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Fiber-seq for flash frozen mouse tissue for IGVF

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IGVF

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

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Abstract

This protocol describes Fiber-seq experiment for frozen mouse tissue and has been optimized from the following protocols:
Nuclei extraction: Protocol to isolate and fix nuclei from flash frozen mouse left cortex and hippocampus for IGVF, Version 2.1 (January 2023)
Labelling: HPRC Fiber-seq protocol on DMSO frozen LCL cells, (March 2024)
DNA extraction: Monarch® HMW DNA Extraction Kit for Tissue (NEB #T3060S/L), Version: 2.1_4/21

Guidelines

Try working quickly to help maintain nuclei integrity and prevent over labelling.

Materials

Reagents

	Name	Manufacturer	Cat #
	PBS	HyClone	SH30256
	7.5% BSA	Life Technologies	15260037
	Monarch® HMW DNA Extraction Kit for Tissue	New England Biolabs	T3060
	EcoGII Methyltransferase	New England Biolabs	M0603S
	Nuclei Extraction Buffer	Miltenyi Biotec	130-128-024
	Spermidine	Thermo Scientific	A19096
	SDS, 20% Solution	Thermo Scientific	AM9820

Supples and Equipment

	Name	Manufacturer	Cat #
	gentleMACS™ C Tube	Miltenyi Biotec	130-093-237
	gentleMACS™ Octo Dissociator	Miltenyi Biotec	130-095-937
	gentleMACS™ Octo Coolers	Miltenyi Biotec	130-130-533
	MACS SmartStrainers (70 um)	Miltenyi Biotec	130-110-916
	MACS SmartStrainers (30 um)	Miltenyi Biotec	130-098-458
	Eppendorf ThermoMixer® C	Eppendorf	-
	Eppendorf SmartBlock 2.0 mL	Eppendorf	5362000035

Set Up

1 Make buffers and solutions

- **Buffer A**

	Final Concentration	Stock Concentration	Volume stock soln
	Nuclease-free H2O	100%	95.8 mL
	15 mM Tris-Cl, pH 8.0	1 M Tris-Cl, pH 8.0	1.5 mL
	15 mM NaCl	5 M NaCl	300 µL
	60 mM KCl	3 M KCl	2 mL
	1 mM EDTA, pH 8.0	0.5 M EDTA, pH 8.0	200 µL
	0.5 mM EGTA, pH 8.0	0.5 M EGTA, pH 8.0	100 µL

This buffer is used in the labelling reaction and is table at room temperature.

- [M] 0.5 Mass Percent **Spermidine** and store at 4 °C
- [M] 20 % (v/v) **Sodium Dodecyl Sulfate (SDS)** and store at Room temperature

2 Pre-chill centrifuge to 4 °C

3 Set two thermomixers at 56 °C and 25 °C

4 Prepare and label the following tubes for DNA extraction (for each labeling reaction):
- Bead strainer in a collection tube
- 2 mL tube for wash step
- 2 mL tube for elution step
- 1.5 tube for final elution

Add 150 µL of **gDNA Elution Buffer II** to the 2 mL elution tubes

5 Prepare an ice bucket and keep the following on ice

- Proteinase k and RNAse A
- Pre-chill **gentleMACS C Tube** and two **5 mL tubes**
- [M] 0.5 Mass Percent Spermidine

Nuclei Extraction

6 Make enough RSB for the total number of samples.

	Reagent	Final conc.	1	2	3	4
	PBS		3.45 mL	6.41 mL	9.37 mL	12.33 mL
	7.5% BSA	0.1%	46.67 µL	86.67 µL	126.67 µL	166.67 µL

Or a large stock can be stored at 4°C for a month.

7 Keep flash frozen tissue samples on dry ice until lysis.

8 Add 2 mL **Nuclei Extraction Buffer** to a chilled gentleMACS C Tube.

9 Drop whole frozen tissue into the chilled gentleMACS C Tube with 2 mL Nuclei Extraction Buffer.

10 Invert ***immediately***, ensuring tissue is not stuck to the bottom or side.
Proceed ***immediately*** to load onto Octo Dissociator and add Octo Cooler over each gentleMACS C Tube.

11 Run the gentleMACS Program **4C_nuclei_1** on the Octo Dissociator (00:05:00). 5m

12 Remove the gentleMACS C Tube and ensure tissue did not get stuck on the sides by tapping the tube.

13 Spin down in 4 °C centrifuge for 00:00:10 . 10s

14 Mix the nuclei suspension thoroughly by pipetting with a 5 mL pipet.

15 Filter nuclei suspension through **70 µm MACS SmartStrainer** into a 5 mL tube.

16 Wash empty, used gentleMACS C Tube with an additional 2 mL **Nuclei Extraction Buffer**. Close the cap and swish to recover any nuclei stuck to the sides.

17 Filter the 2 mL additional Nuclei Extraction Buffer through the 70 µm MACS SmartStrainer into the same 5 mL tube (total volume now 4 mL).

18 Centrifuge the 4 mL nuclei suspension at 350 x g, 4°C, 00:05:00 . 5m

19 Remove the supernatant without disrupting the pellet.

20 Resuspend nuclei pellet in 3 mL **RSB.**

21 Filter nuclei suspension through **30 µm MACS SmartStrainer** into a new 5 mL tube.

22 Make aliquots of 5 million nuclei in 1.5 mL tubes.

Labelling

23 Spin 5 million nuclei at 250 x g, 4°C, 00:05:00 . 5m

24 During the 5 min, mix the following and keep Reaction Master Mix On ice

Buffer A + Spermidine Room temperature

	Number of tubes	1	2	3	4
	Buffer A	500 µL	750 µL	1 mL	2 mL

	Number of tubes	1	2	3	4
	0.5 M Spermidine	0.5 µL	0.75 µL	1 µL	2 µL

This is made fresh on the day of the experiment.

Reaction Master Mix (75 U) On ice

	Number of tubes (+0.2 extra)	1	2	3	4
	Buffer A + Spermidine	277.5 µL	610.5 µL	888 µL	1,165.5 µL
	EcoGII Methyltransferase (200 U)	15 µL	33 µL	48 µL	63 µL
	32 mM SAM	7.5 µL	16.5 µL	24 µL	31.5 µL

This reaction uses 75 units of enzyme and 160 µM SAM with a total reaction volume of 300 µL.

25 Remove supernatant and resuspend the pellet in 300 µL of **Reaction Master Mix** buffer.

26 Incubate on 25 °C heat block for 00:10:00 . 10m

27 Add 15 µL of **20% SDS** to each sample (final concentration of 1%)

28 Thoroughly mix by gently inverting tube. Do not vortex or pipette to mix.

DNA extraction 10m

29 Add 20 µL **Proteinase k** and 10 µL **RNAse A** to each tube

30 Mix gently by pipetting using wide-bore tip.

31 Incubate in a thermal mixer at 2000 rpm, 56°C, 00:10:00 . 10m

32 Add 300 µL **Protein Separation solution**.

33 Mix on vertical rotating mixer for 20 rpm, 00:01:00 . 1m

34 Centrifuge for 16000 x g, 00:10:00 . 10m

35 Using a wide-bore tip, transfer as much of the upper phase containing the DNA to a labeled, empty 2 mL tube without disrupting the pellet at the bottom.

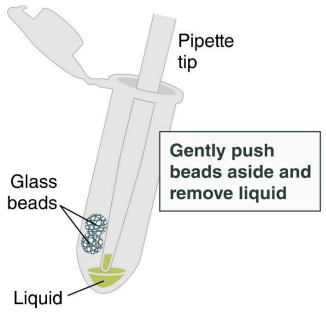
36 Add **2 DNA capture beads** to each sample.

37 Add 425 µL of **isopropanol.**

38 Mix on a vertical rotating mixer at 10 rpm, 00:10:00 to attach DNA to the beads.

39 During the wash steps, work quickly to prevent DNA from drying out on beads!

40 Discard liquid by keeping tube upright, insert pipette tip and gently push beads aside to remove liquid.



41 Add 500 µL **Wash Buffer**.

42 Mix by gently inverting the tube 3 times.

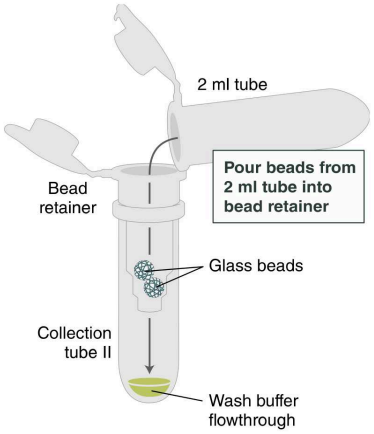
43 Discard liquid by keeping tube upright, insert pipette tip and gently push beads aside to remove liquid.

44 Add 500 µL **Wash Buffer**.

45 Mix by gently inverting the tube 3 times.

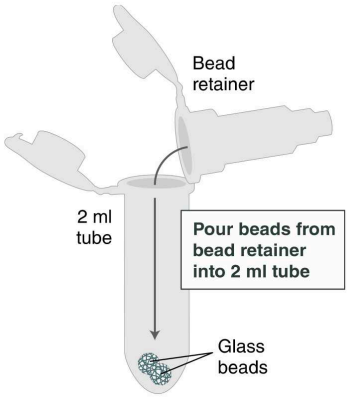
46 Discard liquid by keeping tube upright, insert pipette tip and gently push beads aside to remove liquid.

47 Pour the beads into the bead retainer that is in a collection tube.

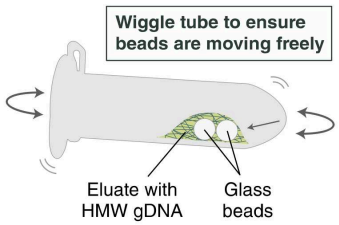


48 Pulse spin (≤ 1 second) the tubes to remove any residual wash buffer from the beads.

49 Separate the bead retainer from the collection tube and pour the beads into the labeled tube with 150 µL of elution buffer and save the bead retainer for a future step.

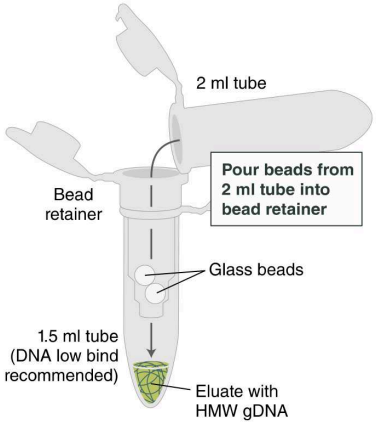


50 Incubate on thermal mixer at 56 °C for 00:10:00 and during incubation, make sure all sides of the beads are exposed to elution buffer by tilting the tube almost horizontally and gently shaking after the first 5 min. 10m



51 Incubate at Room temperature for 01:00:00 or 4 °C Overnight for the DNA to homogenize . 1h 10m

52 Pour the eluate and the glass beads into the bead retainer that is placed in the final labeled tube.



53 Centrifuge for 12000 rpm, 00:00:30 to separate the eluate from the glass beads. 30s

54 Mix gently by pipetting using wide-bore tip.

55 Qubit each tubes and keep on ice until library prep or store in -20°C

Library building

56 Follow "ONT Library Prep for Whole Genome Sequencing" protocol to build libraries for whole genome sequencing using Oxford Nanopore platform.