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SARS-CoV-2 McGill Nanopore sequencing protocol SuperScript IV_42C_ArticV3

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

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ABSTRACT

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cDNA preparation

1

Mix the following components in a 0.2 mL 8-strip tube:

Component	Volume
50 µM random hexamers	1 µl
10 mM dNTPs mix (10 mM 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.

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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 or on hold**

4 Add the following to the annealed template RNA:

Component	Volume
5X SSIV buffer	**4 µl**
100 mM DTT	**1 µl**
RNaseOUT Recombinant RNase Inhibitor (40 U/ul)	**1 µl**
SuperScript IV Reverse Transcriptase (200 U/ul)	**1 µl**
Total	**7 µl 20 ul total with the 13 ul from step 1**

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.

6 Incubate the reaction as follows:

42 °C for **00:50:00**

70 °C for **00:10:00**

Hold at **00:05:00**

Primer pool preparation

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

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https://github.com/sarahreiling/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V3/nCoV-2019_V3only.scheme.bed primers for this protocol were designed to generate overlapping 400 nt amplicons.

8

Generate primer pool stocks by adding **5 µl** of each primer pair to a **1.5 mL** Eppendorf labelled either “Pool 1 (100 µM)” or “Pool 2 (100 µM)”. Total volume should be **490 µl** for Pool 1 (100 µM) and **490 µl** for Pool 2 (100 µM). These are your 100 µM stocks of each primer pool.
Make another primer pool named "Pool LA1 (100 µM)" that contains 5 µl of primer pairs 5, 17, 23, 26, 66, 70, 74, 91, 97, and 10 ul of primer pair 64.

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 10 µM primer stocks. It is recommend that multiple aliquots of each primer pool are made to in case of degradation or contamination.
LA1 primer pool will be diluted to 1 µM primer stock.

Primers need to be used at a final concentration of 0.015 µM per primer. In this case both pools have 98 primers in so the requirement is 3.65 µl primer pools (10 uM) per 25 ul reaction. For other schemes, adjust the volume added appropriately.

Multiplex PCR

10

In the **extraction and sample addition cabinet** add **5 µl** RT product 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.

11

In the mastermix hood set up the multiplex PCR reactions as follows in 1.5 mL low-bind DNA tubes and aliquoted into PCR strip tubes:

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Component	Pool 1 [10 uM primer]	Pool 2 [10 uM]	Pool LA1 [1 uM]
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	12.5 µl	12.5 µl
Primer Pool 1, Pool 2 or Pool LA1)	3.7 µl	3.7 µl	3.7 µl
Nuclease-free water	3.8 µl	3.8 µl	3.8 µl
Total	20 µl	20 µl	20 µl

Add 5 ul RT product to each primer pool as mentioned in step 7.

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

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	36
Annealing	65 °C	00:05:00	36
Hold	4 °C	Indefinite	1

Cycle number should be 25 for Ct 18-21 up to a maximum of 36 cycles for Ct 35

PCR clean-up

14 ⚠

Combine the entire contents of “Pool 1” and “Pool 2” PCR reactions for each biological sample into to a single 1.5 mL Eppendorf tube. **Keep Pool LA1 separate from the combined Pool 1+2 until after the clean-up!!**

15

Clean-up the amplicons using the following protocol:

Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting.
Incubate for 5 min at room temperature.
Pellet on magnet for 5 min. Remove supernatant.
Add 200 ul of 80% ethanol to the pellet and wash twice.
Elute in 30 ul elution buffer.

Amplicon clean-up should be performed in the **post-PCR bench** which should be cleaned with

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decontamination wipes and UV sterilised before and after use.

16 ⚠

Quantify the amplicon pools using a fluorimetric dsDNA assay.

We expect following concentrations:

Pool 1+2 combined:
100-150 ng/ul for Ct 14-24
30-80 ng/ul for Ct 25-29
10-30 ng/ul for Ct 30-36

Pool LA1:
1-10 ng/ul for all Ct

17 ⚠

After quantification of Pool 1+2 and Pool LA1, mix them together in following ratio: 89.8% Pool 1+2 and 10.2% Pool LA1. For this, take a new plate and add 179.6 ng of Pool 1+2 and 20.4 ng of Pool LA1, and add up with nuclease-free water to a total volume of **20 ul (= 10 ng/ul).** From this dilution, take **5 ul (equals 50 ng) per sample** and continue with the next step.

18

Label a **1.5 mL** Eppendorf tube for each sample.

Native barcoding

19

Barcode the amplicon pools using native barcodes.

This is a 'one-pot ligation' protocol for native barcoded ligation libraries. We have seen no reduction in performance compared to standard libraries, and is made faster by using the Ultra II® ligation module which is compatible with the Ultra II® end repair/dA-tailing module removing a clean-up step.

20

Set up the following reaction for each sample:

Component	Volume
DNA amplicons	**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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21

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

Incubate at **65 °C** for **00:05:00**

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

22

Add the following directly to the previous reactions:

Component	Volume
NBXX barcode	**2.5 µl**
Ultra II Ligation Master Mix	**17.5 µl**
Ligation Enhancer	**0.75 µl**
Total	**20.75 µl 35.75 ul total with the 15 ul from step 19.1**

23

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

Incubate at **70 °C** for **00:10:00**

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

The 70°C incubation is to inactivate the DNA ligase to prevent barcode cross-ligation when reactions are pooled in the next step.

24 ⚠

Pool all the samples.

25

Clean-up the native barcodes twice using the following protocol.

Add 0.8X of SPRI beads to the sample tube and mix gently by pipetting.
Incubate for 5 min at room temperature.
Pellet on magnet for 5 min. Remove supernatant.
Add 200 ul of 80% ethanol to the pellet and wash twice.
Elute in **100 ul** elution buffer.

Repeat wash:
Add 0.8X of SPRI beads (80 ul) to the sample tube and mix gently by pipetting.
Incubate for 5 min at room temperature.
Pellet on magnet for 5 min. Remove supernatant.
Add 200 ul of 80% ethanol to the pellet and wash twice.
Elute in **30 ul** elution buffer.

26

Set up the following AMII adapter ligation reaction:

Component	Volume
Barcoded amplicon pools	**30 µl**

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NEBNext Quick Ligation Reaction Buffer (5X)	**10 µl**
AMII adapter mix	**5 µl**
Quick T4 DNA Ligase	**5 µl**
Total	**50 µl**

27

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

28

Clean-up the native barcodes using the following protocol:

Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by pipetting.
Incubate for 5 min at room temperature.
Pellet on magnet for 5 min. Remove supernatant.
Add 200 ul of **SFB** to the pellet and **resuspend beads completely by pipette mixing**.
Pellet on beads, remove supernanant, and repead the was step with another 200 ul SFB.
Elute in 15 ul **EB (provided in the ONT kit)**.
Incubate at room temperature for **00:02:00**
Place on magnetic rack.
Transfer final library to a new 1.5mL Eppendorf tube.

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

29

Quantify the final library using a fluorimetric dsDNA assay.

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.

30 ⚠

Prime the flowcell and load 20 ng to 40 ng sequencing library onto the flowcell. Dilute library in EB if required.

The original protocol says 20 ng, but this leads to only ~50% pore occupancy. Loading 40 ng leads to ~70% pore occupancy but the flow cell needs to be refueled.

30.1

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

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

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Flush tether (FLT

30.2

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

30.3

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.

30.4

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

30.5

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.

30.6

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.

30.7

Wait for **00:05:00**

30.8

Gently lift the SpotON cover to open the SpotON port.

30.9

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.

30.10

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**

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30.11

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

30.12

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.

30.13

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

31

Start the sequencing run using MinKNOW.

31.1

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

31.2

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

31.3

Then select the flowcell so a tick appears.

31.4

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

31.5

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.

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Click 'Start run'.

31.6

Monitor the progress of the run using the MinKNOW interface.

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