STANDARD OPERATING PROCEDURE (SOP)

SOP Title:	**BEADS FREE ONT LIGATION KIT LIBRARY PREPARATION FOR ULTRA-LONG READ SEQUENCING**
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I have read this document and approve its contents.

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A. PURPOSE AND APPLICATION

This SOP covers required equipment, materials, consumables, procedures and anticipated results along with worked example and troubleshooting for Beads Free ONT Ligation Kit based Nanopore Sequencing Library preparation. The described procedures combine different methods developed by nanopore community and ONT standard protocol for ligation kit. The method described in this SOP suits very well for any experiment that require ultra-long reads with significant sequencing throughput. However, this method requires good quality high molecular weight (HMW) DNA (>50kb) or ultra-HMW DNA and takes longer time (5-6 h) than ONT standard protocol.

B. BRIEF SUMMARY OF METHOD

In this method, PEG/NaCl solution and short read eliminator XL (SRE) kit replace AMPure beads while preparing library with Ligation Sequencing Kit (SQK-LSK109) thereby keeping original sample integrity during the library preparation. PEG/NaCl solution is used after the DNA repair and End prep step and SRE after ligation step.

C. DEFINITIONS AND ABBREVIATIONS

PEG: Polyethylene Glycol
NaCl: Sodium Chloride
SRE: Short Read Eliminator
HMW DNA: High Molecular Weight DNA
uHMW DNA: Ultra-High Molecular Weight DNA
LB: Loading Buffer
EB: Elution Buffer
RT: Room Temperature

D. OCCUPATIONAL HEALTH AND SAFETY

No OH&S risk is identified with this SOP.

E. CAUTIONS

User should thaw and quantify DNA sample before starting experiment (use only single thawed sample, fresh sample is ideal) and multiple freezing and thawing degrade HMW DNA. User should use wide-bore pipette tips in all steps.

F. PERSONNEL QUALIFICATIONS, TRAINING AND RESPONSIBILITIES

Training Requirements:

[X] Read and Understand Document [X] Training Required

G. EQUIPMENT AND MATERIALS

Equipment

a. Mini centrifuge
b. Tabletop centrifuge
c. Thermocycler
d. Heatblock
e. Qubit
f. MinION device and IT set up
g. P200 pipette
h. P100 pipette
i. P20 pipette
j. P10 pipette

Materials and Consumables

a. High quality HMW DNA e.g. DNA extracted from Nanobind CBB Big DNA Kit (SKU NB-900-001-01, Circulomics) or equivalent
b. Tris-HCl (pH-8)
c. NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (E7180S, NEB)
d. Ligation Sequencing Kit (SQK-LSK109, ONT)
e. PEG solution (9%PEG 8k (w/v), 1 M NaCl, 10 mM Tris-HCl (pH-8) - [Polyethylene glycol: V3011, Promega; NaCl 71580-500G, Tris T6066-500G, Sigma]
f. Short Read Eliminator XL (SKU SS-100-111-01, Circulomics)
g. Nuclease free water
h. P200 wide bore pipette tips (LC1152-965, Adelab)
i. Qubit dsDNA HS assay kit (Q32854, Life Technologies Australia)
j. MinION Flow Cell (FLO-MIN106, R9.4.1)
k. 1.5 ml Low bind DNA Eppendorf tube
l. Qubit tubes
m. PCR tubes
n. P200 pipette tips (wide bore and regular)
o. P100 pipette (wide bore and regular)
p. P20 pipette tips
q. P10 pipette tips

H. PROCEDURE

A. DNA repair and End-prep

1. Thaw FFPE DNA Repair Buffer and End-prep reaction buffer and mix well by vertexing. Check if any white precipitate appeared.
2. Mix all the following reagents in a 0.2 ml thin-walled PCR tube and mix gently by using P200 wide-bore pipette tips. Try to avoid air bubbles formation which is hard to remove die to highly viscus solution and spin down in a mini centrifuge.

Reagents	Volume (µl)
DNA (4 µg uHMW/HMW)	48
FFPE DNA Repair Buffer	3.5
FFPE DNA Repair Mix	2
End-prep reaction buffer	3.5
End-prep enzyme mix	3
Total	60

3. Incubate the reaction at 20 °C for 10 min and 65 °C for 10 min in a thermocycler.
4. Transfer the reaction solution into a fresh 1.5 ml DNA LowBind Eppendorf tube and add 60 µl of PEG/NaCl solution which contained 9%PEG 8k (e/v), 1 M NaCl, and 10 mM Tris-HCl (pH-8). Mix the solution well by tapping the tube followed by 30 min incubation at room temperature (RT).
5. Spin the reaction solution at 13k rpm at RT for 30 min (place the tube's hinge facing away from the centre of centrifuge). While spinning the tube, prepare 1 ml 70% ethanol in nuclease free water.
6. Remove the supernatant and add 200 µl of 70% ethanol without disturbing the DNA pellet and spin at 13k for 5 min and repeat this step once.
7. Allow DNA to air dry for a 2-3 min and add 33 µl 10 mM Tris-HCl (pH-8) and incubate 37 °C for 15 min.
8. Use 1 µl for qubit quantification.

B. Adaptor Ligation and Clean-up

1. Spin down Adapter mix (AMX) and T4 ligase from NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (E7180S).
2. Thaw Ligation Buffer (LNB) at room temperature (RT), spin down and mix by pipetting. Due to viscosity, vertexing this buffer is ineffective. Place on ice immediately after thawing and mixing.
3. Thaw the Elution Buffer (EB) at RT, mix by vertexing, spin down in a minicentrifuge and place on ice.
4. Thaw Large Fragment Buffer (LFB) at RT, mix well by vertexing, spin down in a minicentrifuge and place on ice.
5. in a 1.5 ml DNA LowBind Eppendorf tube, mix the reagents in following order:

Reagents	Volume (µl)
DNA sample form the previous step	32
Ligation Buffer (LNB)	15
NEBNext Quick T4 DNA Ligase	10
Adapter Mix (AMX)	3
Total	60

6. Mix the reaction solution by pipetting (P200 wide-bore or P100 wide-bore) without introducing air bubbles and spin it down in mini centrifuge. Incubate the reaction for 20 min at RT.
7. Add 60 µl of SRE XL buffer and mix gently by tapping the tube and centrifuge it at 13k for 30 min at RT. (Note: place the tube's hinge facing away from the centre of centrifuge.)
8. Pipette up the supernatant from other side of the tube's hinge region without disturbing the DNA pellet. Add 250 µl LFB and spin it at 13k for 3 min at RT.
9. repeat step 8 once.
10. Allow to dry 2-3 min, but do not completely dry the pellet that makes hard to dissolve DNA pellet.

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11. add 39 µl of EB buffer and incubate at 37 °C for 15 min.
12. Use 1 µl eluate for qubit quantification.

C. Priming and Loading Library

1. Thaw the sequencing buffer (SQB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before placing the tubes on ice as soon as thawing is completed. At the same time, place the flow cell at RT.
2. Mix the SQB, FLT and FB tubes by vortexing and spin down in a mini centrifuge and then return to ice.
3. Perform QC of flow cell according to ONT instruction if that have not performed previously.
4. Prepare the flow cell priming mix by adding 30 µl of FLT to FB tube and mix by pipetting.
5. Load 800 µl priming mix into the flow cell via priming port according to the ONT instructions for MinION flow cell priming. Wait for 5 min. During this time, prepare the library for loading by mixing 37.5 µl SQB with 600 ng DNA library in 37.5 µl EB buffer.
6. Add 75 µl of sample to the flow cell via SpotON sample port according to the ONT instructions for MinION flow cell sample loading. Gently replace the SpotON port cover and close the priming port and wait for at least 10 min before starting the sequencing run.

I. WORKED EXAMPLE

Libraries were prepared from three different samples (mammalian cell origin) and sequencing was performed in MinION flow cell R.9.4.1. 7.2 Gb was final yield in 24 -36 h depending on the flow cells with one-two loading of nuclease flush and fresh library loading.

Table: Sequencing yield and stats of worked samples

Features	Sample1	Sample2	Sample3
Mean read length	16,373.6	17,885.7	16,467
Mean read quality	12.7	13.2	12.7
Median read length	8,794	8,875	6,883
Median read quality	13.1	13.6	13.1
Number of reads	443,137	400,469	339,215
Read length N50	31,756	36,452	39,748
Yield in Gb (24h run)	7.25	7.16	*5.58
Top 5 longest reads (bp) and their mean base call quality score			
1	392706 (11.7)	426575 (10.4)	384770 (14.2)
2	329633 (12.2)	376343 (12.5)	382457 (14.5)
3	305652 (13.0)	360701 (14.7)	366030 (10.9)
4	302711 (11.3)	356412 (11.3)	353636 (10.5)
5	285282 (9.6)	330026 (11.5)	345307 (12.1)

*Used flow cell and sequencing was run for 48 h.

J. SOP VALIDATION DETAILS

The method described in this SOP has been tested in three samples and result showed consistent data quality and quantity.

K. Troubleshooting

Low DNA recovery: Old DNA stock or make sure DIN value ~9 in TapeStation before starting the library preparation. Mix the DNA pellet in step A.7 and B.11 gently by tapping the tube or pipetting using wide bore tips. Make sure DNA pellet was not disturbed by washing step A.6 and B.8–9.

Very Low sequencing throughput: Check the DNA quality in NanoDrop and Qubit. A260/A280 and A260/A230 should > 1.8 and the DNA concentration should closely align between NanoDrop and qubit measurement. Perform Nuclease flush when pore availably become 10-20%.

L. WASTE MANAGEMENT AND DISPOSAL

All the chemicals used in this SOP is very low amount and do not use carcinogenic substances. The chemical wastes are disposed in clinical waste bin and the flow cells are returned to ONT after washing.

M. DATA RECORDS MANAGEMENT

All the generated data both fast5 and fastq files are saved according to the user chose location in the MinION IT system. If the user aims to do base call locally, user must save fast5.

N. REFERENCE DOCUMENTS

Tyson, J. (2020). Rocky Mountain adventures in Genomic DNA sample preparation, ligation protocol optimisation/simplification and Ultra long read generation. dx.doi.org/10.17504/protocols.io.7euhjew

Oxford Nanopore Technologies, Nanopore Protocol, Genomic DNA by ligation (SQK-LSK109) Version: GDE_9063_v109_revT_14Aug2019. https://community.nanoporetech.com/attachments/3390/download

O. QUALITY CONTROL (QC) AND QUALITY ASSURANCE (QA) SECTION

To get a good performance of this SOP, user should –

a. start with good quality intact DNA (DIN value >9)

b. must use wide bore pipette tips throughout the experiment

c. should do nuclease flush and reload fresh library when flow cell occupancy drops to 10 %