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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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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

	A	B	C	D	E
	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 **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 (μL)
Nuclease-free water	26-x
Template DNA	x
10x NEBuffer 2	3
T7 endonulcease I (10 U/ μL)	1
Total volume	30

5

1h

Incubate reactions in a thermal cycler at  37 $^{\circ}\text{C}$ 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 μ L 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 μ L using TE buffer (pH 8), add  35 μ L 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

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 .

30s



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 $^{\circ}\text{C}$ 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 (μL)
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

Incubate reactions in a thermal cycler at  20 $^{\circ}\text{C}$ for  00:30:00 , then  65 $^{\circ}\text{C}$ for  00:05:00 .

35m



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 Incubate at  Room temperature for  00:05:00 . 

- 26 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 Leave tubes on the magnet and wash the pellet by carefully adding  200 μ L 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 Air-dry pellet for up to  00:02:00 . 
- 32 To elute DNA from the beads, remove tubes from the magnetic rack and resuspend the pellets by adding  23 μ L 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

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.

2m



34 Barcode Adapter Ligation:

Prepare the following barcode adapter ligations at  Room temperature

A	B
Reagent	Volume (μL)
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 $^{\circ}\text{C}$ 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

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

10m



51

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

2m



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

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

2m

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 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 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 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 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>