**) tube, mix by pipetting, and return to ice.

- Priming mix: add 30 µl of thawed and mixed Flush Tether (**FLT**) directly to the tube of thawed and mixed Flush Buffer (**FLB**), and mix by pipetting up and down.

- Take out a flow cell from the fridge (you should already have QC'd this)

- Open the sample port. Draw back with the P1000 a few µls of buffer to make sure there is continuous buffer flow from the sample port across the sensor array and that there are no bubbles in the flow cell.

- Load 800 ul of the priming mix slowly through the sample loading port using a P1000 pipette. It is extremely important not to introduce or push any air bubbles into the flow cell. Wait 5 minutes.

- Gently lift the SpotON port cover to make the sample port accessible.

- Load 200 µl of the priming mix as before.

Prepare the library for loading

Component	Amount (µl)
Sequencing Buffer (**SQB**)	37.5
Loading Beads (**LB**), mixed immediately before use	25.5
DNA library (15 ng)	12
Total volume	75

Mix gently by pipetting and spin down. Add the 75 ul diluted library to the flow cell by allowing droplets to fall onto the SpotON port using a P200 pipette without the pipette touching the port. Droplets will be drawn into the flow cell by capillary action. If the library is not drawn into the flow cell, close the SpotON port and perform a further 200 ul prime via the sample inlet port and try loading the library again.

Gently replace the SpotON port cover, making sure the bung enters the SpotON.

8. Start sequencing run.

By default, you will need an Internet connection before the sequencing script can be started, although off-line versions of MinKNOW can be requested from the manufacturer if an Internet connection is not available.

Check the folder ‘INSTRUCTIONS’ on your Desktop for instructions on how to set up the MinION run.

Note: In addition to the notes in the instructions, the starting voltage can vary depending on how long you have previously run that flow cell for. Approximate run voltages are: for the first 10 hours -180 v, after 10 hours -190 v, after 200v. An ‘hours_voltage_1’ file and ‘hours_voltage_2’.

After running, keep the library in the fridge until the next sequencing.

9. Bioinformatics:

Please follow as: https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.htm