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Nuclear extraction using opti-prep gradient centrifugation for 10X Genomics Multiome Assay

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We use this protocol and it's working

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Abstract

This protocol describes the nuclear extraction by using opti-prep gradient centrifugation for 10X Genomics Multiome Assay.

Materials

Equipment and Materials:

- Bench-top centrifuge
- Swing-bucket rotor ultracentrifuge (Beckman, L-70)
- Swing-bucket rotor (Beckman, SW 28)
- Ultracentrifuge tube (4 ml thick-walled polycarbonate tube, Beckman Coulter 355645)

Equipment	
4 mL, Open-Top Thickwall Polycarbonate Tube	NAME
Centrifuge tube	TYPE
Beckman Coulter	BRAND
355645	SKU
https://www.mybeckman.in/supplies/tubes-and-bottles/open-top-tubes/355645	LINK

- Ultracentrifuge rotor adaptor (13mm Diameter Delrin Tube Adapter, Beckman Coulter 303392)

Equipment	
13mm Diameter Delrin Tube Adapter	NAME
Ultracentrifuge Rotors	TYPE
Beckman Coulter	BRAND
303392	SKU
https://www.mybeckman.in/supplies/adapters/303392	LINK

- 2 ml Dounce Tissue Grinder (Sigma-Aldrich, D8938-1SET)

Equipment

Dounce tissue grinder set	NAME
Tissue grinder	TYPE
KIMBLE	BRAND
D8938	SKU
https://www.sigmaaldrich.com/IN/en/product/sigma/d8938?srsltid=AfmBOor_tbRsOVPIEmWp6R9nh7fQDqXh4WQFaYkkhyTHiIWGNG1n7RVw	LINK

- 5 ml DNA LoBind tube (Eppendorf, 0030108310)

Equipment

DNA LoBind® Tubes, 5 mL	NAME
Eppendorf Tubes	TYPE
Eppendorf	BRAND
0030108310	SKU
https://www.eppendorf.com/in-en/Products/Laboratory-Consumables/Tubes/DNA-LoBind-Tubes-p-0030108310	LINK

- 2 ml DNA LoBind tube (Eppendorf, 022431048)
- 1.5 ml DNA LoBind tube (Eppendorf, 022431021)
- 5 mL Round Bottom High Clarity PP Test Tube

Equipment

5 mL Round Bottom High Clarity PP Test Tube	NAME
Tube	TYPE
Falcon®	BRAND
352063	SKU
https://ecatalog.corning.com/life-sciences/b2b/DE/en/General-Labware/Tubes/Tubes,-Round-Bottom/Falcon%C2%AE-Round-Bottom-High-clarity-Polypropylene-Tube/p/352063''	LINK

- CellTrics® filters (50 um, Sysmex, 04-004-2327)
- Pipettes and pipette tips
- Hemocytometer

Reagents:

- Sucrose **Fisher Scientific** **Catalog #**AAJ6514836
- PBS, 10X Solution **Fisher Scientific** **Catalog #**AAJ75889AE
- Ultrapure Water **Thermofisher** **Catalog #**10977023
- Tris-HCl (ph7.4) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T2194-100ML
- Tris (1 M) pH = 8 RNase free **Invitrogen - Thermo Fisher** **Catalog #**AM9855G
- 5M NaCl **Invitrogen - Thermo Fisher** **Catalog #**AM9760G
- 2M KCl **Invitrogen - Thermo Fisher** **Catalog #**AM9640G
- 1M MgCl2 **Invitrogen - Thermo Fisher** **Catalog #**AM9530G
- 10% Tween 20 **Bio-Rad Laboratories** **Catalog #**1662404
- Surfact-Amps NP-40 **Thermo Fisher Scientific** **Catalog #**28324
- Digitonin (5%) **Thermo Fisher** **Catalog #**BN2006
- 10% Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**93443-100ML
- Optiprep (Iodixanol) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D1556-250ML
- Bovine Serum Albumin **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**126625

- Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**3335402001
- DAPI Solution (1 mg/mL) **Thermo Fisher Scientific Catalog #**62248
- 7-ADD Ready Made Solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**SML1633-1ML
- 1M DL-Dithiothreitol solution (DTT) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**646563
- Nuclei Buffer 20X **10x Genomics Catalog #**2000153/2000207

	A	B	C	D
	PBS	Stock	Final	50 mL
	Nuclease-free Water			45 mL
	PBS	10X	1X	5 mL

	A	B	C	D
	NIM (Nuclear isolation media)	Stock	Final	100 mL
	Nuclease-free Water			47.25 mL
	Sucrose	0.5 M	0.25 M	50 mL
	KCl	2 M	25 mM	1250 µL
	MgCl2	1 M	5 mM	500 µL
	Tris-HCl, pH 8.0	1 M	10 mM	1000 µL

	A	B	C	D
	Diluent	Stock	Final	50 mL
	Nuclease-free Water			41.75 mL
	KCl	2 M	150 mM	3.75 mL
	MgCl2	1 M	30 mM	1.5 mL
	Tris-HCl, pH 8.0	1 M	60 mM	3 mL

	A	B	C	D
	Homogenization Buffer	Stock	Final	1100µL per sample
	NIM			1060 µL
	BSA	30%	1%	37 µL
	RNase inhibitor	40 U/ µL	0.08 U/ µL	2.2 µL

	A	B	C	D
	Resuspension Buffer	Stock	Final	1100µL per sample
	PBS	1X	1X	1060 µL
	BSA	30%	1%	37 µL
	RNase inhibitor	40 U/ µL	0.08 U/ µL	2.2 µL

	A	B	C	D
	Nuclei Permeabilization Buffer	Stock	Final	1 ml per 8 samples
	Nuclease-free Water			963 µL
	Tris-HCl (pH 7.4)	1M	10 mM	10 µL
	NaCl	5M	10 mM	2 µL
	MgCl2	1M	3 mM	3 µL
	Tween-20	10%	0.1%	10 µL
	NP40	10%	0.1%	10 µL
	Digitonin	5%	0.01%	2 µL

	A	B	C	D
	Diluted Nuclei Buffer	Stock	Final	55µL per sample
	Nuclease-free Water	-	-	520 µL
	Nuclei Buffer (20X)	20X	1X	27.5 µL
	DTT	1M	1mM	0.55 µL
	RNase inhibitor	40U/ µL	0.08 U/ µL	1.1 µL

Opti-prep gradients per sample:

	A	B
	50% iodixanol	3 mL per sample
	Opti-prep	2500 µL
	Diluent	500 µL

	A	B
	40% iodixanol	1100 µL per sample
	50% iodixanol	880 µL
	NIM	220 µL
	30% BSA	37 µL
	RNase inhibitor	2.2 µL

	A	B
	30% iodixanol	1100 µL per sample
	50% iodixanol	660 µL
	NIM	440 µL
	30% BSA	37 µL
	RNase inhibitor	2.2 µL

Before start

One day prior to the experiment:

1. Wipe clean the working area with 70% ethanol and RNase Zap.
2. PBS, NIM, and Diluent can be prepared in advance and stored at 4 °C for up to 3 months. Filter NIM with 0.2 µm filter.
3. Clean douncers, scissors, and forceps with 70% ethanol, and rinse them with nuclease-free water for a total of 2 times.

On the day of the experiment

1h

1 Gather equipment, materials, and reagents.

1.1 Thaw and dissolve DTT by incubating the tube at Room temperature.

1.2 Place SW28 rotor into the ultracentrifuge, start test run at 8900 rpm, 4°C, 01:00:00.

1h

1.3 Pre-chill the bench-top centrifuge to 4 °C.

1.4 Place douncers On ice.

2 Prepare buffers and store them On ice.

3 Gather samples and store on dry ice.

4 Layer in ultracentrifuge tube 1 ml 40% at bottom of tube, and then slowly add on top of it, 1 mL 30% iodixanol solutions carefully. Slowly pipette just above 40% layer against the wall allowing it to form two distinct layers. Leave this On ice while you prepare the sample. Pre-indent ice with another tube to avoid disrupting layers.

Sample processing

1m 5s

5 Take one sample out from dry ice and thaw On ice for 00:01:00.

1m

6 Add 1 mL of 1X PBS to the original tube, pipet mix 10 times and vortex for 00:00:05 to wash off OCT.

5s

7 Quick spin and aspire out PBS.

8 Repeat Steps 6 and 7 one more time.

9 Using the forceps to transfer the tissue to a 5 mL centrifuge tube.

10 Add 1 mL of homogenization buffer and 10 µL of 10% NP40.

11 Break down tissues by chopping the sample 30 times with a clean scissor until all sections have been trimmed into small pieces.

Homogenization

12 Transfer samples to a pre-chilled douncer.

13 Insert the loose pestle (“A”) into the homogenizer and perform 15 strokes. Perform additional 15 strokes with the tight pestle (“B”). Try to make less bubbles as possible.

> Note
>
> **Optional:** if big chunks are still visible, remove the pestle and let the douncer sit On ice for 00:01:00 allowing chunks to precipitate. Perform additional 5 strokes with the tight pestle (“B”). At the end of each stroke, rotate the pestle back and forth to further grind the sample.

14 Filter the homogenate directly with a 50 µm Sysmex filter into a 2 mL centrifuge tube.

> Note
>
> If there is abundant debris, filtration can be aided by taking your thumb and covering the top of the filter opening. This will create positive pressure that pushes the filtrate through the filter.

15 Bring the sample volume up to 1 mL using resuspension buffer.

16 Vortex 50% iodixanol. Add 1 mL of freshly-vortexed 50% iodixanol to the homogenate to make 25% iodixanol.

17 Cap the tube and keep On ice. Repeat all sample preparation steps and Homogenization steps for additional samples.

Centrifugation

28m

18 Invert sample tubes 10 times to mix up nuclear suspensions in 25% iodixanol.

19 Using a 1 ml pipette, slowly pipette 2 mL of nuclear resuspensions on top of the 30% layer in the ultracentrifuge tube. Slowly pipette just above 30% layer against the wall allowing it to form two distinct layers.

20 Adjust volume of samples with nuclease-free water to match same level.

21 Insert the ultracentrifuge tube into the adaptor. Seal the top with parafilm.

22 Spin: 10000 x g, 00:18:00 (8,900 rpm with SW28 rotor).

18m

23 When spin is finished, aspirate out 1.5 mL of 25% iodixanol layer in order to get better access to the interlayer.

24 Stab P200 pipette the interface of 30% and 40%. While moving pipette tip over the entire surface of this interface, aspirate 200 µL of interlayer and transfer to a 2 mL centrifuge tube.

Note

Dots of nuclei may be visible interface between 30% iodixanol and 40% iodixanol layers.

25 Repeat step 24 for a total of 3 times to collect 600 µL from the interlayer.

26 Dilute iodixanol with 600 µL of Resuspension Buffer + 6 µL of 7-AAD ready-made solution (1 µL stock concentration, 5 µL final concentration). Pipet mix 10 times.

27 Centrifuge the tubes at 500 x g, 4°C, 00:10:00 . 10m

Note

If you start with a small amount of tissues, the pellet may not be visible after centrifugation. Therefore, it's important to note the position the tubes in the centrifuge so that you know where the pellet is supposed to be after centrifugation.

Note

Using a centrifuge with swinging bucket rotor will improve efficiency.

28 Gently remove one tube at a time from the centrifuge. Remove the supernatant by inverting the tube. Leave behind 50 µL of supernatant as not to disturb nuclei pellet.

29 Resuspend the pellets in each tube in 150 µL of resuspension buffer. Pipet mix 10 times.

Nuclei Permeabilization 15m

30 Add 20 µL of Nuclei Permeabilization Buffer to a final concentration of 0.1 X. Mix by inverting tube 5 times.

31 Incubate tubes On ice for 00:03:00 . 3m

32 Add 400 µL Diluted Nuclei Buffer to dilute Nuclei Permeabilization Buffer and facilitate buffer exchange. Mix by inverting tube 5 times.

33 Centrifuge tubes at 500 x g, 4°C, 00:10:00 . 10m

34 Remove most of the supernatant without disrupting the nuclei pellet.

35 Centrifuge at 500 x g, 4°C, 00:02:00 to spin down excessive supernatant. 2m

36 Carefully remove the supernatant without disturbing the pellet.

Nuclei count and dilution

37 Resuspend pellet in 10 µL of chilled Diluted Nuclei Buffer. Pipet mix 10 time and pulse vortex for one second to resuspend the nuclei pellet.

38 For each sample, Dilute 1 µL of nuclei in 11 µL of Diluted Nuclei Buffer. Load 10 µL on a hemocytometer.

39 Inspect and count on an epifluorescence microscope with a 7-AAD filter.

> Note
>
> 7-AAD has its peak excitation at ~550 nm and peak emission at ~650 nm.