Nuclei Preparation from Frozen Tissue for 10X Multiome using gentleMACS Homogenization and FANS

Reagents List:

Reagent	Concentration	Vendor	Catalog Number
$CaCl_2$	1M	Thermo Scientific	J63122.AE
EDTA, pH 8.0	0.5M	Invitrogen	AM9260G
$MgCl_2$	1M	Invitrogen	AM9530G
Mg acetate (MgAc)	1M	Thermo Scientific	J60041.AD
Tris-HCl, pH 7.5	1M	Invitrogen	15567-027
Tris-HCl, pH 8.0	1M	Invitrogen	15568-025
DTT (DL-Dithiothreitol)	-	Sigma	D9779-10G
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets	-	Sigma	5056489001
Recombinant RNasin (Ribonuclease Inhibitor), 10000 U	-	Promega	N2515
Molecular biology water	-	Corning	46-000-CV
IGEPAL CA-630	-	Sigma	I8896-50ML
Tween-20	10%	BioRad	1662404
NaCl	5M	Invitrogen	AM9760G
Fatty acid-free BSA	-	Lampire Biological Laboratories	7500804
7-AAD	-	Invitrogen	A1310

PBS	-	Corning	21-040-CV
Trypan Blue	0.4%	Invitrogen	T10282
DMSO (Dimethyl Sulfoxide)	-	MP Biomedicals	ICN19481980

Equipment:

- Sony Cell Sorter (SH800)
- Eppendorf tabletop swing-bucket centrifuge (Eppendorf, 5920R)
- gentleMACS Octo Dissociator with Heaters (Miltenyi Biotec)

Consumables

- Sony Sorting Chip-100 µm for SH800 and MA900 (Sony, LEC3210)
- Thermo Scientific™ NERL™ Diluent 2 Hematology Reagent for Flow Cytometry (Fisher Scientific, 23-029-361)
- 30 µm CellTrics (Fisher Scientific, NC9682496)
- gentleMACS M tubes, sterile packed as 4x25 pieces (Miltenyi Biotec,130-096-335)
- Standard Line Sterile Centrifuge Tubes with Flat Caps, Conical-Bottom, 15 mL (VWR, 10025-686)
- 1.5 mL Lo Bind Centrifuge tubes (Eppendorf, 022431021)
- 5 mL Eppendorf DNA LoBind tubes (Eppendorf, 0030108310)
- Thermo Scientific™ SoftFit-L™ Filtered Pipette Tips in Hinged Racks, 200 µL (Fisher Scientific, 21-402-561)
- Thermo Scientific™ SoftFit-L™ Filtered Pipette Tips in Hinged Racks, 20 µL (Fisher Scientific, 21-402-550)
- xTIP4™ Racked Pipette Tips, Rainin® LTS® Pipette Compatible, Biotix, 1000 µL (Fisher Scientific, 76266-146)
- Olympus Plastics 0.2 mL 8-Strip PCR Tubes, Flex Free Individual Attached Flat Caps (Genesee Scientific, 27-125U)
- Serological Pipets, 10 mL, Sterile, Individually Wrapped (Genesee Scientific, 12-104)

Reagent preparation:

1. Prepare buffers fresh and leave on ice.
    a. The volume of MACS Buffer prepared depends on the input mass of each sample. An additional 1 mL should be prepared for rinsing in addition to the homogenization volume. Refer to the chart below:

Recommended MACS Buffer Homogenization Volumes	
Tissue Mass	Volume per Sample
10-40 mg	2 mL
40-100 mg	3 mL
100-200 mg	4 mL
> 200 mg	5 mL

MACS Buffer			
Reagent	**Stock Concentration**	**Final Concentration**	**for 1 mL**
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	40 µl
DTT	200 mM	0.6 mM	3 µl
$CaCl_2$	250mM	5 mM	20 µl
EDTA	500mM	5 mM	10 µl
Tris-HCl, pH 8.0	1M	10 mM	10 µl
MgAc	300 mM	3mM	10 µl
Recombinant RNasin	40U/µl	1U/µl	25 µl
Molecular biology water	-	-	1.1 mL

Sort Buffer (SB)			
Reagent	**Stock Concentration**	**Final Concentration**	**For 4 samples**
Fatty acid-free BSA in PBS	10%	1%	200 µL
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	80 µL
7-AAD (10% in DMSO)	1 mM	2 µM	4 µL
Recombinant RNasin	40 U/µl	1 U/µl	50 µL
PBS			1666 µL
TOTAL	-	-	2000 µL

Collection Buffer (CB)			
Reagent	**Stock Concentration**	**Final Concentration**	**For 4 samples**
Fatty acid-free BSA in PBS	10%	5%	200 µL
Recombinant RNasin	40 U/µL	5 U/µL	50 µL
PBS	-	-	150 µL
TOTAL	-	-	400 µL

Nuclear Permeabilization Buffer (NPB)			
Reagent	**Stock Concentration**	**Final Concentration**	**1 mL**
Fatty acid-free BSA in PBS	-	5%	50 mg
IGEPAL-CA630	10%	0.20%	2 µL
DTT	200 mM	1 mM	5 µL
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	40 µL
Recombinant RNasin	40 U/µL	1 U/µL	25 µL
PBS			928 µL

Wash Buffer (WB)			
Reagent	**Stock Concentration**	**Final Concentration**	**Volume per Sample**
Fatty acid-free BSA in PBS	10%	1%	200 µL
Roche cOmplete, EDTA-free Protease Inhibitor Cocktail	25X	1X	80 µL
Tris-HCl, pH 7.5	1M	10mM	20 µL
DTT	200 mM	1 mM	10 µL
$MgCl_2$	1M	3mM	6 µL
NaCl	5M	10mM	4 µL
Tween-20	10%	0.01%	2 µL
Recombinant RNasin	40 U/µL	1 U/µL	50 µL
Molecular biology water	-	-	1628 µL

Nuclei Preparation

1. Pre-chill a large, swing-bucket tabletop centrifuge to 4°C.
2. Add the pre-determined homogenization volume of MACS buffer to each MACS tube on ice.
3. Immediately transfer samples to MACS tubes. If needed, resuspend tissue first with MACS buffer from the MACS tube and then transfer the full volume to the tube.
4. Tighten the cap of each MACS tube and flip the tube upside down. Ensure that all tissue is submerged in the buffer.
5. Thaw on ice for 1 min.
6. Homogenize samples using the gentleMACS Octo Dissociator in the 4 °C cold room.
7. Run the protocol “protein_01_01” for gentleMACS M tubes (~1 min).
8. Quick spin M tubes to bring all liquid to the bottom of the tubes.
9. Filter each sample into a 15 mL tube using a 30 um (green) Celltrics filter.
10. Rinse the sides of each MACS tube with 1 mL MACS buffer.
11. Quick spin M tubes to bring all liquid to the bottom of the tubes.
12. Transfer the rinse to each Celltrics filter.
13. Centrifuge homogenized samples for 5 min at 500 rcf, 4°C, and 3/3 acceleration/deceleration.
14. Discard supernatants. A small volume of supernatant can be left in each tube to avoid disturbing the pellet.
15. Gently resuspend each pellet in 500 µL sort buffer.
16. Transfer the full sample volume to a pre-chilled 1.5 mL LoBind tube.
17. Incubate on ice, protected from light, for 10 min.

18. Aliquot 90 uL of 5X collection buffer into a 1.5 mL LoBind tube for each sample.

19. Sort 120,000 nuclei into the 90 µL of collection buffer for each sample.

20. Centrifuge for 5 min at 500 rcf, 4°C, and 3/3 acceleration/deceleration.

21. Discard supernatant.

22. Gently resuspend pellet in 100 µL of NPB.

23. Incubate on ice for 1 min.

24. Add 900 µL of Wash buffer to each sample

25. Centrifuge for 5 min at 500 rcf, 4°C, and 3/3 acceleration/deceleration.

26. Carefully discard supernatants. Switch to a P20 pipette once the volume reaches ~40 µL. Do NOT disturb the pellet.

27. Gently resuspend in 12 µL of 1X Nuclei Buffer (prepared from 10X Genomics protocol).

28. Stain an aliquot of nuclei with 0.4% Trypan Blue. Load 10 µL into one chamber of a hemocytometer.

29. Count nuclei in four quadrants. Average the count and determine the nuclei concentration (nuclei/µL).

30. Capture images from the microscope field at 10X and 20X magnification.

31. Follow the 10X Genomics protocol **"Chromium Next GEM Single Cell Multiome ATAC + Gene Expression" (CG000338, Rev F)** for the remainder of the experiment. Input 18,000 nuclei for each tagmentation reaction for a targeted recovery of ~10,000 nuclei.