🌐 nCoV-2019 McGill Nanopore LibPrep Protocol, 10 ng NB

Forked from Artic nCoV-2019 McGill modified Lunascript Reverse Transcriptase Nanopore sequencing protocol

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Dec 21, 2020

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

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ABSTRACT

This protocol works for 12 native barcodes, 24 native barcodes, and 96 native barcodes.

DOI

dx.doi.org/10.17504/protocols.io.bjktkkwn

PROTOCOL CITATION

FORK NOTE

FORK FROM

Forked from Artic nCoV-2019 McGill modified Lunascript Reverse Transcriptase Nanopore sequencing protocol, Shu-Huang Chen

KEYWORDS

Cov-19 Nanopore Sequencing

LICENSE

CREATED

Aug 11, 2020

LAST MODIFIED

Dec 21, 2020

PROTOCOL INTEGER ID

40307

ABSTRACT

This protocol works for 12 native barcodes, 24 native barcodes, and 96 native barcodes.

Native barcoding

1

Barcode the amplicon pools using native barcodes.

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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.

2

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

3

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

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

Incubate on ice for 🕐**00:01:00**

4

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**

5

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.

6 ⚠

Clean-up the native barcodes using the following protocol (**before pooling**):

Add 0.8X of SPRI beads (28.6 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 **20 ul** elution buffer.

7 Quantify the barcodes using a fluorimetric dsDNA assay.

8 Normalize the barcodes to **10 ng** each and pool (except neg. ctrl - which will be equal volume).

9 Clean-up the barcode pool 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 **30 ul** elution buffer.

10 Pool the samples.

11 Repeat the clean-up step to avoid any unligated barcode carryover to the next ligation reaction.

Add 0.8X of SPRI beads to the pooled 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.

12 **Optional:** Run a tapestation to verify that no unligated barcodes are present in the elute.

13 Set up the following AMII adapter ligation reaction:

Component	Volume
Barcoded amplicon pools	🥛**30 µl**
NEBNext Quick Ligation Reaction Buffer (5X)	🥛**10 µl**
AMII adapter mix	🥛**5 µl**
Quick T4 DNA Ligase	🥛**5 µl**
Total	🥛**50 µl**

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

15 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 wash step with another 200 ul of **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 1.5 mL Eppendorf tube.

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

16 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.

17 ⚠️

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.

17.1

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

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

17.2

Add 🔲**30 µl** FLT to the FLB tube (1.16 mL) and mix well by vortexing.

17.3

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.

17.4

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

17.5

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.

17.6

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.

17.7

Wait for ⏱**00:05:00**

17.8

Gently lift the SpotON cover to open the SpotON port.

17.9

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.

17.10

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**

17.11

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

17.12

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.

17.13

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

18

Start the sequencing run using MinKNOW.

18.1

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

18.2

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

18.3

Then select the flowcell so a tick appears.

18.4

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

18.5

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'.

18.6

Monitor the progress of the run using the MinKNOW interface.