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Oxford Nanopore Technologies (ONT) library preparation and sequencing of DNA prepared using droplet Multiple Displacement Amplification (dMDA)

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We use this protocol and it's working

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Disclaimer

Abstract

Debranching of DNA generated by Multiple Displacement Amplification (MDA) is an essential step prior to nanopore sequencing. This protocol describes an optimised T7-based debranching protocol for genomic DNA amplified from single nuclei using droplet MDA (dMDA) obtained according to Kalef-Ezra et al 2023 (https://www.protocols.io/view/manual-isolation-of-nuclei-from-human-brain-using-kxygxzjjov8j/v1). Subsequent library preparation and sequencing steps are also outlined here.

Materials

	A	B	C	D	E
	Item	Supplier	Catalogue Number	Preparation prior to use	Storage
	Nuclease-free water	Sigma Aldrich	W4502	Aliquot	RT
	T7 Endonuclease I (10U/ul)	NEB	M0302S	Place on ice	-20 °C
	10X NEBuffer 2	NEB	M0302S	Thaw on ice	-20 °C
	AMPure XP Beads	Beckman Coulter	A63882	Equilibrate at RT for 30 min	4 °C
	1 M Tris-HCl pH 8	Thermo Scientific	J22638-AE		RT
	0.5 M EDTA pH 8, nuclease-free	Thermo Fisher Scientific	AM9260G		RT
	5M NaCl	Thermo Fisher Scientific	AM9759		RT
	PEG 8000 (40% w/v)	Sigma Aldrich	P1458		4 °C
	TE Buffer pH 8, 2 um filtered	Invitrogen	AM9858		RT
	Native Barcoding Kit 96 V14	ONT	SQK-NBD114.96	See before starting notes	-20 °C
	NEBNext Ultra II End repair/dA-tailing Module	NEB	E7546	Thaw on ice	-20 °C
	NEBNext FFPE Repair Mix	NEB	M6630	Thaw on ice	-20 °C
	NEB Blunt/TA Ligase Master Mix	NEB	M0367	Thaw on ice	-20 °C
	NEBNext Quick Ligation Module	NEB	E6056	Thaw on ice	-20 °C
	Qubit Assay Tubes	ThermoFisher	Q32856		RT
	Qubit 1X dsDNA HS Assay Kit	ThermoFisher	Q33231	Equilibrate at RT for 30 min	4 °C
	Genomic DNA ScreenTape Assay (optional)	Agilent	5067-5365; 5067-5366	Equilibrate at RT for 30 min	4 °C
	TapeStation strips and caps (optional)	Agilent	401428; 401425		RT
	TapeStation Loading tips (optional)	Agilent	5067-5598		RT
	PromethION R10.4.1M flow cell	ONT	FLO-PRO114M	Equilibrate at RT for 20 min	4 °C
	Flow Cell Wash Kit	ONT	EXP-WSH004 or EXP-WSH004-XL		-20 °C

General consumables:

- Gloves
- Pipettes: P10, P20, P200 and P1000 (any brand)
- Pipette filter tips: 10 ul, 200 ul and 100 ul (any brand)

- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf LoBind tubes
- 2 ml Eppendorf LoBind tubes
- 80 % Ethanol, prepared with nuclease-free water
- 70 % Ethanol, prepared with nuclease-free water

Equipment:

- HulaMixer (ThermoFisher) or rotating tube mixer
- Magnetic rack for 0.2 ml PCR tubes
- Magnetic stand for 1.5 ml Eppendorf tubes, e.g. DynaMag-2 Magnet (ThermoFisher; 12321D)
- Microfuge
- Thermal cycler
- Ice bucket and ice
- Benchtop Cooler Rack for holding 1.5-2 ml tubes, with -20 °C temperature range
- Vortex mixer
- Qubit fluorometer (or equivalent)
- Agilent TapeStation (optional)
- Vortex mixer IKA MS3 with 96-well sample plate adapter (optional)
- PromethION sequencer (ONT)
- Heat block or thermomixer, set to 37 °C

Equipment

Magnet

NAME

DynaMag™-2 Magnet

TYPE

Thermo Fisher

BRAND

12321D

SKU

https://www.thermofisher.com/order/catalog/product/12321D

LINK

Nuclease-free Water **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**W4502-1L

T7 Endonuclease I - 250 units **New England Biolabs Catalog #**M0302S

AMPure XP Beads **Beckman Coulter Catalog #**A63882

Tris-HCl pH 8.0 **Thermo Scientific Catalog #**J22638-AE

- EDTA (0.5 M, pH 8.0, nuclease-free) **Thermo Fisher Scientific** **Catalog #**AM9260G
- 5M NaCl solution **Thermo Fisher Scientific** **Catalog #**AM9759
- 40% Polyethylene Glycol MW 8000 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P1458
- TE, pH 8.0, RNase-free **Thermo Fisher** **Catalog #**AM9858
- Native barcoding kit (96) **Oxford Nanopore Technologies** **Catalog #**SQK-NBD114.96
- NEBNext® Ultra™ II End Repair/dA-Tailing Module **New England Biolabs** **Catalog #**E7546
- NEBNext FFPE Repair Mix **New England Biolabs** **Catalog #**M6630
- NEB Blunt/TA Ligase Master Mix **Contributed by users** **Catalog #**M0367
- NEBNext Quick Ligation Module **New England Biolabs** **Catalog #**E6056S
- Qubit assay tubes **Thermo Fisher Scientific** **Catalog #**Q32856
- Genomic DNA ScreenTape **Agilent Technologies** **Catalog #**5067-5365
- Optical tube strips (8x Strip) **Agilent Technologies** **Catalog #**401428
- Genomic DNA Reagents **Agilent Technologies** **Catalog #**5067-5366
- Qubit™ 1X dsDNA HS Assay Kit **Thermo Fisher** **Catalog #**Q33231
- Optical tube strip caps (8x strip) **Agilent Technologies** **Catalog #**401425
- TapeStation Loading tips **Agilent Technologies** **Catalog #**5067-5598
- PromethION R10.4.1M flow cell **Oxford Nanopore Technologies** **Catalog #**FLO-PRO114M
- ONT Flow Cell Wash Kit **Oxford Nanopore Technologies** **Catalog #**EXP-WSH004
- Flow Cell Wash Kit **Oxford Nanopore Technologies** **Catalog #**EXP-WSH004-XL

Before start

Section 1: DNA QC

Before starting:

- Pre-warm Qubit 1X dsDNA HS reagent to room temp for 00:30:00 prior to using.
- Pre-warm Genomic ScreenTape assay reagents to room temp for 00:30:00 prior to using.

Section 2: T7 debranching

Before starting:

- Thaw 10x NEBuffer 2 On ice, vortex and keep On ice until needed.
- Pre-warm AMPure XP beads at room temperature for 00:30:00 and vortex to thoroughly mix immediately prior to use.
- Prepare custom AMPure buffer as follows, by adding the following and pipette mixing:

	A	B
	Reagent	Volume (ul)
	1 M Tris-HCl pH 8	20
	0.5 M EDTA pH 8	4
	5 M NaCl	640
	PEG 8000 (40% w/v)	550
	Nuclease-free water	778

Section 3: Library preparation

Before starting:

- Thaw all buffers On ice, vortex to mix and ensure there is no precipitate. If precipitate is visible, vortex until it has fully mixed into solution. Keep on ice until needed. Briefly spin tubes in microfuge before opening.
- Keep enzymes and Native Adapter (NA) at -20 °C until needed. Just before use, transfer enzymes to Benchtop Cooler. Mix enzymes by very gently flicking the tubes, spin tubes briefly in microfuge and keep in Benchtop Cooler.
- Pre-warm AMPure XP beads at room temperature for 00:30:00 and vortex immediately prior to using.
- Thaw NBD114.96 barcode plate (or NDB114.24 barcode tubes) at room temperature. Centrifuge plate or tubes before opening and prepare 1.25 ul aliquots of individual barcodes in 0.2 ml PCR tubes and keep on ice.
- Thaw NEB Blunt/TA Ligase Master Mix on ice, mix by inversion and keep On ice.
- Thaw Short Fragment Buffer (SFB) at room temperature, vortex, and centrifuge briefly before using.

Section 4: Flow cell loading

Before starting:

- Thaw Flow Cell Tether (FCT), Flow Cell Flush (FCF), Sequencing Buffer (SB) and Library Solution (LIS) at Room temperature. Mix by vortex and centrifuge briefly before use.

Remove PromethION flow cell from 4 °C and equilibrate to Room temperature for 00:20:00 before loading.

Section 1: DNA QC

1

2m 10s

Measure the concentration of each dMDA sample using the Qubit Fluorometer. Mix 1 µL of DNA with 199 µL of 1X dsDNA HS reagent, in a 0.5 ml Qubit tube. Vortex for 00:00:10, spin briefly in a microfuge and incubate at Room temperature for 00:02:00 and measure concentration.

2

(Optional) Assess the DNA integrity and fragment-length profile of each sample using the Agilent TapeStation and Genomic ScreenTape Assay. Use 1 µL of DNA, at a concentration of 10-100 ng/µL for TapeStation analysis.

Section 2: T7 debranching

1h 2m

3

Aliquot 500 ng of each dMDA sample into a 0.2 ml thin-walled PCR tube and make the volume up to 26 µL with nuclease-free water.

4

Prepare the following reaction:

	A	B
	Reagent	Volume (uL)
	Nuclease-free water	26-x
	Template DNA	x
	10x NEBuffer 2	3
	T7 endonulcease I (10 U/uL)	1
	Total volume	30

5

1h

Incubate reactions in a thermal cycler at 37 °C for 01:00:00

6

Prepare **custom SPRI beads**, by replacing the buffer of AMPure XP beads with custom AMPure XP buffer (according to the ONT protocol: Ligation sequencing gDNA – whole genome amplification (SQK-LSK110) Version: WAL_9115_v110_revH_10Nov2020).

6.1 Mix AMPure XP beads by vortexing thoroughly and transfer two 1 ml aliquots into two 1.5 ml Eppendorf LoBind tubes.

6.2 Place tubes in a magnetic rack for 00:02:00, to pellet the beads and then remove the supernatant. 2m

6.3 Remove the tubes from the magnet and resuspend the beads by adding 1 mL of nuclease-free water and vortexing, then return the tubes to the magnet rack.

6.4 Repeat the above wash step, for a total of 2 washes.

6.5 Briefly centrifuge the tubes, return to the magnet, and use a P20 pipette to remove any remaining water.

6.6 Resuspend and pool the two pellets in 200 µL of custom AMPure buffer, then transfer the resuspended beads to the 2 ml tube containing the remaining custom AMPure buffer.

6.7 Vortex thoroughly and make sure beads are thoroughly mixed again just before use.

7 Adjust debranching reactions to 50 µL using TE buffer (pH 8), add 35 µL of **custom SPRI beads** and incubate on a Hula-Mixer at 9 rpm, 00:20:00. 20m

8 Pellet beads on a magnetic rack for 00:02:00, or until beads are completely separated from solution and remove the supernatant. 2m

9 30s

Leave tubes on the magnet and wash the pellet by carefully adding 200 µL of 70 % ethanol. Do not disturb the bead pellet and remove ethanol after 00:00:30 .

10 Repeat the above wash step, for a total of two washes.

11 Briefly centrifuge the samples, return to the magnet, and use a P20 pipette to remove any remaining ethanol.

12 Air-dry pellet for up to 00:02:00 . 2m

13 To elute DNA from the beads, resuspend the pellet by adding 20 µL of nuclease-free water and flicking the tubes to thoroughly resuspend the beads.

14 Briefly spin the tubes in a microfuge for 1-2 s.

15 Incubate in a thermal cycler at 50 °C for 00:01:00 . 1m

16 Incubate at Room temperature for 00:09:00 . 9m

17 Pellet beads on a magnetic rack for 00:02:00 . 2m

18 Transfer the supernatant, containing eluted DNA, to a new 0.2 ml thin-walled PCR tube.

19 Quantify 1 µL of eluted DNA by Qubit.

Section 3: Library Preparation

56m 30s

20

The following library preparation steps follow the protocol Ligation sequencing gDNA – Native Barcoding Kit 24 V14; Version: NBE_9169_v114_revQ_15Sep2022, with some modifications. For each sample, use all recovered debranched dMDA DNA from step 18.

End-prep:

Prepare the following reaction for each sample, in a 0.2 ml thin-walled PCR tube:

	A	B
	Reagent	Volume (uL)
	Nuclease-free water	5
	Debranched dMDA sample	19
	NEBNext FFPE DNA Repair Buffer	1.75
	Ultra II End-prep Reaction Buffer	1.75
	Ultra II End-prep Enzyme Mix	1.5
	NEBNext FFPE DNA Repair Mix	1
	Total volume	30

21

Mix samples by pipetting and briefly centrifuge.

22

35m

Incubate reactions in a thermal cycler at 20 °C for 00:30:00, then 65 °C for 00:05:00.

23

Mix AMPure XP beads by vortexing and add 60 µL to each reaction.

24

Mix the AMPure beads with the reaction solution by thoroughly flicking and inverting the tubes.

25

5m

Incubate at Room temperature for 00:05:00.

26 2m

Briefly centrifuge the tubes then place on a magnetic rack for ~ 00:02:00 , or until the beads have completely pelleted.

27

Remove the supernatant.

28 30s

Leave tubes on the magnet and wash the pellet by carefully adding 200 µL of 80 % ethanol. Do not disturb the bead pellet and remove ethanol after 00:00:30 .

29

Repeat the above wash step, for a total of two washes.

30

Briefly centrifuge the samples, return to the magnet, and use a P20 pipette to remove any remaining ethanol.

31 2m

Air-dry pellet for up to 00:02:00 .

32 10m

To elute DNA from the beads, remove tubes from the magnetic rack and resuspend the pellets by adding 23 µL of nuclease-free water and flicking the tubes to thoroughly resuspend the beads. Briefly spin the tubes in a microfuge for 1-2 s and incubate in a thermal cycler at 37 °C for 00:10:00 .

33 2m

Pellet beads on a magnetic rack for 00:02:00 and then transfer 22.5 µL of supernatant, containing eluted DNA, to a new 0.2 ml thin-walled PCR tube.

34 **Barcode Adapter Ligation:**

Prepare the following barcode adapter ligations at Room temperature

	A	B
	Reagent	Volume (uL)

	A	B
	End-prepped DNA	22.5
	Native Barcode (NB01-24)	2.5
	Blunt/TA Ligase Master Mix	25
	Total volume	50

35 Gently pipette mix to thoroughly mix reactions and briefly centrifuge.

36 Incubate at Room temperature for 00:30:00 . 30m

37 Mix AMPure XP beads by vortexing and add 100 µL to each reaction.

38 Mix the AMPure beads with the reaction solution by thoroughly flicking and inverting the tubes and incubate at Room temperature for 00:05:00 . 5m

39 Briefly centrifuge the tubes then place on a magnetic rack for ~ 00:02:00 , or until the beads have completely pelleted. 2m

40 Remove the supernatant.

41 Leave tubes on the magnet and wash the pellet by carefully adding 200 µL of 80 % ethanol. Do not disturb the bead pellet and remove ethanol after 00:00:30 . 30s

42 Repeat the above wash step, for a total of two washes.

43 Briefly centrifuge the samples, return to the magnet, and use a P20 pipette to remove any remaining ethanol.

44 Air-dry pellet for up to 00:02:00 . 2m

45 To elute DNA from the beads, remove tubes from the magnetic rack and resuspend the pellets by adding 16 µL of nuclease-free water and flicking the tubes to thoroughly resuspend the beads. Briefly spin the tubes in a microfuge for 1-2 s and incubate in a thermal cycler at 37 °C for 00:10:00 . 10m

46 Pellet beads on a magnetic rack for 00:02:00 and then transfer the supernatant, containing eluted DNA, to a new 0.2 ml thin-walled PCR tube. 2m

47 Quantify 1 µL of eluted DNA by Qubit.

48 **Sequencing Adapter Ligation:**
Combine barcoded samples in equal amounts, in a 1.5 ml LoBind tube, and use the pooled sample to prepare the below reaction. Add reagents in the listed order:

	A	B
	Reagent	Volume (uL)
	Barcoded sample pool	30
	Native Adapter	5
	NEBNext Quick Ligation Reaction Buffer (5x)	10
	Quick T4 DNA Ligase	5
	Total volume	50

49 Incubate ligation reaction at Room temperature for 00:30:00 . 30m

50 10m

Add 100 µL of resuspended AMPure beads and incubate on a HulaMixer for 00:10:00 at 9 rpm .

51 2m

Briefly centrifuge the tube then place on a magnetic rack for ~ 00:02:00 , or until the beads have completely pelleted.

52

Remove the supernatant.

53

Remove tube from the magnetic stand and wash the beads by adding 125 µL of Short Fragment Buffer (SF) and thoroughly resuspend beads by flicking the tube.

54 2m

Return tube to the magnetic stand and pellet beads for ~ 00:02:00 .

55

Repeat the above wash steps, for a total of two washes.

56

Briefly centrifuge the samples, return to the magnet, and use a P20 pipette to remove any remaining SFB.

57 30s

Air-dry pellet for up to 00:00:30 .

58

To elute DNA from the beads, remove the tube from the magnetic rack and resuspend the pellet by adding 25 µL of Elution Buffer (EB) and flicking the tube to thoroughly resuspend the beads.

59 10m

Briefly spin the tube in a microfuge for 1-2 s and incubate in a heat block or water bath at 37 °C for 00:10:00 .

60 2m

Pellet beads on a magnetic rack for 00:02:00 and then transfer the supernatant, containing eluted DNA, to a new 1.5 ml LoBind tube.

61

Quantify 1 µL of eluted DNA by Qubit.

Section 4: Flow cell loading 3d

62

Prepare and load PromethION flow cell according to the protocol: **Ligation sequencing gDNA – Native Barcoding Kit 24 V14; Version: NBE_9169_v114_revQ_15Sep2022**.

63

Start run in MinKNOW using default parameters for the NBD114.24 or NBD114.96 library prep kit.

64 3d

Monitor number of available pores during the run and perform nuclease flush and reloads as needed, using the Flow Cell Wash Kit (EXP-WSH004 or EXP-WSH004-XL) and the protocol: **Flow Cell Wash Kit (EXP-WSH004 or EXP-WSH004-XL) Version: WFC_9120_v1_revQ_08Dec2020**. A 72:00:00 run will normally require 1-2 flush and reloads to maximise the output of the flow cell.

Protocol references

Ester Kalef-Ezra, Diego Perez-Rodriguez, Christos Proukakis 2023. Manual isolation of nuclei from human brain using CellRaft device and single nucleus Whole Genome Amplification. **protocols.io**https://dx.doi.org/10.17504/protocols.io.kxygxzjjov8j/v1