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ONT Library Prep for Split-seq cDNA

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol outlines the preparation of single-cell barcoded cDNA for Oxford Nanopore long-read sequencing. The input cDNA was initially barcoded using the Parse Biosciences Evercode platform for single-nucleus or single-cell RNA-seq (refer to the protocol “Evercode WT v2.2.1” at **dx.doi.org/10.17504/protocols.io.eq2lyj9relx9/v1** or “Evercode WT Mega v2.2.1" at **dx.doi.org/10.17504/protocols.io.8epv5xxrng1b/v1**).

Since we typically perform single-nucleus RNA-seq rather than single-cell RNA-seq, we enrich the cDNA for exome-containing fragments to reduce the number of intronic reads. This is achieved using Twist Biosciences exome panels and a modified version of the Parse Biosciences Gene Capture protocol (refer to the protocol “cDNA Exome Capture v1.0.1” at **dx.doi.org/10.17504/protocols.io.36wgq3b83lk5/v1**).

The resulting product of this protocol is full-length, barcoded cDNA, excluding intron-only fragments, with Nanopore adapters added to both ends, creating a final library ready for sequencing. The first part of the protocol after setup, End Prep, involves using NEBNext reagents to repair the cDNA ends, resulting in 5' phosphorylated, 3' dA-tailed ends, followed by a bead-based cleanup. The next part, Adapter Ligation, adds Nanopore adapters to the end-prepped cDNA, followed by a bead-based cleanup and final elution.

Please see the attachment for the original protocol.

Attachments

PDF

Ligation Sequencing ...

108KB

Materials

REAGENTS

	Item	Supplier	Part Number	Notes
	Ligation Sequencing Kit V14	Oxford Nanopore Technologies	SQK-LSK114	The Long Fragment Buffer (LFB) will NOT be used because it selects for fragments that are 3kb and longer.
	NEBNext Ultra II End repair/dA-tailing Module	New England BioLabs	E7546	
	NEBNext Quick Ligation Module	New England BioLabs	E6056	NEBNext Quick Ligation Reaction Buffer will NOT be used.
	AMPure® XP Reagent	Beckman Coulter	A63880 (5 mL) A63881 (60 mL)	
	Nuclease-Free Water	Sigma-Aldrich	W4502	Or equivalent nuclease-free water.
	Ethyl Alcohol, Pure	Sigma-Aldrich	459844	Or equivalent 100% non-denatured ethanol.

EQUIPMENT

	A	B	C	D
	Thermomixer	Various Suppliers	Varies	Compatible with 1.5 mL tubes.
	Microcentrifuge	Various Suppliers	Varies	Compatible with 1.5 mL tubes.
	Single Channel Pipettes: P2, P20, P200, P1000	Various Suppliers	Varies	
	Vortex-Genie 2	Scientific Industries	SI-0236	Or an equivalent vortex mixer.
	6-Tube Magnetic Separation Rack	New England Biolabs	S1506S	Or an equivalent magnetic rack for 1.5 mL tubes.
	Qubit Flex Fluorometer	Thermo Fisher Scientific	Q33327	Or an equivalent fluorometer.

SUPPLIES

	A	B	C	D
	Pipette Tips TR LTS 10 µL, 20 µL, 200 µL, 1,000 µL	Rainin	17014961 17014963 17014967	Or appropriate sterile, DNA low-binding, and filtered pipette tips. We do not recommend using wide bore tips.

	A	B	C	D
	DNA LoBind® Tubes, 1.5 mL, Snap Cap	Eppendorf	022431021	Or equivalent DNA low-binding, nuclease-free 1.5 mL tubes.
	Qubit dsDNA HS (High Sensitivity) Assay Kit	Thermo Fisher Scientific	Q33230 (100 assays) Q33231 (500 assays)	Or equivalent fluorescent DNA dye based quantification kit.
	Ice bucket	Various Suppliers	Varies	

Before start

Input:

This protocol needs 100-200 fmol of full-length cDNA. We recommend using 200 fmol to get the highest yield. This concentration could be calculated with average basepair length and weight using an **online calculator**. cDNA fragments with average basepair length between 1-1.5 kb will need between 130-200ng of total cDNA as input. Library yields will range between 3-6.5 ng/µL.

Preparation

1 **Thaw the following reagents:**

1.1

Item	Location	Handling and Storage
DNA Control Sample (DCS)	Ligation Sequencing Kit V14 (-20°C)	Thaw on ice and mix by pipetting.
Ligation Adapter (LA)	Ligation Sequencing Kit V14 (-20°C)	Thaw on ice and mix by pipetting, do NOT vortex.
Ligation Buffer (LNB)	Ligation Sequencing Kit V14 (-20°C)	Due to viscosity, vortexing this buffer is ineffective. Spin down and mix by pipetting.
Short Fragment Buffer (SFB)	Ligation Sequencing Kit V14 (-20°C)	Thaw on ice and mix well by vortexing.
Elution Buffer (EB)	Ligation Sequencing Kit V14 (-20°C)	Thaw on ice and mix well by vortexing.
Ultra II End-Prep Enzyme Mix	NEBNext Ultra II End repair/dA-tailing Module (-20°C)	Thaw on ice and mix by pipetting, do NOT vortex.
Ultra II End-Prep Reaction Buffer	NEBNext Ultra II End repair/dA-tailing Module (-20°C)	Thaw on ice and mix well by vortexing. Make sure there is no precipitate.
Ultra II End Prep Enzyme Mix	NEBNext Quick Ligation Module (-20°C)	Thaw on ice and mix by pipetting, do NOT vortex.
AMPure XP Beads (AXP)	4°C	Equilibrate to room temperature.

2 **Prepare the following:**

2.1 For each sample: Adjust **100-200 fmol** of cDNA volume to **49 µL** with **Nuclease-free water** in a 1.5 mL tube and mix thoroughly by pipetting up and down.

2.2 Prepare fresh **80% ethanol** for the wash steps. Each sample will need 400 µL

3 Set heat block to **65°C**.

End-prep

4 End-prep Reaction

4.1 In a 1.5 mL tube, mix the following for each sample:

	Item	Volume (µL)
	DNA CS	1
	cDNA sample	49
	Ultra II End-prep Reaction Buffer	7
	Ultra II End-prep Enzyme Mix	3

4.2 Thoroughly mix by pipetting and avoid creating bubbles.

4.3 Incubate at **room temperature** for **5 minutes** on bench top and **65°C** for **5 minutes** on thermomixer.

5 Clean-Up

5.1 Vortex AMPure XP Beads (AXP).

5.2 Add **60 µL** of the resuspended **AMPure XP Beads (AXP)** to the end-prep reaction(s) and mix by pipetting.

5.3 Incubate tube(s) on thermomixer at **300 rpm** for **5 minutes** at **room temperature**.

5.4 Spin down the tube(s) and pellet on a magnet.

5.5 Keep the tube(s) on the magnet and pipette off the supernatant.

5.6 Keep the tube(s) on the magnet and wash the beads with **200 µL** of freshly prepared **80% ethanol** without disturbing the pellet.

5.7 Remove the ethanol using a pipette and discard without disturbing the pellet.

5.8 Keep the tube(s) on the magnet and wash the beads with **200 µL** of freshly prepared **80% ethanol** without disturbing the pellet (again).

5.9 Remove the ethanol using a pipette and discard without disturbing the pellet (again).

5.10 Allow to dry for ~30 seconds, but do NOT dry the pellet to the point of cracking.

5.11 Remove tube(s) from magnet and resuspend the pellet in **61 µL Nuclease-free water**.

5.12 Incubate tube(s) for **5 minutes** at **room temperature**.

5.13 Pellet the beads on a magnet.

5.14 Remove and retain **60 µL** of eluate into a new 1.5 mL tube(s).

Adapter Ligation

6 **Adapter ligation reaction**

6.1 In the same 1.5 mL tube, mix in the following order for each sample:

	Item	Volume (µL)
	cDNA sample (from the previous step)	60
	Ligation Adapter (LA)	5
	Ligation Buffer (LNB)	25
	NEBNext Quick T4 DNA Ligase	10

6.2 Thoroughly mix by gently pipetting and avoid creating bubbles.

6.3 Incubate tube(s) at room temperature for 10 minutes.

7 **Clean-up**

7.1 Vortex AMPure XP Beads (AXP).

7.2 Add **40 µL** of the resuspended **AMPure XP Beads (AXP)** to the reaction(s) and mix by pipetting.

7.3 Incubate tube(s) on the thermomixer at **300 rpm** for **5 minutes** at **room temperature**.

7.4 Spin down the tube(s) and pellet on a magnet.

7.5 Keep the tube(s) on the magnet and pipette off the supernatant.

7.6 Remove tube(s) from magnet and wash the beads by adding **250 µL Short Fragment Buffer (SFB)**.

7.7 Resuspend the beads by pipetting.

7.8 Pellet the beads on a magnet and pipette off the supernatant.

7.9 Remove tube(s) from magnet and wash the beads by adding **250 µL Short Fragment Buffer (SFB)** (again).

7.10 Resuspend the beads by pipetting (again).

7.11 Pellet the beads on a magnet and pipette off the supernatant (again).

7.12 Allow to dry for ~30 seconds, but do NOT dry the pellet to the point of cracking.

7.13 Remove tube(s) from magnet and resuspend the pellet in **15 µL Elution Buffer (EB)**.

7.14 Incubate for **5 minutes** at **room temperature**.

7.15 Pellet the beads on a magnet.

7.16 Remove and retain **15 µL** of eluate into a new 1.5 mL tube(s).

7.17 Qubit each library and keep on ice until loading or store in -20°C.

Protocol references

Please see the attachment for the original Oxford Nanopore Technologies protocol.