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High-Performance Protocol for ONT Library Preparation V.1

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We use this protocol and it's working

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Abstract

This protocol presents a refined version of the Oxford Nanopore Technologies (ONT) Ligation Sequencing amplicons V14 (SQK-LSK114) protocol, optimized for the sequencing of short and ultra-short DNA fragments. It maintains the use of reagents required for standard ONT library preparation while enhancing DNA yield and improving sequencing performance.

The protocol can be successfully used for all types of Flow Cells, but the Flow Cell priming and loaded library volumes must be adjusted accordingly.



Materials

- 0.2 mL PCR tube
- 1.5 mL RNase- and DNase-free low-bind tube
- Milli-Q water (Thermo Fisher Scientific)
- Ultra II End-Prep Reaction Buffer (E7546S, NEB)
- Ultra II End-Prep Enzyme Mix (E7546S, NEB)
- Ligation Buffer (LNB, ONT)
- Quick T4 DNA Ligase (E6056S, NEB)
- Ligation Adapters (LA, ONT)
- Small Fragment Buffer (SFB, ONT)
- Elution Buffer (EB, ONT)
- Qubit HS dsDNA kit (Thermo Fisher Scientific)
- Flongle (ONT) flow cell
- MinION Mk1B device (ONT)
- Flow Cell Flush (FCF, ONT)
- Flow Cell Tether (FCT, ONT)
- Sequencing Buffer (SB, ONT)
- Library Beads (LIB, ONT)

 NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #E7546S**

 NEBNext Quick Ligation Module **New England Biolabs Catalog #E6056S**

Before start

- Thaw all the reagents and keep them on ice throughout the protocol.
- Prior to use, thoroughly mix all components by vortexing, except for the End-Prep Enzyme Mix and Quick T4 DNA Ligase.
- Perform all centrifugation steps at room temperature.

Note

Note: "ONT" refers to reagents from the standard SQK-LSK114 kit provided by Oxford Nanopore Technologies.



DNA End Preparation

10m

- 1 Transfer **[M] 250 Mass Percent** of the dsDNA to a clean 0.2 mL PCR tube, adjusting the volume based on DNA concentration. 
- 2 Add the following components sequentially, mixing by pipetting approximately 10 times after each addition: 

A	B
Component	Volume
dsDNA	X μ L (250 fmol)
Milli-Q water (Thermo Fisher Scientific)	X μ L (to 50 μ L)
Ultra II End-Prep Reaction Buffer (E7546S, NEB)	7 μ L
Ultra II End-Prep Enzyme Mix (E7546S, NEB)	3 μ L
Total volume:	60 μL

- 3 Briefly centrifuge the tube and place it into a thermal cycler with a heated lid.
- 4 Run the following thermal cycling program:

 20 °C for  00:05:00 .

 65 °C for  00:05:00 .

10m

Adapter Ligation

20m

- 5 After incubation, transfer the reaction mixture to a 1.5 mL RNase- and DNase-free low-bind tube.
- 6 Add the following components in the specified order, mixing by pipetting approximately 10 times after each addition: 

A	B
Component	Volume



A	B
End-prepped DNA	60 µL
Ligation Buffer (LNB, ONT)	25 µL
Quick T4 DNA Ligase (E6056S, NEB)	10 µL
Ligation Adapters (LA, ONT)	5 µL
Total volume	100 µL

- 7 Incubate the reaction at Room temperature for 00:20:00 .

20m



Purification and Elution

23m

- 8 Add AMPure XP Beads (AXP, ONT) to the reaction tube based on the DNA length:



- 90 µL for fragments < 100 bp.
- 50 µL for fragments > 100 bp.

- 9 Mix by inverting the tube for 00:05:00 .

5m



- 10 Briefly centrifuge the tube, then place it on a magnetic rack for 00:03:00 to pellet the beads.

3m



- 11 While keeping the tube on the magnet, carefully remove the supernatant.

- 12 Add 250 µL of Small Fragment Buffer (SFB, ONT) to the tube.



- 13 Remove the tube from the magnet and gently rotate for at least 00:03:00 , occasionally flicking to ensure even bead distribution.

3m

- 14 Briefly centrifuge the tube and place it back on the magnet for 00:03:00 , then remove the supernatant.

3m





- 15 Repeat the wash step with  250 μL of SFB following the same procedure.
- 16 After the final wash, briefly centrifuge the tube, then place it on the magnet to remove any remaining supernatant. 
- 17 Allow the bead pellet to dry for  00:01:00 before removing the tube from the magnet. 
- 18 Add  12 μL of Elution Buffer (EB, ONT) to the tube and mix thoroughly by pipetting. 
- 19 Incubate at  Room temperature for  00:05:00 . 

- 20 Place the tube back on the magnet for  00:03:00 to pellet the beads. 
- 21 Transfer the supernatant containing the prepared library to a new 1.5 mL RNase- and DNase-free tube and place  On ice . 
- 22 Measure DNA concentration using the Qubit HS dsDNA kit (Thermo Fisher Scientific).

Flongle Flow Cell Loading and Sequencing: Flow Cell Preparation

- 23 Insert a Flongle (ONT) flow cell into a MinION Mk1B device (ONT).
- 24 Perform a quality check using MinKNOW software.
- 25 Select only flow cells with ≥ 50 active pores for sequencing.

Flongle Flow Cell Loading and Sequencing: Flow Cell Priming

5m



- 26 Prepare the flow cell priming mix by combining the following components in a 1.5 mL tube, then immediately place  On ice :

A	B
Component	Volume
Flow Cell Flush (FCF, ONT)	117 μ L
Flow Cell Tether (FCT, ONT)	3 μ L
Total volume:	120 μL

- 27 Peel back the seal tab of the Flongle flow cell to expose the sample port.
- 28 Secure the seal tab by adhering it to the MinION lid using its adhesive strip.
- 29 Carefully load the priming mix into the flow cell, ensuring no air bubbles are introduced.
- 30 Incubate the flow cell for  00:05:00 .

5m



Flongle Flow Cell Loading and Sequencing: Library Preparation for Sequencing

10m

- 31 During the incubation period, prepare the sequencing mix by combining the following components:

A	B
Component	Volume
Sequencing Buffer (SB, ONT)	15 μ L
Library Beads (LIB, ONT)	10 μ L
Prepared Library	X μ L (50 fmol)
Elution Buffer (EB, ONT)	X μ L
Total volume:	35 μL



32 Incubate at  Room temperature for  00:10:00 before initiating sequencing run.

10m

