Imperial College London

MULTI-'OMICS ATLAS PROJECT

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Nuclei Isolation Method for 10x Single Cell Multiome ATAC + Gene Expression Sequencing (80µm sectioned frozen human brain tissue)

Adapted from 10x Genomics

I. Preparation

Can be prepared ahead of time:

- 29% and 50% Iodixanol (Nature protocol above bench)

Preparation:

- Chill centrifuge (4°C)
- Clean RNA zap workspace (+UV dounces, pipettes and chip cover)
- Shake BSA coated tubes @RT
- Digitonin at 65°C for 15 mins to dissolve precipitate before making 1X Lysis Buffer.

To be made fresh (on ice) before starting:

- NP40 Lysis Buffer
- BSA Buffer

To be made fresh (on ice) during the last FACS sort:

- Lysis Dilution Buffer
- 1X Lysis Buffer
- 0.1X Lysis Buffer
- Wash Buffer
- Diluted nuclei buffer

II. Notes

- Before making diluted nuclei buffer take nuclei buffer (20X) out of -20 and let equilibrate to RT.
- Transposition mix from Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide (CG000338) needs to be made at a similar time as the diluted nuclei buffer.

LYSIS DILUTION BUFFER	**Volume (µL)**	**Final Concentration**
Tris-HCl (pH 7.4)	10	10mM
NaCl 5M	2	10mM
$MgCl_2$ 1M	3	3mM
MACS BSA 10%	100	1%
DTT	1	1mM
RNase Inhibitor 40U/ul	25	1 U/µL

Nuclease-Free Water	859	-
Total volume	1mL	-

WASH BUFFER	**Volume (µL) 1 sample**	**Volume (µL) 2 samples**	**Volume (µL) 3 samples**	**Volume (µL) 4 samples**	**Final Concentration**
Tris-HCl (pH 7.4)	12	24	36	48	10mM
NaCl 5M	2.4	4.8	7.2	9.6	10mM
$MgCl_2$ 1M	3.6	7.2	10.8	14.4	3mM
MACS BSA 10%	120	240	360	480	1%
Tween-20	12	24	36	48	0.1%
DTT	1.2	2.4	3.6	4.8	1mM
RNase Inhibitor 40U/ul	30	60	90	120	1U/µL
Nuclease-free water	1020	2040	3060	4090	-
Total Volume	1.2 mL	2.4mL	3.6mL	4.8mL	-

1X LYSIS BUFFER	**Volume (µL)**	**Final Concentration**
Tris-HCl (pH 7.4)	1	10mM
NaCl 5M	0.2	10mM
$MgCl_2$ 1M	0.3	3mM
Tween-20 1M	1	0.1%
Nonidet P40 Substitute (10%)	1	0.1%
Digitonin 5% *(incubate 65°C to dissolve precipitate before use)*	0.2	0.01%
MACS BSA 10%	10	1%
DTT	0.1	1mM
RNase Inhibitor 40U/ul	2.5	1 U/µM
Nuclease-free water	83.5	-
Total volume	100	-

0.1X LYSIS BUFFER	**Volume (µL) 1 sample**	**Volume (µL) 2 samples**	**Volume (µL) 3 samples**	**Volume (µL) 4 samples**	**Final Concentration**
1X Lysis Buffer	11	22	33	44	0.1X
Lysis Dilution Buffer	99	198	297	384	-
Total Volume	110µL	220µL	330µL	428µL	-

NP40 LYSIS BUFFER	**Volume (µL) 1 sample**	**Volume (µL) 2 samples**	**Volume (µL) 3 samples**	**Volume (µL) 4 samples**	**Final Concentration**

Tris-HCl (pH 7.4)	15	30	45	60	10mM
NaCl 5ML	3	6	9	12	10mM
$MgCl_2$ 1M	4.5	9	13.5	18	3mM
Nonidet P40 Substitute (10%)	15	30	45	60	0.1%
DTT	1.5	3	4.5	6	1mM
RNase inhibitor 40U/ul	37.5	75	112.5	150	1 U/µM
Nuclease-free water	1425	2850	4275	5700	-
Total Volume	1500	3000	4500	6000	-

BSA BUFFER (PBS+ 1% BSA+ 1U/ µL RNase Inhibitor)	**Volume (µL) 1 sample**	**Volume (µL) 2 samples**	**Volume (µL) 3 samples**	**Volume (µL) 4 samples**	**Final Concentration**
MACS BSA 10%	176	330	506	660	1%
RNase Inhibitor	44	82.5	126.5	165	1U/ µL
PBS (pH 7.4)	1540	2887	4427	5774	-
Total Volume	1.76mL	3.3mL	5mL	6.6mL	-

DILUTED NUCLEI BUFFER	**Volume (µL) 5-6 samples**	**Final Concentration**
Nuclei Buffer (20X)	5	1x
DTT	0.1	1mM
RNase inhibitor 40U/ul	2.5	1 U/µM
Nuclease-free water	92.4	-
Total Volume	100µL	-

III. Nuclei Isolation

In Hood(Microbiological Safety Cabinet)

a. Place a pre-labelled Eppendorf on ice.

b. On ice, add 700µL NP40 Lysis Buffer to tissue sample.

c. Pipette tissue, transfer to 2mL dounce.

d. Rinse tube with 100µL NP40 Lysis Buffer and add to the dounce.

e. 5 strokes pestle A

f. 10 strokes pestle B

g. Transfer homogenate to the labelled Eppendorf.

h. Add 500µL NP40 Lysis Buffer to the tube

i. Incubate for 5 mins on ice (Pipette mix a few times during incubation)

Out of Hood

j. Centrifuge at 1,000rcf for 8 mins at 4°C.

-During this time: Add 500µl 29% iodixanol to new Eppendorfs (2 Eppendorfs per sample). Add 500µl 50% iodixanol to a 50mL falcon. Place 70µm filter on top of 50mL falcon.

k. Remove most of the supernatant.

l. Add 500 ul BSA Buffer (**DO NOT MIX**).

m. Incubate for 5mins on ice.

n. Pipette mix to resuspend the pellet and filter through 70µm filter cap.

o. Pipette to mix 50% iodixanol and cell suspension (total volume 1000ul)

p. Layer ½ (500ul) of the nuclei/sucrose mix on top of each of the 2 tubes with 29% iodixanol.

n. Centrifuge 13,000g for 40min at 4^0c

o. After centrifugation, remove the supernatant, starting at the top.

p. Resuspend with 1 ml BSA Buffer, FACS filter (combining the 2 individual sample tubes).

q. Pipette mix and FACS filter again (total of 2 times).

r. Take 10µL nuclei from FACS tube, load onto cell imaging slide. View on microscope (EVOS 20X) to assess debris and clumping.

IV. Staining and FACS

a. Add 10µL 7-AAD ready-made solution per 1ml sample stepwise during FACS.

b. Incubate on ice for 20 mins.

d. FACS Sort into 5mL FACS tubes coated with 100µL BSA Buffer.

e. Split into 1.5mL tubes.

V. Nuclei Permeabilization

a. Centrifuge at 500 rcf for 5 min at 4°C.

b. Remove most of the supernatant and resuspend each sample in 100µL 0.1X Lysis Buffer while combining individual tubes.

c. Pipette mix 5x and incubate for 2 mins on ice.

d. Add 1mL Wash Buffer and pipette mix 10x.

e. Centrifuge at 500rcf for 5mins at 4°C.

f. Remove the supernatant without disrupting the pellet. Leave 30ul cell nuclei suspension.

g. Count cells on Luna Cell counter. In a tube strip prepare for each sample:

-1µl Propidium Iodide stain

-9µl nuclei suspension

-Transfer 10ul stained nuclei to a Cell Counting Slide chamber.

- Save each count - take USB and save data to PC

- Calculate: # cells/1000 = cells per µL. (we want 7000 cells recovery)

i. Proceed immediately to Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide (CG000338).

j. After getting samples in PCR for Isothermal Incubation, take 10ul nuclei and load on slide + coverslip. View on EVOS 40X and save 1-2 pictures per sample.

APPENDIX

I. NUCLEI CONCENTRATION GUIDELINES

Nuclei Concentration Guidelines

Based on the Targeted Nuclei Recovery, resuspend the nuclei in Diluted Nuclei Buffer to get corresponding Nuclei Stock Concentrations (see Table). This enables pipetting volumes of the Nuclei Stock for Transposition (step 1.1) to be 2-5 µl. Higher Nuclei Stock Concentrations will result in lower pipetting volumes that may increase nuclei input variability.

Targeted Nuclei Recovery	Nuclei Stock Concentration (nuclei/µl)
500	160-400
1,000	320-810
2,000	650-1,610
3,000	970-2,420
4,000	1,290-3,230
5,000	1,610-4,030
6,000	1,940-4,840
7,000	2,260-5,650
8,000	2,580-6,450
9,000	2,900-7,260
10,000	3,230-8,060

Calculate volume of Nuclei Stock and Diluted Nuclei Buffer for a total volume of 5 µl

$$\text{Volume of Nuclei Stock (µl)} = \frac{\text{Targeted Nuclei Recovery x 1.61 (Recovery efficiency factor)}}{\text{Nuclei Stock Concentration (nuclei/ µl)}}$$

Volume of Diluted Nuclei Buffer* (µl) = **5 µl** - volume of Nuclei Stock (µl)
*Use ONLY Diluted Nuclei Buffer (Dilute 20X Nuclei Buffer (PN-2000207) 1:20 in nuclease-free water)

Example Calculation

Targeted Nuclei Recovery = 4000 nuclei
Nuclei Stock Concentration = 2500 nuclei/µl
Recovery efficiency factor 1.61

Volume of Nuclei Stock (µl) =

$$\frac{\text{Targeted Nuclei Recovery x 1.61 (Recovery efficiency factor)}}{\text{Nuclei Stock Concentration (nuclei/µl)}} = \frac{4000 \times 1.61}{2500} = \mathbf{2.58\ µl}$$

Volume of Diluted Nuclei Buffer = 5 µl - 2.58 µl = **2.42 µl**

Add calculated volumes of Diluted Nuclei Buffer and Nuclei Stock to the Transposition Mix in step 1.1

II. BSA COATING OF TUBES

For each sample:

- 6x 1.5ml tubes (500ul 0.5% BSA)- usually we need 5 per sample but a 6th one may be needed depending on the volume of sorted nuclei.
- 1x 50ml Falcon (2ml 0.5% BSA)
- 2x 5ml FACS tubes with filter (500ul 0.5% BSA)
- 1x 5ml FACS tubes no filter (500ul 0.5% BSA)

0.5% BSA Solution

Reagent	Volume (µL) 1 sample	Volume (µL) 3 samples	Final Concentration
PBS (pH 7.4)	7125	21375	
BSA (10%)	375	1125	0.5%
Total	7500	22500	