Oxford Nanopore Technologies library preparation

The following amplification-free library preparation protocols are available for Oxford Nanopore Technology (ONT) sequencing:

1) Ligation kit
2) Ligation kit + T7 Endonuclease I
3) Rapid Sequencing Kit

Samplix recommends using **2) Ligation kit + T7 Endonuclease I** for library preparation of Samplicons (Samplix enriched DNA samples). This is also the protocol recommended by ONT for whole genome amplification (WGA) products. Both WGA and Samplix enrichment with Xdrop™ use an isothermal amplification (Xdrop™ uses Multiple Displacement Amplification in droplets), which creates branched DNA structures. The T7 Endonuclease I treatment reduces DNA branching by cleavage.

Ligation Kit + T7 Endonuclease I

To perform the Ligation library preparation with the T7 endonuclease I please follow the ONT "**Premium whole genome amplification protocol**" (SQK-LSK109) (note that you might need to log in to be able to access protocols from ONT community).

This protocol is intended for library preparation of small amounts of DNA, and thus is initiated with a WGA step using the Qiagen REPLI-g Midi kit. For Library preparation of Samplicons **skip the WGA step and proceed to the T7 Endonuclease I digestion.**

Library preparation overview

- Digest the amplified DNA with T7 Endonuclease I to remove branching
- Size-select for longer fragments using AMPure XP beads (Beckman Coulter, ONT recommended) or HighPrep beads (MagBio, Samplix recommended)
- Prepare the DNA ends for adapter attachment
- Attach sequencing adapters supplied in the Ligation kit to the DNA ends
- Prime the flow cell, and load your DNA library into the flow cell

Special consideration for Samplix library preparation with the Ligation kit +T7 Endonuclease I

As the ligation Ligation kit +T7 Endonuclease I library preparation protocol involves several bead-based DNA cleaning steps, an input DNA amount of 1-1,5 µg for whole genome amplification is required. However, for DNA fragments below 10kb, less DNA is required (~100-200 fmol of DNA), e.g. 300-600 ng for 5 kb and 600-1200 ng for 10 kb DNA fragments.

Here the formula:

mass of dsDNA (g) = moles of dsDNA (mol) x ((length of dsDNA (bp) x 617.96 g/mol) + 36.04 g/mol)

Note: the average molecular weight of a base pair is 617.96 g/mol, and 36.04 g/mol accounts for the 2 -OH and 2 -H added to the ends.

The DNA concentration of Samplicons depends on the number of positive droplets sorted during the Xdrop™ enrichment protocol, but usually falls within the DNA concentrations required.

In case the amount of DNA recovered is below 100 fmol, Samplix recommends to:

1. Reduce DNA loss by omitting the AMpure XP bead clean-up of the repaired/end-repaired DNA sample (note that this can reduce subsequent adapter ligation efficiency, increase the prevalence of chimeric reads, and reduce pores availability for sequencing).
2. After loading 10µl of Samplix broken positive droplets, some volume is left. This volume can be loaded into an additional dMDA lane to increase the DNA output.
3. Enrich in parallel the same sample in multiple Xdrop™ lanes and then pool those replicates after sorting Samplix droplets.

Note that if AMpure XP bead clean-up is omitted, incubation of the ligation reaction should be no longer than 10 min, as per protocol.

Depending on the DNA concentration, the Ligation kit + T7 Endonuclease I library preparation can be attempted anyway, although with possible lower sequencing efficiency and without success guarantees from neither ONT nor Samplix.

Note that resulting libraries of the Ligation Kit + T7 Endonuclease I may reduce fragment size, due to the DNA shearing during the Endonuclease I digestion step.

Consumables needed for Samplicon library preparation with the Ligation kit +T7 Endonuclease I

Materials

- Samplicons
- Ligation Sequencing Kit (SQK-LSK109)
- Flow Cell Priming Kit (EXP-FLP002)

Consumables

- Agencourt AMPure XP beads*
- NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (cat # E7180S). Alternatively, you can use the three NEBNext® products below:
    - NEBNext FFPE Repair Mix (M6630)
    - NEBNext End repair / dA-tailing Module (E7546)
    - NEBNext Quick Ligation Module (E6056) • Covaris g-TUBE
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 70% ethanol in nuclease-free water
- T7 Endonuclease I (NEB, #M0302) • TE buffer: 10 mM Tris (pH 8.0), 0.1 mM EDTA

Equipment

- Hula mixer (gentle rotator mixer)
- Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- Microfuge
- Vortex mixer
- Heating block at 37° C capable of taking 1.5 ml tubes
- Thermal cycler
- ~~Ice bucket with ice~~ Freezing block**
- Timer
- P1000, P200, P100, P10, and P2 pipette and tips

* Samplix has also successfully tested MagBio HighPrep beads.

** Samplix recommends using a freezing block if available.

Notes to the protocol (version WAL_9070_v109_revM_14Aug2019)

- Start the protocol at page 11, at the following step: “In a clean 0.2 ml PCR tube, mix the reagents in the following order:”
- After preparing custom buffer (page 12), Samplix recommends making 200 µl aliquots and store at -20 ºC.
- Recommends preparing beads fresh every time: scale down volumes from the protocol to 35ul of beads + 35 ul of custom buffer per sample.
- Resuspend in 50 µl of nuclease-free water instead of 49 µl (second step on page 13). Use the extra µl to run the sample on Tapestation, on top of the recommended Quibit quantification.