Sep 24, 2020

McGill Nanopore Native Barcoding LibPrep Protocol,10 ng NB

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1 Works for me dx.doi.org/10.17504/protocols.io.bmpsk5ne

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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.bmpsk5ne

PROTOCOL CITATION

Sarah J Reiling, Anne-Marie Roy, Shu-Huang Chen, Ioannis Ragoussis 2020. McGill Nanopore Native Barcoding LibPrep Protocol,10 ng NB. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bmpsk5ne

KEYWORDS

nanopore native barcoding

CREATED

Sep 24, 2020

LAST MODIFIED

Sep 24, 2020

PROTOCOL INTEGER ID

42450

Before you start

1

For native barcoding library preparations, it is highly recommended to always add a libprep negative control. Because we have two subsequenc ligation reactions, any leftover native barcodes from the first ligation step may bind to end-prepped DNA strands in the second ligation step.
To reduce the presence of leftover native barcodes, we perform two bead cleanup steps after the first ligation reaction. If in doubt, run a gel or Tapestation to verify that no barcodes are present before continuing with the second ligation step.

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Native barcoding

2

Barcode the samples using native barcodes. ONT recommends to load 100 - 200 fmol total concentration. We recommend to start with 100-fold this concentration at the beginning of the library preparation due to losses and normalization steps.

This is a 'one-pot ligation' protocol for native barcoded ligation libraries, which means that we do not clean up the end-prepped product; instead, it is added as is to the native barcoding step. 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.

3

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**

4

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

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

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

5

Add the following directly to the previous reactions:

Component	Volume
NBXX barcode	**2.5 µl**
Ultra II Ligation Master Mix	**10 µl**
Ligation Enhancer	**0.3 µl**
Total	**17 µl 20 ul total with 3 ul from step 3**

6

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.

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7

Clean-up the native barcoded samples using the following protocol:

Add 0.8X of SPRI beads (16 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.

8

Quantify the barcodes using a fluorimetric dsDNA assay.

9

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

10

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.

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

12

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**

13

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

14

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 or LFB (depending on your desired fragment size)** to the pellet and **resuspend beads completely by pipette mixing**.

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Pellet on beads, remove supernanant, and repead the wash step with another 200 ul of **SFB or LFB**.
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.

15 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.

16 ⚠

Prime the flowcell and load 100 - 200 fmol sequencing library onto the flowcell. Dilute library in EB if required.

The original ONT protocol says to load 20 ng for 400 bp amplicons, but this leads to only ~50% pore occupancy. Loading 40 ng leads to ~70% pore occupancy but the flow cell needs to be refueled after 24 hrs. This guideline can be used for both MinION and PromethION fc, however, it needs to be adjusted for Flongle fc.

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

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

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

16.3 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.

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

16.5 Take a P1000 pipette and tip and set the volume to **800 µl** . Place the tip in the inlet port and

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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.

16.6

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.

16.7

Wait for **00:05:00**

16.8

Gently lift the SpotON cover to open the SpotON port.

16.9

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.

16.10

In a new tube prepare the library dilution for sequencing:

Component	MinION fc	PromethION fc
SQB	**37.5 µl**	**75 µl**
LB	**25.5 µl**	**51 µl**
Final library (adjust with EB)	**12 µl**	**24 µl**
Total	**75 µl**	**150 µl**

16.11

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

16.12

Add the sequencing library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop siphons into the port before adding the next.

16.13

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

17

Start the sequencing run using MinKNOW.

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17.1

Monitor the progress of the run using the MinKNOW interface.

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