ENCODE Oxford Nanopore Library Protocol for Split-Seq Single-Nucleus cDNA v1

Prepared by Jasmine Sakr
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Ali Mortazavi Lab, University of California, Irvine

Contact Information

Jasmine Sakr
2300 Biological Sciences III
University of California, Irvine
Irvine, CA 92697-2300
Telephone: (714) 906-1978
Email: jsakr@uci.edu

Ali Mortazavi
2218 Biological Sciences III
University of California, Irvine
Irvine, CA 92697-2300
Telephone: (949) 824-6762
Email: ali.mortazavi@uci.edu

INTRODUCTION

This document describes the steps used to prepare Oxford Nanopore libraries from Split-Seq single-nucleus cDNA. Samples were loaded on R9.4.1 flow cells and MinION Mk1B instrument was used for sequencing. Kits and other reagents used can be found in Table 1.

Table 1:

Name	Cat. No	Available from
Agencourt AMPure XP Beads	A63881	https://www.beckman.com/search?msclkid=4aff6d9a792a1458fce30c6c74f7c71d#q=A63881&t=coveo-tab-techdocs
NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing	E7180S	https://www.neb.com/products/e7180-nebnext-companion-module-for-oxford-nanopore-technologies-ligation-sequencing#Product%20Information
Ligation Sequencing Kit	SQK-LSK110	https://store.nanoporetech.com/us/ligation-sequencing-kit110.html
Flow Cell (R9.4.1)	FLO-MIN106D	https://store.nanoporetech.com/us/flow-cell-r9-4-1.html

DNA Repair & End-Prep

1. Thaw NEBNext® Companion Module, spin down, mix by pipetting, and place on ice.
2. Thaw DNA CS (DCS) at RT, spin down, mix by pipetting, and place on ice.
3. Prepare the DNA in Nuclease-free water:
    - Adjust volume of 200 fmol DNA sample to 47 µL with Nuclease-free water.
    - Mix thoroughly by flicking the tube.
    - Spin down briefly in a microfuge.
4. In a PCR tube, mix the following:
    - 1 µL DNA CS
    - 47 µL DNA sample
    - 3.5 µL NEBNext FFPE DNA Repair Buffer
    - 2 µL NEBNext FFPE DNA Repair Mix
    - 3.5 µL Ultra II End-prep reaction buffer
    - 3 µL Ultra II End-prep enzyme mix
5. Mix gently and spin down.
6. Using a thermal cycler, incubate at 20°C for 5 mins and 65°C for 5 mins.

AMPure XP Bead Clean-Up

1. Resuspend the AMPure XP beads by vortexing.
2. Transfer the DNA sample to a new 1.5 mL tube.
3. Add 60 µL of resuspended AMPure XP beads to the end-prep reaction and mix.
4. Incubate on a mixer for 5 minutes at 300 rpm at RT.
5. Prepare fresh 70% ethanol in Nuclease-free water.
6. Pellet sample on a magnet until solution is clear and colorless.
7. Keep the tube on the magnet and pipette off the supernatant.
8. While tube is still on the magnet, wash the beads with 200 µL of freshly prepared 70% ethanol without disturbing the pellet.
    - Remove the ethanol using a pipette and discard.
9. Repeat the previous wash step.
10. Spin down and place the tube back on the magnet.
11. Pipette off any residual ethanol and allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.
12. Remove the tube from the magnet and resuspend the pellet in 61 µL Nuclease-free water.
13. Incubate for 2 minutes at RT.
14. Pellet the beads on a magnet until the solution is clear and colorless.
15. Remove and retain 60 µL of eluate into a new 1.5 mL tube.

Adapter Ligation & Clean-Up

1. Thaw the Adapter Mix F (AMX-F) and Quick T4 Ligase, spin down then place on ice.
2. Thaw Ligation Buffer (LNB) at RT, spin down and mix by pipetting.
    - NOTE: Due to viscosity, vortexing this buffer is ineffective.
    - Place on ice immediately after thawing and mixing.
3. Thaw the Elution Buffer (EB) at RT, mix by vortexing, spin down and place on ice.
4. *We used the SFB wash buffer to generate our libraries
    - Thaw the Short Fragment Buffer (SFB) at RT, mix by vortexing, spin down and place on ice.
5. In a 1.5 mL tube, mix in the following order:
    - 60 µL DNA sample from the previous step
    - 25 µL Ligation Buffer (LNB)
    - 10 µL NEBNext Quick T4 DNA Ligase
    - 5 µL Adapter Mix F (AMX-F)
6. Mix well and spin down.
7. Incubate reaction for 10 minutes at RT.

AMPure Purification

1. Resuspend the AMPure XP beads by vortexing.
2. Add 40 µL of resuspended AMPure XP beads to the reaction and mix.
3. Incubate on a mixer for 5 minutes at 300 rpm at RT.
4. Pellet sample on a magnet until solution is clear and colorless.
5. While tube is on the magnet, pipette off the supernatant.
6. Wash the beads by adding 250 µL Short Fragment Buffer (SFB):
    - Mix the beads to resuspend and spin down.
    - Return the tube to the magnet and allow the beads to pellet.
    - Remove the supernatant using a pipette and discard.
7. Repeat the previous SFB wash step.
8. Spin down and place the tube back on the magnet.
9. Pipette off any residual supernatant and allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.
10. Remove the tube from the magnet and resuspend the pellet in 15 µL Elution Buffer (EB).
11. Incubate for 10 minutes at RT.
12. Pellet the beads on a magnet until the solution is clear and colorless.
13. Remove and retain 15 µL of eluate containing the DNA library into a new 1.5 mL tube.
14. Quantify 1 µL of eluted sample using a Qubit fluorometer.
15. Keep the library on ice until ready to load.

Priming & Loading the R9.4.1 Flow Cell

1. Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII), Flush Tether (FLT) and one tube of Flush Buffer (FB) per sample at RT.
2. Mix the Sequencing Buffer II (SBII), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing and spin down at RT.
3. Prepare the flow cell priming mix:
    - Add 30 µL of thawed and vortexed Flush Tether (FLT) *directly* to the tube of thawed and vortexed Flush Buffer (FB).
    - Mix by vortexing again at RT.
4. Load the flow cell priming mix via the primming port on the R9.4.1 flow cell.
    - See nanopore website for detailed loading instructions.
5. In a new tube, prepare the library for loading by mixing the following:
    - 37.5 µL Sequencing Buffer II (SBII)
    - 25.5 µL Loading Beads II (LBII) mixed immediately before use
    - 12 µL of 50 fmol DNA library
6. Mix the prepared library gently by pipetting up and down just prior to loading.
7. Add 75 µL of library to the flow cell via the SpotON sample port in a dropwise fashion.

Sequencing

1. Samples were sequenced on MinION Mk1B instrument:
    - MinKNOW version 21.10.4
    - MinKNOW Core version 4.4.3

References

1. Genomic DNA by Ligation protocol version GDE_9108_v110_revF_10Nov2020 (Last update: 10/06/2021) downloaded from Oxford Nanopore website https://store.nanoporetech.com/us/ligation-sequencing-kit110.html