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One-pot native barcoding of amplicons

Josh Quick[1]

[1]University of Birmingham

1 Works for me dx.doi.org/10.17504/protocols.io.sg2ebye

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Josh Quick

ABSTRACT

This is a 'one-pot ligation' protocol for Oxford Nanopore native barcoded ligation libraries using shearing.

EXTERNAL LINK

http://lab.loman.net/protocols/

One-pot native barcoding protocol (1).pdf

GUIDELINES

Scope:

There has been no evidence of a reduction of performance compared to standard libraries, yet it can be 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.

The FFPE DNA repair step is optional. If you have the time, we recommend using the double incubation times in **bold**. If you are in a hurry, the times in *italic* are a good compromise between speed and efficiency.

Required:

g-TUBEs (optional)
SQK-LSK108 1D ligation kit
Native barcoding kit
Ultra II End Repair/dA-Tailing Module
Ultra II Ligation Module
FFPE DNA Repair Mix (optional)
Ampure XP beads
80% ethanol
EB (10 mM Tris-HCl pH 8)

SAFETY WARNINGS

See SDS (Safety Data Sheet) for safety warnings and hazards.

1 Set up the following reaction for each sample:

Component	Volume
DNA amplicons	10 µl
Ultra II End Prep Reaction Buffer	1.4 µl
Ultra II End Prep Enzyme Mix	0.6 µl
Total	12 µl

2 Incubate at room temperature for 00:10:00

Incubate at 65 °C for 00:05:00

Incubate on ice for 00:01:00

3 Add the following directly to the previous reactions:

Component	Volume
NBXX barcode	2.5 µl
Ultra II Ligation Master Mix	14.5 µl
Ligation Enhancer	0.5 µl
Total	29.5 µl

Use one native barcode from the EXP-NBD104 (1-12) or EXP-NBD114 (13-24) per sample. Use from 6 to 24 barcodes in a library, any fewer and there will be insufficient total material to achieve good yields.

4 Incubate at room temperature for 00:20:00

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.

5 Pool all barcoded fragments together into a new 1.5 ml Eppendorf tube.

Amplicon clean-up using SPRI beads
by Josh Quick
PREVIEW RUN

5.1 ⚠

Vortex SPRI beads thoroughly to ensure they are well resuspended, the solution should be a homogenous brown colour.

Agencourt AMPure XP
by Beckman Coulter
Catalog #: A63880

5.2 Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. For example add **50 µl** SPRI beads to a **50 µl** reaction.

5.3 Pulse centrifuge to collect all liquid at the bottom of the tube.

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

5.5 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear.

5.6 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

5.7 Add **200 µl** of room-temperature **[M]70 % volume** ethanol to the pellet.

5.8 Carefully remove and discard ethanol, being careful not to touch the bead pellet.

5.9 **go to step #7** and repeat ethanol wash.

5.10 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.

5.11 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).

5.12 Resuspend pellet in **30 µl** Elution Buffer (EB), mix gently by either flicking or pipetting and incubate for **00:02:00** .

Elution Buffer (EB)
by Qiagen
Catalog #: 19086

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

5.14 Quantify **1 µl** product using the Quantus Fluorometer using the ONE dsDNA assay.

QuantiFluor(R) ONE dsDNA System, 100rxn
by Promega
Catalog #: E4871

Quantus
Fluorometer
Promega E6150

6 Quantify the barcoded amplicon pools using the Quantus Fluorometer using the ONE dsDNA assay.

DNA quantification using the Quantus fluorometer PREVIEW RUN
by Josh Quick

6.1 Remove Lambda DNA 400 ng/µL standard from the freezer and leave on ice to thaw. Remove ONE dsDNA dye solution from the fridge and allow to come to room temperature.

QuantiFluor(R) ONE dsDNA System, 500rxn
by Promega
Catalog #: E4870

6.2 Set up two **0.5 ml** tubes for the calibration and label them 'Blank' and 'Standard'

6.3 Add **200 µl** ONE dsDNA Dye solution to each tube.

6.4 Mix the Lambda DNA standard 400 ng/µL standard by pipetting then add **1 µl** to one of the standard tube.

6.5 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

6.6 Allow both tubes to incubate at room temperature for **00:02:00** before proceeding.

6.7 Selection 'Calibrate' then 'ONE DNA' then place the blank sample in the reader then select 'Read Blank'. Now place the standard in the reader and select 'Read Std'.

6.8 Set up the required number of **0.5 ml** tubes for the number of DNA samples to be quantified.

Use only thin-wall, clear, 0.5mL PCR tubes such as Axygen #PCR-05-C

6.9 Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading.

6.10 Add **199 µl** ONE dsDNA dye solution to each tube.

6.11 Add **1 µl** of each user sample to the appropriate tube.

Use a P2 pipette for highest accuracy.

6.12 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

6.13 Allow all tubes to incubate at room temperature for **00:02:00** before proceeding.

6.14 On the Home screen of the Quantus Fluorometer, select `Protocol`, then select `ONE DNA` as the assay type.

If you have already performed a calibration for the selected assay you can continue, there is no need to perform repeat calibrations when using ONE DNA pre diluted dye solution. If you want to use the previous calibration, skip to step 11. Otherwise, continue with step 9.

6.15 On the home screen navigate to 'Sample Volume' and set it to **1 µl** then 'Units' and set it to ng/µL.

6.16 Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.

6.17 Repeat step 16 until all samples have been read.

6.18 The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory notebook.

7 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**

The input of barcoded amplicon pools will depend on the number of barcoded pools and should be between 50 ng (6 barcodes) and 200 ng (24 barcodes).

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

9 Add **50 µl** (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting.

Vortex SPRI beads thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown colour.

10 Pulse centrifuge to collect all liquid at the bottom of the tube.

11 Incubate for 00:05:00 at room temperature.

12 Place on magnetic rack and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear.

13 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

14 Add 200 µl SFB and resuspend beads completely by pipette mixing.

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

15 Pulse centrifuge to collect all liquid at the bottom of the tube.

16 Remove supernatant and discard.

17 Repeat steps 14-16 to perform a second SFB wash.

18 Pulse centrifuge and remove any residual SFB.

You do not need to allow to air dry with SFB washes.

19 Add 15 µl EB and resuspend beads by pipette mixing.

20 Incubate at room temperature for 00:02:00 .

21 Place on magnetic rack.

22 Transfer final library to a new 1.5mL Eppendorf tube.