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One-pot native barcoding of amplicons 🖘

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ABSTRACT

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

EXTERNAL LINK

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

ATTACHMENTS

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 | ⊒ 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 |

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:

ComponentVolumeNBXX barcode□2.5 μlUltra II Ligation Master Mix□17.5 μlLigation Enhancer□0.5 μlTotal□35.5 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 **© 00:15:00**

```
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.

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



5.1

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



- 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 $\Box 50 \ \mu I$ SPRI beads to a $\Box 50 \ \mu I$ 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 $200 \,\mu l$ 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 **5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5**
- 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.



- 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 $\Box 1 \mu I$ product using the Quantus Fluorometer using the ONE dsDNA assay.





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



6.1 Remove Lambda DNA 400 $ng/\mu L$ 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.



| 0.2 | Set up two U.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/ μ L standard by pipetting then add $\Box 1 \mu I$ 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 11 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. |
| | |

| 0.15 | on the nome screen havigate to sample volui | The and set it to [] i μi then onits and set it to hg/με. | |
|------|----------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|--|
| 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 $ng/\mu L$, 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 | |
| 9 | | | |
| 10 | Pulse centrifuge to collect all liquid at the bottom of the tube. | | |
| 11 | Incubate for ③ 00:05:00 at room temperate | ure. | |
| 12 | Place on magnetic rack and incubate for © 0 | 0:02:00 or until the beads have pelleted and the supernatant is completely clear. | |
| 13 | Carefully remove and discard the supernatant | t, being careful not to touch the bead pellet. | |
| | | | |

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. Remove supernatant and discard. 16 Repeat steps 14-16 to perform a second SFB wash. 17 Pulse centrifuge and remove any residual SFB. 18 You do not need to allow to air dry with SFB washes. Add 15 µl EB and resuspend beads by pipette mixing. 20 Incubate at room temperature for **© 00:02:00**. Place on magnetic rack. 21 Transfer final library to a new 1.5mL Eppendorf tube.

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