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Midbrain sample preparation for 10xGenomics Multiome Protocol

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We use this protocol and it's working

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Abstract

This protocol describes the processing of human frozen postmortem midbrain tissue for isolating single nuclei. The nuclei are purified and sorted and subjected to 10X single-nuclei multiome library generation and sequencing (RNAseq + ATAC-seq). Four samples can be processed simultaneously with this protocol.

Materials

1. 20X Nuclei Buffer (10x Genomics, 2000207, Supplied in Multiome Kit), -20°C
2. Sigma Protector RNase Inhibitor (Sigma,3335402001), -20°C
3. Digitonin (Thermo Fisher, BN2006), 4°C, incubate at 65 °C before use
4. 1000mM DTT (Sigma, 43816-10ml), 4°C, freshly dilute to 100mM with water
5. 10% MACS BSA Stock Solution (Miltenyi Biotec, 130-091-376), 4°C
6. 1mg/ml 7-ADD Solution (Invitrogen, A1310)
7. RNase-Free Disposable Pellet Pestles (Fisher Scientific, 12-141-368), RT
8. DPBS (Gibco, 14190-144), RT
9. Sodium Chloride (Fisher, S671), RT
10. Magnesium Chloride (Sigma, M9272), RT
11. 1M pH7.4 Trizma Hydrochloride Solution (Sigma, T2196), RT
12. 100% Nonidet P40 Substitute (Sigma, 74385), RT, dilute to 10% with water
13. 10% Tween 20 (Bio-Rad, 1662404), RT
14. 70µm Nylon Mesh (Fisher, 22363548), RT
15. 40µm Flowmi Cell Strainer (Bel-Art, 136800040), RT
16. Wide bore tips (Thermo Scientific, 2079G), RT

	A	B	C
	All Buffer Amounts Sufficient for Processing 4 Samples		
	NP40 Lysis Buffer (NPLB)		
		Stock	**Amount**
	pH 7.4 Tris-HCl	1M	57µl
	NaCl	1M	57µl
	MgCl2	1M	17.1µl
	Nonidet P40 Substitute	10% (Diluted from 100% with water before use)	57µl
	DTT	100mM (Diluted from 1M with water before use)	57µl
	RNase Inhibitor	40U/µl	142.5µl
	Nuclease-free Water		5.31ml
	Total		**5.7mL**

	A	B	C
	PBS Buffer (PBSB)		
		Stock	**Amount**
	BSA	10%	1ml
	RNase Inhibitor	40U/µl	250µl
	DPBS		8.75ml
	Total		**10ml**
	1X Lysis Buffer (LB)		
		Stock	**Amount**
	pH 7.4 Tris-HCl	1M	10µl
	NaCl	1M	10µl
	MgCl2	1M	3µl
	Tween-20	10%	10µl
	Nonidet P40 Substitute	10% (Diluted from 100% with water before use)	10µl
	Digitonin (Incubate at 65°C before use)		2µl
	BSA	10%	100µl
	DTT	100mM (Diluted from 1M with water before use)	10µl
	Nuclease-free Water		845µl
	Total		**1ml**
	Lysis Dilution Buffer (DLB)		
		Stock	**Amount**
	pH 7.4 Tris-HCl	1M	10µl
	NaCl	1M	10µl

	A	B	C
	MgCl2	1M	3µl
	BSA	10%	100µl
	DTT	100mM (Diluted from 1M with water before use)	10µl
	Nuclease-free Water		867µl
	Total		**1ml**
	0.1X Lysis Buffer (0.1XLB)		
		Stock	**Amount**
	LB		44µl
	Rnase Inhibitor	40U/µl	11µl
	DLB		385µl
	Total		**440µl**
	Wash Buffer (WB)		
		Stock	**Amount**
	pH 7.4 Tris-HCl	1M	22µl
	NaCl	1M	22µl
	MgCl2	1M	6.6µl
	BSA	10%	220µl
	Tween-20	10%	22µl
	DTT	100mM (Diluted from 1M with water before use)	22µl
	Rnase Inhibitor	40U/µl	55µl
	Nuclease-free Water		1.83ml
	Total		**2.2ml**

	A	B	C
	Diluted Nuclei Buffer (DNB)		
		Stock	**Amount**
	20X Nuclei Buffer		5µl
	DTT	100mM (Diluted from 1M with water before use)	1µl
	Rnase Inhibitor	40U/µl	2.5µl
	Nuclease-free Water		91.5µl
	Total		**100µl**

Protocol materials

7-AAD (7-Aminoactinomycin D) **Thermo Fisher Catalog #**A1310

Troubleshooting

Cryosectioning

1 Frozen Brain Samples were received from the brain bank - Four 50µm sections were collected in a 1.5 ml microcentrifuge tube and shipped on dry ice to the Lab.

Prepare Buffers and Equipment

2 All buffers are prepared the day of the experiment. DTT and RNAse inhibitor are added to the buffers immediately before using. Prepare & maintain all buffers On ice . See all buffer recipes in the Materials tab.

3 Pre-cool the microcentrifuge to 4 °C

Nuclei Isolation and Sorting 25m

4 Add 650 µL **NPLB** to the tube with tissue and homogenize 15x using a pestle On ice .

5 Add 650 µL **NPLB** to the tube and incubate for 00:05:00 On ice , pipette mix a few times during incubation with wide bore tips (use in step 4, 5 and 6). 5m

6 Pass the suspension through a 70 µm Nylon Mesh into a pre-chilled 50 ml Falcon tube On ice .

7 Transfer the filtered lysate to a pre-chilled Lo-Bind 2 ml tube On ice .

8 Centrifuge at 500 rcf, 4°C, 00:05:00 . 5m

9 Remove most of the supernatant, leaving around 50 µL .

10 Add 1 mL **PBSB**, DO NOT mix. Incubate for 00:05:00 On ice .

11 Pipette mix to resuspend the pellet

12 Centrifuge at 500 rcf, 4°C, 00:05:00 . 5m

13 Remove most of the supernatant, leaving around 50 µL .

14 Resuspend with 1.4 mL **PBSB**.

15 Add 1.4 µL 7-AAD (7-Aminoactinomycin D) **Thermo Fisher Catalog #**A1310 to the 1.4 mL sample, mix by inverting.

16 Incubate for 00:05:00 On ice . 5m

17 Pass the suspension through a 40 µm FlowMi strainer into a pre-chilled 5 ml FACS tube On ice :

17.1 Aspirate 700 µL of the suspension into a regular 1 mL pipette tip. Add the FlowMi filter to the end of the tip. Press the plunger slowly to dispense into the new tube. Discard tip and filter.

17.2 Repeat step 17.1 one additional time for the remaining volume.

18 Pre-load 1.5 ml microcentrifuge tubes with 15 µL **PBSB** for nuclei collection. Maintain On ice .

19 Sort nuclei using a 100 µm nozzle on BD FACSAria or similar. Collect sorted nuclei in chilled, pre-loaded 1.5 ml microcentrifuge tubes.

Note

Gate first by SSC and FSC to remove doublets. Then, **collect 7-AAD positive events - these are nuclei.**

Nuclei Permeabilization

12m

20 Centrifuge sorted nuclei at 500 rcf, 4°C, 00:05:00 5m

21 Remove the supernatant without disrupting the nuclei pellet.

22 Resuspend the pellet in 100 µL **0.1XLB** and pipette mix 5x.

23 Incubate for 00:02:00 On ice . 2m

24 Add 500 µL **WB** and pipette mix 5x.

25 Centrifuge at 500 rcf, 4°C, 00:05:00 . 5m

26 Remove the supernatant without disrupting the nuclei pellet.

27 Based on the nuclei count post-sorting & targeted nuclei recovery, resuspend in chilled **DNB**.

Note

See table in Step 1.1 of the 10X Multiome User Guide (Rev E) for help with calculating **DNB** volume.

Note

Based on empirical data, actual nuclei counts are approximately 1/3rd of collected FANS events.

28 Proceed immediately to Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide (CG000338 Rev E, page 30). Library preparation and sequencing for both RNAseq + ATAC-seq is performed follwing strictly 10x Genomics protocol (CG000338 Rev E).