DEMONSTRATED PROTOCOL

Nuclei Isolation for Single Cell ATAC Sequencing

Overview

This protocol outlines how to isolate, wash, and count nuclei suspensions for use with the Chromium Single Cell ATAC Solution. Cryopreserved primary cells (PBMCs) and cell lines (GM12878 cells; EL4 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. Optimization of some protocol steps (e.g. lysis time, centrifugation speed/time and filtration steps) may be needed based on cell type.

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 ATAC Solution performance. Failure to adhere to these guidelines may result in compromised microfluidics chip operation.

DO NOT use this protocol 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
EL4	Mouse	ATCC
Normal Peripheral Blood MNC (PBMC)	Human	AllCells

Preparation – Buffers

Diluted Nuclei Buffer Maintain at 4°C	Stock	Final	1 ml
Nuclei Buffer* (20X) (10x Genomics, PN-2000153/ 2000207)	20X	1X	50 µl
Nuclease-free Water	-	-	950 µl

See Appendix for DNase Treatment specific reagents & buffers

Wash 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
BSA	10%	1%	200 µl
Tween-20	10%	0.1%	20 µl
Nuclease-free Water	-	-	1.75 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
Nuclease-free Water	-	-	1.726 ml

Additional Buffers

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

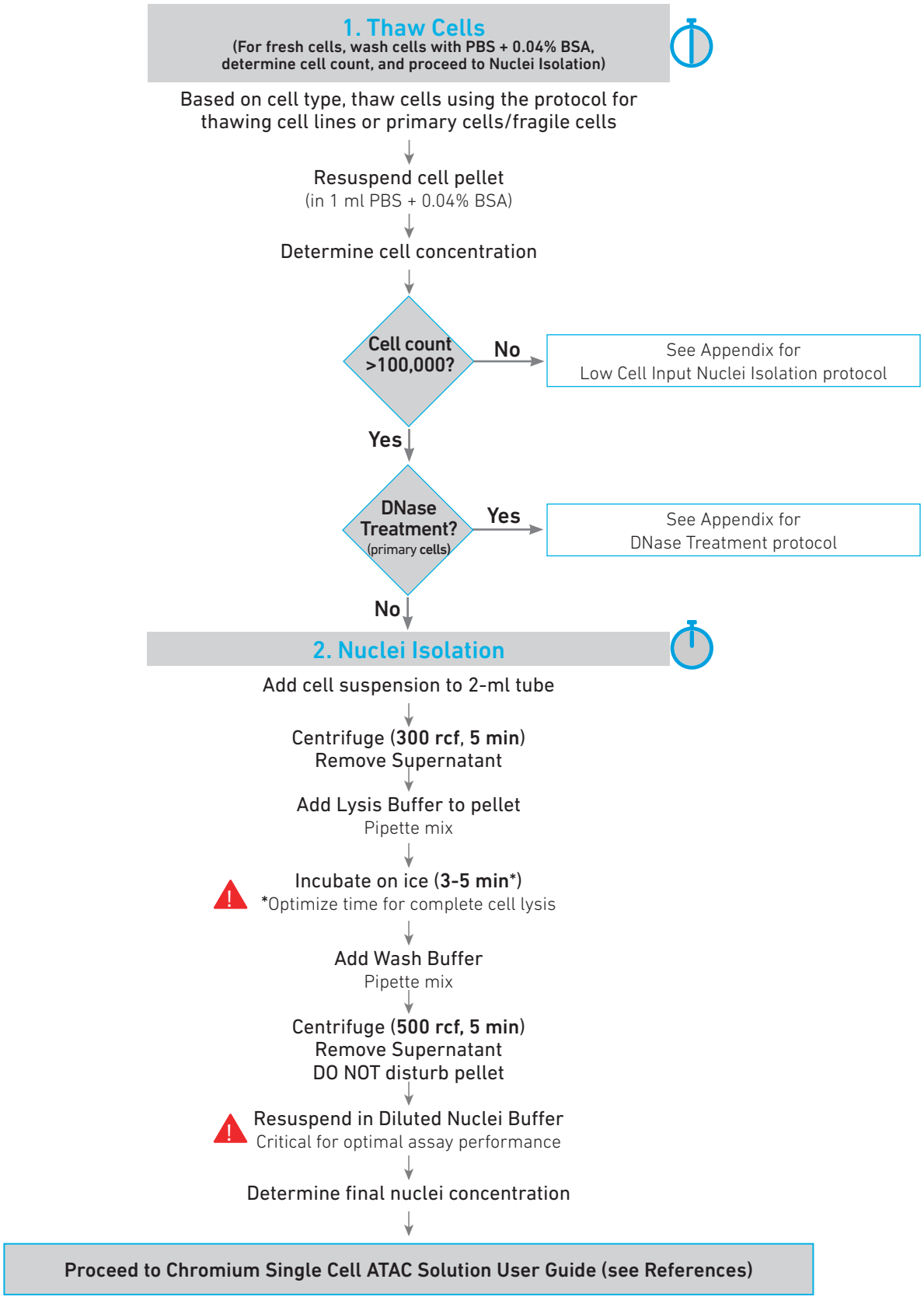
PBS + 0.04% BSA (maintain at **4°C**)

Specific Reagents & Consumables

Vendor	Item	Part Number
10x Genomics	Nuclei Buffer* (20X)	2000153/ 2000207
Thermo Fisher Scientific	Digitonin Tubes, 0.2 ml, flat cap tube**	BN2006 AB0620
Fisher Scientific	Sorvall Microtube Adapters**	76003750
Sigma-Aldrich	Trizma Hydrochloride Solution, pH 7.4 Sodium Chloride Solution, 5 M Magnesium Chloride Solution, 1M Nonidet P40 Substitute IGEPAL CA-630	T2194 59222C M1028 74385 i8896
Corning	1X Phosphate-Buffered Saline, pH 7.4	21-040-CV
Miltenyi Biotec	MACS BSA Stock Solution	130-091-376
Bel-Art	Flowmi Cell Strainer, 40 µm	H13680-0040

*Included in the 10x Genomics Single Cell ATAC Library Kits
**ONLY for Low Cell Input Nuclei Isolation protocol

Protocol Overview



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 cryopreserved cells)

For cell lines (used for GM12878 and EL4 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.

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).

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.

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).

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. Viability >70% is recommended before starting nuclei isolation.

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. 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.

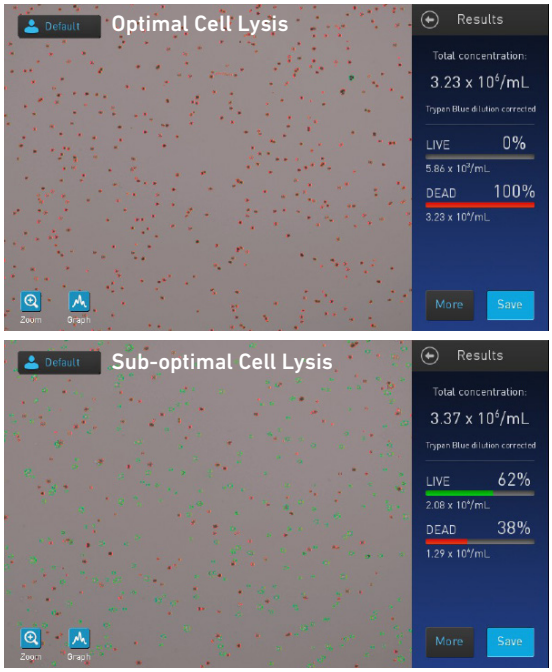
The resuspension in Diluted Nuclei Buffer is critical for optimal Single Cell ATAC assay performance. The composition of the Tris-based Diluted Nuclei Buffer, including Magnesium concentration, has been optimized for the Transposition and Barcoding steps in the Single Cell ATAC protocol. Suspension of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

i. **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.

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

k. Proceed **immediately** to Chromium Single Cell ATAC Solution User Guide (see References).

Results



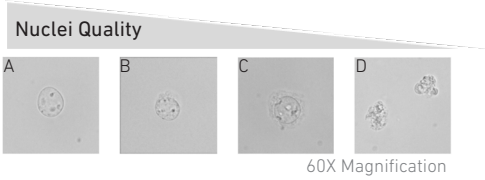
Troubleshooting

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

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).

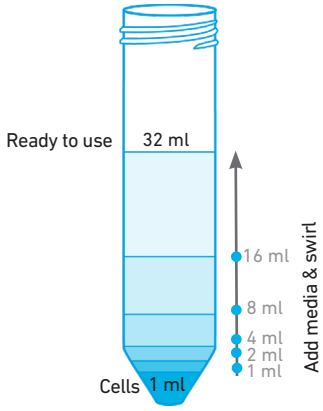
Nuclei Quality - Representative Images (Panel A: recommended quality)



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	155-390
1,000	310-780
2,000	610-1,540
3,000	925-2,300
4,000	1,230-3,075
5,000	1,540-3,850
6,000	1,850-4,600
7,000	2,150-5,400
8,000	2,460-6,150
9,000	2,770-6,900
10,000	3,080-7,700

Example Calculation

Cell count at step 2a: **200,000**
Estimated nuclei count at step 2h (~50% loss):**100,000**
If targeting 5,000 Nuclei Recovery, nuclei pellet at step 2h may be resuspended in **30 µl** Diluted Nuclei Buffer for Nuclei Stock Concentration of 1,540-3,850 nuclei/µl (see Table above)

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. If number of cells is >40,000 and <100,000, the sample can either be used for one low input prep or be split for two replicate low input preps, depending on desired recovery.

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

Cell Input	Expected Nuclei Recovery (after cell lysis)	Expected Nuclei Recovery (ATAC sequencing)
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.

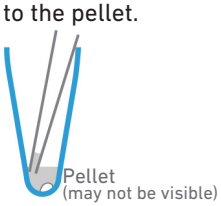
h. Centrifuge at **500 rcf** for **5 min** at **4°C**.

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 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 ATAC 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. The nuclei suspension is now diluted 1:5. Mix **10 µl** Trypan Blue to determine the cell concentration by a Countess II FL Automated Cell Counter (see Appendix) or a hemocytometer.

The 1:5 diluted nuclei suspension should have a minimum final nuclei concentration of 30 nuclei/µl (actual concentration 150 nuclei/ul) for a Targeted Nuclei Recovery of 500 nuclei.

o. Proceed **immediately** to Chromium Single Cell ATAC Solution User Guide (see References).

Appendix

DNase Treatment

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 the Single Cell ATAC 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. Centrifuge cells at **300 rcf** for **10 min** at **4°C**.

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

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

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

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

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

References

- Chromium Single Cell ATAC Reagent Kits User Guide (CG000168)
- Chromium Next GEM Single Cell ATAC Reagent Kits v1.1 User Guide (CG000209)
- Chromium Next GEM Single Cell ATAC Reagent Kits v2 User Guide (CG000496)

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