DEMONSTRATED PROTOCOL

Nuclei Isolation for Single Cell Multiome ATAC + Gene Expression Sequencing

Overview

This protocol outlines how to isolate, wash, and count nuclei suspensions for use with the Chromium Next GEM Single Cell Multiome ATAC + Gene Expression (GEX) protocol (CG000338). Cryopreserved primary cells (PBMCs) and cell lines (GM12878 cells; 3T3 cells) were used to develop this protocol. PBMCs were cryopreserved in IMDM + 40% FBS + 15% DMSO. Cell lines were cryopreserved in RPMI + 15% FBS + 5% DMSO.

For optimal assay performance, nuclei isolation should be performed using this protocol and not the standalone protocols for nuclei isolation for ATAC or RNA sequencing only. The recommended buffer compositions, final nuclei suspension concentration, and the wash step guidelines presented in this protocol for nuclei sample preparation are critical for optimal Chromium Single Cell Multiome ATAC + GEX assay performance. Failure to adhere to these guidelines may result in suboptimal assay performance.

DO NOT use this protocol for isolating nuclei from tissue-derived cells. Refer to Demonstrated Protocols- Nuclei Isolation from Embryonic Mouse Brain for Single Cell Multiome ATAC + Gene Expression Sequencing (CG000666) & Nuclei Isolation from Complex Tissues for Single Cell Multiome ATAC + Gene Expression Sequencing (CG000375) for isolating nuclei from tissue-derived cells.

Additional Guidance

Consult Demonstrated Protocol Cell Preparation Guide (Document CG00053) for Tips & Best Practices.

Cells carry potentially hazardous pathogens. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage and disposal of biological materials.

Cell Sourcing

Cell Type	Species	Supplier
GM12878	Human	Coriell Institute
3T3	Mouse	ATCC
Normal Peripheral Blood MNC (PBMC)	Human	AllCells

Optimization Recommendations

The following demonstrated protocol was performed using the indicated sample types. Optimization of some protocol steps may be needed for other cell types.

- **Lysis time:**
  Perform a lysis timeline to determine appropriate lysis incubation time for a specific cell type.
  For optimization experiments, RNAse inhibitor may be omitted from the buffer and instead of the 10x Genomics' Nuclei Buffer, PBS may be used for nuclei resuspension. However for the actual experiment, ensure that RNAse inhibitor and the 1x Nuclei Buffer are used as recommended.

- **Lysis buffer strength:**
  If nuclei quality is poor at short lysis times, buffer strength can be decreased for a gentler lysis

- **Sample cleanup steps:**
  Additional cleanup steps such as washes, filtering, density gradient centrifugation, and FACS may be necessary to clean up excess debris present in the sample

See Appendix for additional optimization and troubleshooting guidance

Preparation – Buffers

Diluted Nuclei Buffer Prepare fresh, maintain at 4°C	Stock	Final	1 ml
Nuclei Buffer* (20X)	20X	1X	50 µl
DTT	1000 mM	1mM	1 µl
RNase inhibitor (check vendor-specific stock concentration)	40 U/µl	1 U/µl	25 µl
Nuclease-free Water	-	-	924 µl

Wash Buffer Prepare fresh, maintain at 4°C	Stock	Final	4 ml
Tris-HCl (pH 7.4)	1 M	10 mM	40 µl
NaCl	5 M	10 mM	8 µl
$MgCl_2$	1 M	3 mM	12 µl
BSA	10%	1%	400 µl
Tween-20	10%	0.1%	40 µl
DTT	1000 mM	1 mM	4 µl
RNase inhibitor	40 U/µl	1 U/µl	100 µl
Nuclease-free Water	-	-	3.40 ml

Lysis Buffer Prepare fresh, maintain at 4°C	Stock	Final	2 ml
Tris-HCl (pH 7.4)	1 M	10 mM	20 µl
NaCl	5 M	10 mM	4 µl
$MgCl_2$	1 M	3 mM	6 µl
Tween-20	10%	0.1%	20 µl
Nonidet P40 Substitute (alternatively, use IGEPAL CA-630) If using Sigma 74385 or i8896, prepare a 10% stock	10%	0.1%	20 µl
Digitonin (incubate at 65°C to dissolve precipitate before use)	5%	0.01%	4 µl
BSA	10%	1%	200 µl
DTT	1000 mM	1 mM	2 µl
RNase inhibitor 40 U/µl	40 U/µl	1 U/µl	50 µl
Nuclease-free Water	-	-	1.67 ml

Additional Buffers
RPMI + 10% FBS (maintain at **4°C**, pre-warm at **37°C** before use)
PBS + 0.04% BSA (maintain at **4°C**)

Specific Reagents & Consumables

Vendor	Item	Part Number
10x Genomics	Nuclei Buffer* (20X)	2000153/2000207
Thermo Fisher	Digitonin	BN2006
	Tubes, 0.2 ml, flat cap tube**	AB0620
	Sorvall Microtube Adapters**	76003750
Sigma-Aldrich	Trizma Hydrochloride Solution, pH 7.4	T2194
	Sodium Chloride Solution, 5 M	59222C
	Magnesium Chloride Solution, 1M	M1028
	Nonidet P40 Substitute (alternatively, use IGEPAL CA-630)	74385 i8896
	Sigma Protector RNase inhibitor (substitution is not recommended)	3335402001
	DTT	646563
Miltenyi Biotec	MACS BSA Stock Solution	130-091-376
Bel-Art	Flowmi Cell Strainer, 40 µm	H13680-0040
Bio-Rad	Tween 20	1662404
Corning	1X Phosphate-Buffered Saline, pH 7.4	21-040-CV

*Included in the 10x Genomics Single Cell Multiome ATAC Kit A
**ONLY for Low Cell Input Nuclei Isolation protocol

See Appendix for DNase Treatment specific reagents & buffers

Protocol Overview

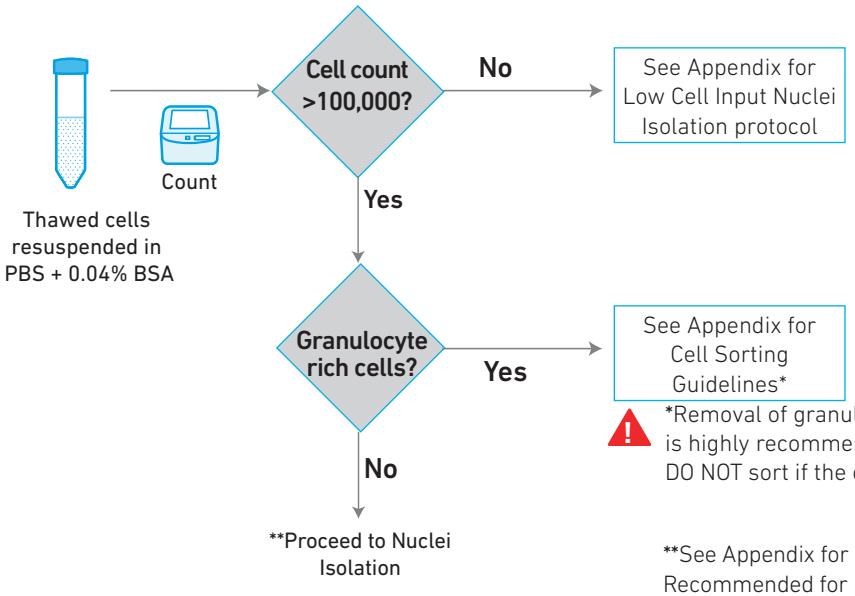
Frozen Cells:

Thaw cells using the protocol for thawing cell lines or primary/fragile cells (step 1)

Fresh Cells:

Wash cells with PBS + 0.04% BSA, determine cell count, and proceed to Nuclei Isolation (step 2)

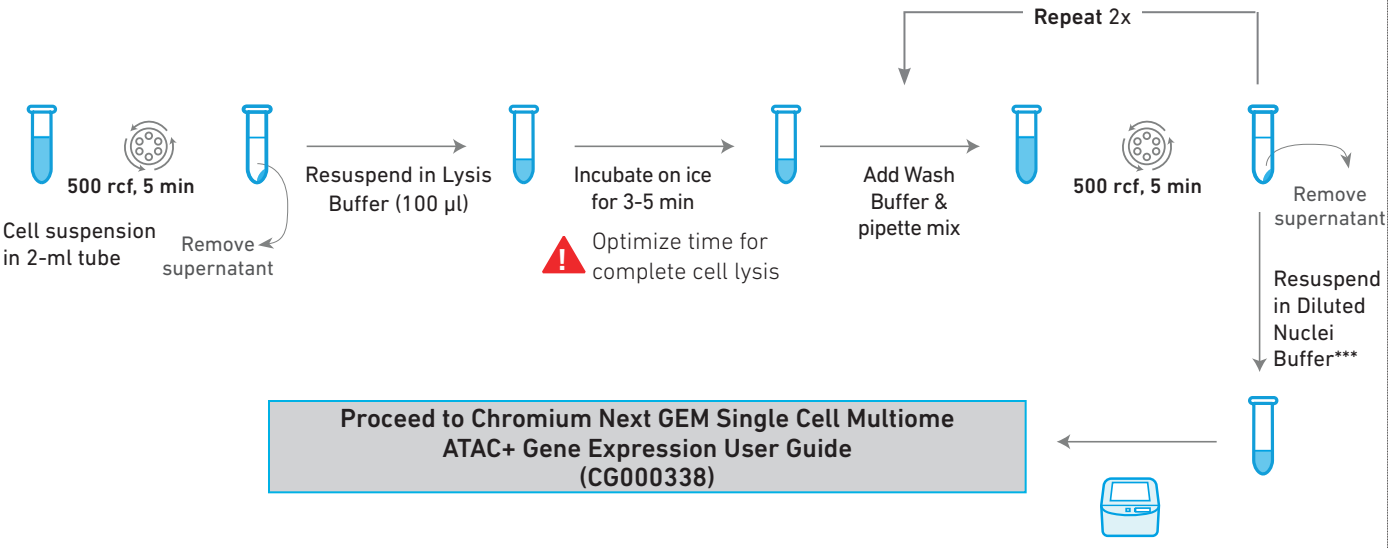
1. Thaw Cells



*Removal of granulocytes by cell sorting is highly recommended (PBMCs, BMMCs). DO NOT sort if the cell count is <100,000.

**See Appendix for DNase Treatment protocol. Recommended for primary cells that have not been sorted.

2.Nuclei Isolation



***Critical for optimal assay performance

Protocol

If using fresh cells, perform 1-2 washes with PBS + 0.04% BSA, determine cell count, and proceed to Nuclei Isolation (step 2).

1. Thaw Cells (if using frozen cells)

For cell lines (used for GM12878 and 3T3 cells):

a. Remove cryovials from storage, thaw in the water bath at **37°C** for **1-2 min**. Remove from the water bath when a tiny ice crystal remains.

b. Pipette mix the cells and transfer to a 15-ml conical tube containing **10 ml** pre-warmed media (RPMI + 10% FBS).

c. Centrifuge at **300 rcf** for **5 min**.

d. Remove the supernatant without disrupting the cell pellet and resuspend in **1 ml** PBS + 0.04% BSA. Transfer to a 2-ml microcentrifuge tube. Rinse the 15-ml tube with **0.5 ml** PBS + 0.04% BSA and transfer the rinse to the 2-ml tube containing the cells.

e. Centrifuge cells at **300 rcf** for **5 min**.

f. Remove the supernatant without disrupting the cell pellet and resuspend in **1 ml** PBS + 0.04% BSA.

g. Pass cell suspension through a **40 µm** Flowmi Cell Strainer.

h. Determine the cell concentration using a Countess II FL Automated Cell Counter (see Appendix) or a hemocytometer.

i. Proceed to Nuclei Isolation (step 2).
If cell count is <100,000, nuclei may be isolated using the Low Cell Input Nuclei Isolation protocol (see Appendix).

For primary cells/fragile cells (used for PBMCs):

a. Remove cryovial from storage, thaw in the water bath at **37°C** for **1-2 min**. Remove from the water bath when a tiny ice crystal remains.

b. Transfer the thawed cells to a 50-ml conical tube. Rinse the cryovial with **1 ml** pre-warmed media (RPMI + 10% FBS) and add the rinse drop-wise to the 50-ml conical tube while gently shaking the tube (**300 rpm** at **37°C** on the thermomixer).

c. Sequentially dilute cells in the 50-ml conical tube by incremental 1:1 volume additions of media for a total of 5 times (including dilution at step b), with ~**1 min** wait between additions (see Appendix). Add media (RPMI + 10% FBS) at a speed of **1 ml/3-5 sec** to the tube and swirl.

d. Centrifuge at **300 rcf** for **5 min**.

e. Remove most of the supernatant, leaving ~**1 ml** and resuspend cell pellet in this volume.

f. Add an additional **9 ml** media (**1 ml/3-5 sec**) to achieve a total volume of ~**10 ml**.

g. Centrifuge at **300 rcf** for **5 min**.

h. Remove the supernatant without disrupting the cell pellet and resuspend in **1 ml** PBS + 0.04% BSA, gently pipette mix 5x.

i. Transfer to a 2-ml microcentrifuge tube. Rinse the 50-ml tube with **0.5 ml** PBS + 0.04% BSA and transfer the rinse to the 2-ml tube containing the cells. Mix by gently inverting the tube.
OPTIONAL Primary/fragile cells may have high amounts of ambient/background DNA. Treating the cells with DNase I prior to nuclei isolation can remove the ambient DNA, improving the quality of Single Cell ATAC libraries (see Appendix for protocol). DNase treatment is not necessary if cells are being sorted prior to nuclei isolation.

j. Centrifuge cells at **300 rcf** for **5 min**.

k. Remove the supernatant without disrupting the cell pellet and resuspend in **1 ml** PBS + 0.04% BSA.

l. Pass cell suspension through a **40 µm** Flowmi Cell Strainer.

m. Determine the cell concentration using a Countess II FL Automated Cell Counter (see Appendix) or a hemocytometer.

Cell sorting for granulocyte removal before nuclei isolation is highly recommended for granulocyte-rich samples, such as PBMCs and BMMCs (see Appendix for Cell Sorting Guidelines). Removing RNase-rich granulocytes prevents degradation of mRNA and excludes highly transposed DNA from Neutrophil Extracellular traps (NETs), resulting in cleaner data.

DO NOT sort cells if the cell count is <100,000.

n. Proceed to Nuclei Isolation (step 2).
If cell count is <100,000, nuclei may be isolated using the Low Cell Input Nuclei Isolation protocol (see Appendix).

Protocol

2. Nuclei Isolation

If using fresh cells, perform 1-2 washes with PBS + 0.04% BSA and determine cell count, before proceeding to step 2a. Nuclei may be isolated from 100,000-1,000,000 cells using this protocol.

a. Add 100,000-1,000,000 cells to a 2-ml microcentrifuge tube. Centrifuge at **300 rcf** for **5 min** at **4°C**.

b. Remove ALL the supernatant without disrupting the cell pellet.

c. Add **100 µl** chilled Lysis Buffer. Pipette mix 10x.

d. Incubate for **3-5 min*** on ice.
*Cryopreserved PBMCs were incubated for **3 min**
*Cryopreserved cell lines were incubated for **5 min**

Optimize incubation time based on cell type. Sub-optimal or prolonged lysis times can both alter assay performance. Assess lysis efficacy via the Countess II FL Automated Cell Counter/microscopy (see Results).

e. Add **1 ml** chilled Wash Buffer to the lysed cells. Pipette mix 5x.

f. Centrifuge at **500 rcf** for **5 min** at **4°C**.

g. Remove the supernatant without disrupting the nuclei pellet.

h. Repeat steps e-g two more times for a total of 3 washes.

i. Based on cell concentration step 2a and assuming ~50% nuclei loss during cell lysis, resuspend in chilled Diluted Nuclei Buffer. See Nuclei Stock Concentration Table and Example Calculation in Appendix. Maintain on ice.

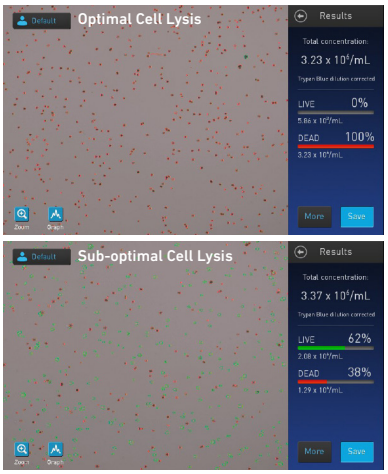
The resuspension in Diluted Nuclei Buffer is critical for optimal Single Cell ATAC assay performance. The composition of the Tris-based Diluted Nuclei Buffer, including Magnesium concentration, has been optimized for the Transposition and Barcoding steps in the Single Cell Multiome ATAC + GEX protocol. Suspension of nuclei in a different buffer may not be compatible.

j. **OPTIONAL** If cell debris and large clumps are observed, pass through a cell strainer. For low volume, use a **40 µm** Flowmi Cell Strainer to minimize volume loss.

k. Determine the nuclei concentration using a Countess II FL Automated Cell Counter (see Appendix) or a hemocytometer.

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

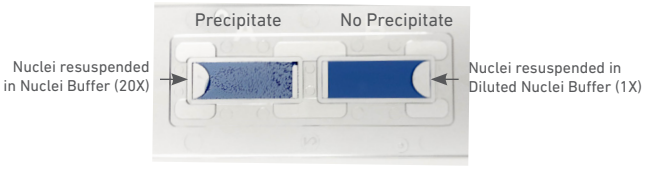
Results



Troubleshooting

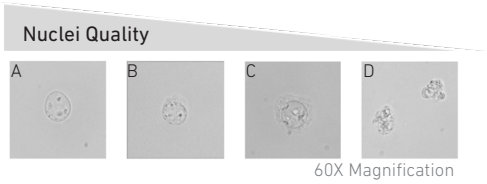
Problem	Possible Solution
High fraction of non-viable cells in input material prior to starting nuclei isolation	Optimize cell thawing to enhance sample quality Reduce fraction of dead cells. Refer to Demonstrated Protocol Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing (Document CG000093) Sort cells using flow cytometry Gently handle cell suspensions by following best practices and reduce cell processing times
High fraction of viable cells post cell lysis	Incrementally increase the lysis time and monitor lysis efficacy microscopically
Difficult to count nuclei/excess debris	Use a fluorescent dye (ethidium-homodimer-1) and fluorescence compatible cell counter or microscope
Excessive debris	Sample may be cleaned by extra washes/ filtering/desnity centrigugation/FACS (7-AAD stain)
Low nuclei recovery	Use a swing-bucket rotor for centrifugation steps

Trypan Blue Precipitate in the Countess II Slide



DO NOT use nuclei resuspended in 20X Nuclei Buffer. Repeat nuclei isolation and resuspend in Diluted Nuclei Buffer (1X).

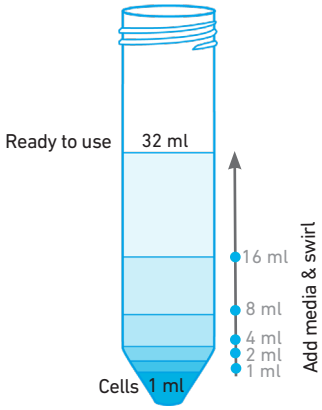
Nuclei Quality - Representative Images (Panel A: recommended quality)



Appendix

Illustrative Overview of Incremental1:1 Volume Additions

Incrementally add media volumes at the indicated points & swirl



Nuclei Stock Concentration Table

Based on the Targeted Nuclei Recovery, prepare the nuclei suspension in Diluted Nuclei Buffer to achieve the corresponding Nuclei Stock concentrations.

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

Appendix

Nuclei Counting and Viability

Countess II FL Automated Cell Counter is recommended for determining nuclei concentrations. The optimal range of cell concentration for Cell Counter is 1,000-10,000 cells/µl. Refer to manufacturer's instructions for details on operations.

a. Vortex 0.4% trypan blue stain, centrifuge briefly and aliquot **10 µl** per tube.

b. Pipette mix the nuclei suspension. Immediately add **10 µl** nuclei suspension to **10 µl** aliquot of 0.4% trypan blue stain. Gently pipette mix 10x.

c. Transfer **10 µl** trypan blue stained nuclei to a Countess II Cell Counting Slide chamber.

d. Insert the slide into the Countess II FL Cell Counter, and determine the nuclei concentration and viability. <5% of input cells should be viable. Optimize focusing and light exposure.

Low Cell Input Nuclei Isolation

Nuclei may be isolated from 2,000-100,000 cells using this protocol. If cell count is <40,000, centrifuge cell suspension at **300 rcf** for **5 min** at **4°C** and resuspend the cell pellet in **50 µl** PBS + 0.04% BSA. Transfer **50 µl** cell suspension to a 0.2-ml tube. Proceed directly to **step c**.

a. Centrifuge cell suspension at **300 rcf** for **5 min** at **4°C**. Remove supernatant and resuspend pellet in PBS + 0.04% BSA for 1,000 cells/µl cell suspension.

b. Add 2,000–40,000 cells to a 0.2-ml tube in a total volume of **50 µl** PBS + 0.04% BSA.

Approximately 25% of the cell input is expected to be recovered during Chromium Single Cell Multiome ATAC + GEX sequencing. Always determine nuclei counts after nuclei isolation.

Cell Input	Expected Nuclei Recovery (after cell lysis)	Expected Nuclei Recovery (ATAC + GEX)
40,000	16,000	10,000
20,000	8,000	5,000
10,000	4,000	2,500
4,000	1,600	1,000
2,000	800	500

c. Centrifuge at **300 rcf** for **5 min** at **4°C**.

d. Remove **45 µl** supernatant without touching the bottom of the tube to avoid dislodging the cell pellet.

e. Add **45 µl** chilled Lysis Buffer. Gently pipette mix 3x.

f. Incubate for **3-5 min*** on ice.

*Cryopreserved PBMCs were incubated for **3 min**
*Cryopreserved cell lines were incubated for **5 min**

Optimize incubation time based on cell type. Sub-optimal or prolonged lysis times can both alter assay performance. Assess lysis efficacy via the Countess II FL Automated Cell Counter/microscopy. See Results for optimal cell lysis.

g. Add **50 µl** chilled Wash Buffer to the tube. DO NOT mix.

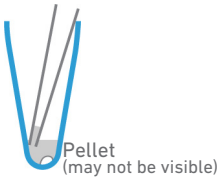
h. Centrifuge at **500 rcf** for **5 min** at **4°C**.

i. Remove **95 µl** supernatant without disrupting the nuclei pellet.

j. Add **45 µl** chilled Diluted Nuclei Buffer to the pellet. DO NOT mix.

k. Centrifuge at **500 rcf** for **5 min** at **4°C**.

l. Remove the supernatant without touching the bottom of the tube to avoid dislodging the nuclei pellet.



The supernatant may be removed in two steps, first with a 100-µl pipette (set to 40 µl), followed by removal with a 10-µl pipette (set to 10 µl).

m. Resuspend the nuclei pellet in **7 µl** chilled Diluted Nuclei Buffer (pellet may not be visible).

The use of the Diluted Nuclei Buffer for nuclei suspension is critical for optimal assay performance. The composition of the Tris-based Diluted Nuclei Buffer, including Magnesium concentration, has been optimized for the Transposition and Barcoding steps in the Single Cell Multiome ATAC + GEX protocol. Suspension of nuclei in a different buffer may not be compatible with these protocol steps.

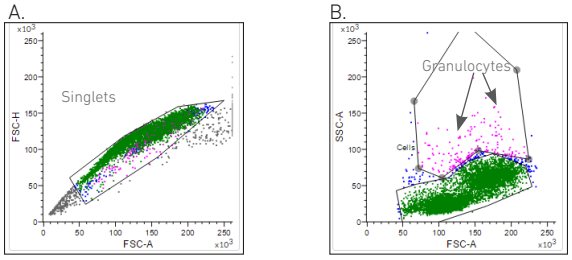
n. Use **2 µl** nuclei suspension mixed with **8 µl** Diluted Nuclei Buffer and **10 µl** Trypan Blue to determine the cell concentration by a Countess II FL Automated Cell Counter (see Appendix) or a hemocytometer.
A final nuclei concentration of 30 nuclei/µl is needed for Targeted Nuclei Recovery of 500.

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

Appendix

Cell Sorting Guidelines

- Removal of granulocytes by cell sorting is highly recommended before nuclei isolation from granulocyte-rich samples, such as PBMCs and BMMCs.
- Cell sorting is not recommended if cell count is <100,000.
- After thawing and counting cells (step 1m), cells can be sorted using a 100 µm nozzle (BD FACSMelody or comparable). No stain is needed for cell sorting. Granulocytes can be identified using side scatter.
- The first collection of sorted cells is for singlets as shown in the representative plot A.
- The second collection of sorted cells separates lymphocytes and monocytes (green cells) from granulocytes (pink cells) as shown in the representative plot B.



- The lymphocytes and monocytes fraction should be collected and used for isolating nuclei.
- Collect the sorted cells in a 5-ml FACS tube containing **500 µl** PBS + 0.04% BSA.
- Centrifuge the collected cells at **300 rcf** for **5 min** at **4°C**.
- Remove the supernatant without disrupting the cell pellet and resuspend in **500 µl** PBS + 0.04% BSA.
- Determine the cell concentration using a Countess II FL Automated Cell Counter (see Appendix) or a hemocytometer.
- Proceed **directly** to Nuclei Isolation (step 2).

References

- Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide (CG000338)

DNase Treatment

Recommended for primary cells prior to nuclei isolation. DNase treatment is not necessary if cells are being sorted prior to nuclei isolation.

Specific Reagents

DNase I, RNase-free (includes 10x Reaction Buffer with $MgCl_2$) from ThermoFisher Scientific, Part Number-EN0521

Preparation – Buffers

10X TBS	Stock	Final	5 ml
Tris-HCl (pH 7.4)	1 M	200 mM	1 ml
NaCl	5 M	1.5 M	1.5 ml
Nuclease-free Water	-	3 mM	2.5 ml
DNase Solution Prepare fresh, maintain at 4°C	**Stock**	**Final**	**1 ml**
TBS	10X	1X	100 µl
10X Reaction Buffer with $MgCl_2$	10X	1X	100 µl
DNase I	1 U/µl	0.1 U/µl	100 µl
Nuclease-free Water	-	-	700 µl

Primary cells/fragile cells may have high amounts of ambient/background DNA. Treating the cells with DNase I prior to nuclei isolation can reduce the ambient DNA, which may improve the quality of libraries.

a. Centrifuge the cells in a 2-ml microcentrifuge tube at **300 rcf** for **10 min** at **4°C**.

Using a 2-ml microcentrifuge tube and centrifuging for a longer time (10 min) is critical in maintaining an equal proportion of all cell types.

b. Remove supernatant without disrupting the pellet and resuspend the pellet in **300 µl** DNase Solution.

c. Pipette mix 5x and incubate on **ice** for **5 min**.

d. Add **1 ml** PBS + 0.04% BSA.

e. Centrifuge cells at **300 rcf** for **10 min** at **4°C**.

f. Remove supernatant without disrupting the pellet and resuspend the pellet in **1 ml** PBS + 0.04% BSA.

g. **Repeat** steps d-e for a total of 2 washes.

h. Pass cell suspension through a **40 µm** Flowmi Cell Strainer.

i. Determine the nuclei concentration using a Countess II FL Automated Cell Counter (see Appendix) or a hemocytometer.

j. Proceed **directly** to Nuclei Isolation (step 2).

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