DEMONSTRATED PROTOCOL

Nuclei Isolation from Complex Tissues for Single Cell Multiome ATAC + Gene Expression Sequencing

Overview

This protocol outlines how to isolate, wash, and count nuclei suspensions from complex tissues for use with the Chromium Next GEM Single Cell Multiome ATAC + Gene Expression (GEX) protocol (CG000338). Fresh frozen human malignant lymphoma, glioblastoma, and normal brain tissue were used to develop this protocol. This approach may also be used for fresh tissue that is difficult to dissociate and a high level of debris is expected.

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.

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
Malignant Lymphoma	Human	BioIVT
Glioblastoma	Human	BioIVT
Normal Brain	Human	BioIVT

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.

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	40 U/µl	1 U/µl	25 µl
Nuclease-free Water	-	-	924 µl

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
DTT	1000 mM	1 mM	2 µl
RNase inhibitor	40 U/µl	1 U/µl	50 µl
Nuclease-free Water	-	-	1.67 ml

1X 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 (if using Sigma (74385) 100% solution, 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

NP40 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
Nonidet P40 Substitute (if using Sigma (74385) 100% solution, prepare a 10% stock)	10%	0.1%	20 µl
DTT	1000 mM	1 mM	2 µl
RNase inhibitor 40 U/µl	40 U/µl	1 U/µl	50 µl
Nuclease-free Water	-	-	1.9 ml

Lysis Dilution Buffer May be prepared ahead	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
DTT	1000 mM	1 mM	2 µl
RNase inhibitor	40 U/µl	1 U/µl	50 µl
Nuclease-free Water	-	-	1.718 ml

0.1X Lysis Buffer Prepare fresh, maintain at 4°C	Stock	Final	2 ml
1X Lysis Buffer	1X	0.1X	200 µl
Lysis Dilution Buffer	-	-	1.8 ml

Additional Buffers
PBS + 1% BSA + 1U/µl RNase I
BSA Stock Solution

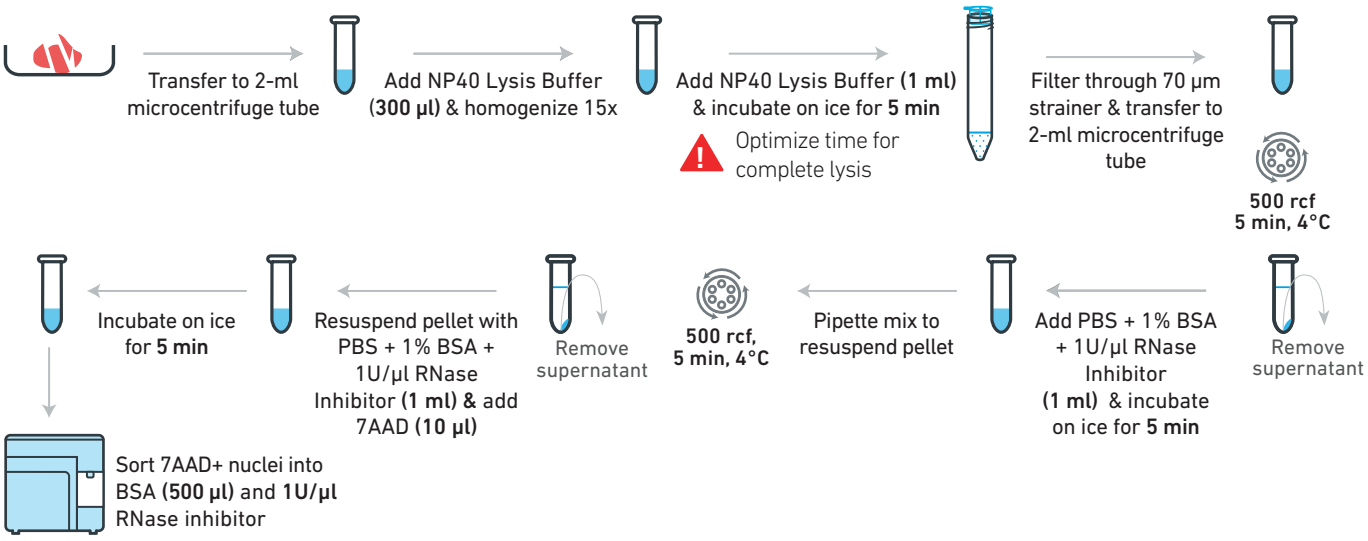
Specific Reagents & Consumables

Vendor	Item	Part Number
10x Genomics	Nuclei Buffer* (20X)	2000153/ 2000207
Thermo Fisher Scientific	Digitonin	BN2006
Sigma-Aldrich	Trizma Hydrochloride Solution, pH 7.4	T2194
	Sodium Chloride Solution, 5 M	59222C
	Magnesium Chloride Solution, 1M	M1028
	Nonidet P40 Substitute	74385
	Sigma Protector RNase inhibitor† (substitution is not recommended)	3335402001
	DTT	646563
	7-AAD Ready Made Solution	SML1633-1ML
Miltenyi Biotec	MACS BSA Stock Solution	130-091-376
Fisher Scientific	RNase-Free Disposable Pellet Pestles	12-141-368
Bel-Art	Flowmi Cell Strainer, 70 µm	H13680-0070
Bio-Rad	Tween 20	1662404

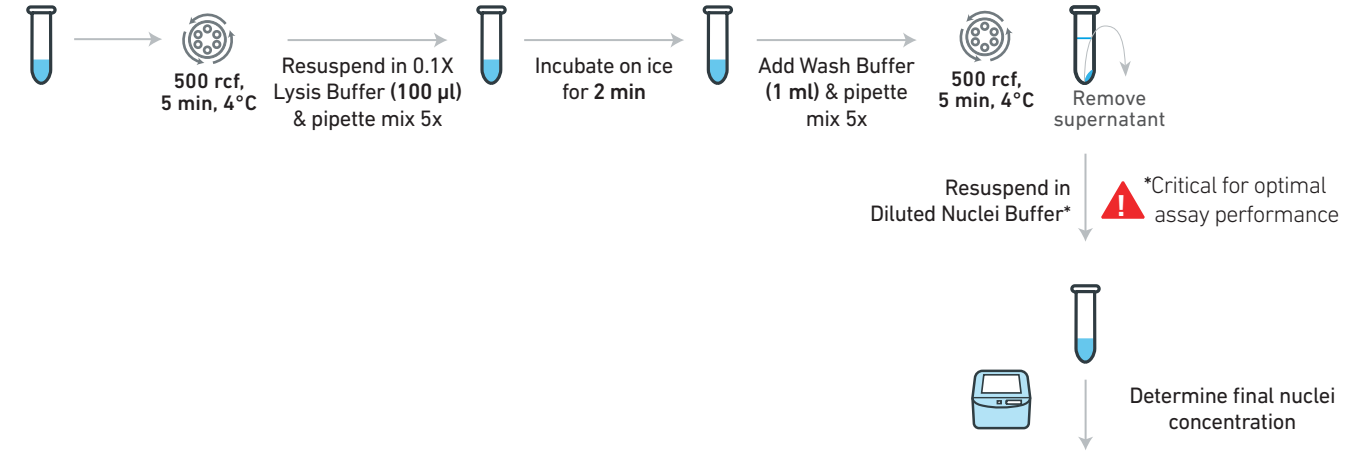
*Included in the 10x Genomics Single Cell Multiome ATAC Kit A
†Two of this part number are required

Protocol Overview

Nuclei Isolation



Nuclei Permeabilization



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

Protocol: Nuclei Isolation

1.1 Nuclei Isolation

If using frozen tissue, DO NOT thaw tissue prior to lysis.

a. Cut tissue into small pieces (size of rice grain) and transfer to a 1.5-ml microcentrifuge tube.

b. Add **300 µl** NP40 Lysis Buffer and homogenize 15x using a Pellet Pestle on ice.

c. Add **1 ml** NP40 Lysis Buffer.

d. Incubate for **5 min** on ice. Pipette mix a few times during incubation with a wide-bore pipette tip (regular-bore pipette tip may be used if tissue disintegrates easily). *OPTIMIZE based on cell type*

e. Pass the suspension through a **70 µm** strainer into a 15-ml conical tube.

f. Transfer the collected flowthrough to a 2-ml microcentrifuge tube.

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

h. Remove most of the supernatant, leaving ~**50 µl.**

i. Add **1 ml** PBS + 1% BSA + 1U/µl RNase Inhibitor. **DO NOT mix**.

j. Incubate for **5 min** on ice.

k. Pipette mix to resuspend the pellet.

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

m. Remove the supernatant.

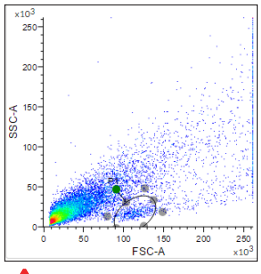
n. Resuspend with **1ml** PBS + 1% BSA + 1U/µl RNase Inhibitor (volume may be adjusted as needed for nuclei sorting).

o. Add **10 ul** 7AAD ready-made solution to 1-ml sample.

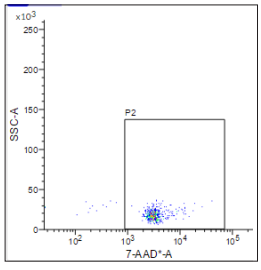
p. Incubate for **5 min** on ice.

q. 7AAD+ nuclei can be sorted using a 100 µm nozzle and a flow rate of 3 on a BD FACSMelody (or equivalent) into a 5-ml FACS tube containing **500 µl** BSA with **5,000U** RNase inhibitor. After sorting, assuming the final volume is 5-ml, the final concentration of the nuclei suspension should be 1% BSA and 1U/ul RNase inhibitor. If sorting yields less or more than 5-ml of final volume, adjust BSA and RNAse inhibitor concentration accordingly.

Step 1: Sorted nuclei are separated based on size and granularity



Step 2: 7AAD stained nuclei are separated from background and debris



Nuclei sorting is not recommended if user cannot retrieve at least 500,000 nuclei post-sorting.

r. Determine the cell concentration using a Countess II FL Automated Cell Counter or a hemocytometer.

1.2 Nuclei Permeabilization

a. Transfer sorted nuclei to a 15-ml conical tube and centrifuge at **500 rcf** for **5 min** at **4°C**.

b. Resuspend the pellet in **100 ul** 0.1X Lysis Buffer and pipette mix 5x.

c. Incubate for **2 min** on ice.

d. Add **1 ml** Wash Buffer and pipette mix 5x.

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

f. Remove the supernatant without disrupting the nuclei pellet.

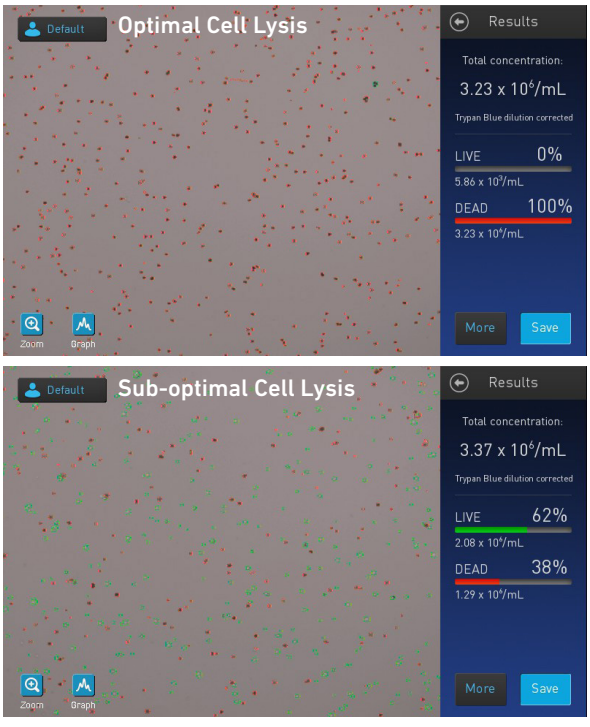
g. Based on the nuclei concentration estimated by the cell sorter and count post-sorting, resuspend in chilled Diluted Nuclei Buffer. See Nuclei Stock Concentration Table and Example Calculation in Appendix. Maintain on ice.

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.

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

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

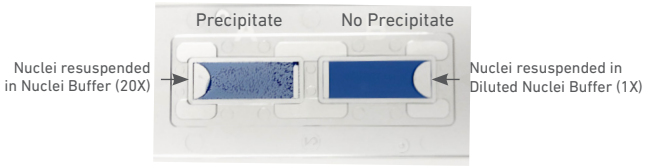
Results



Troubleshooting

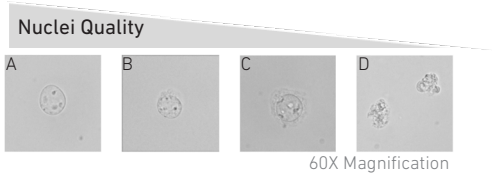
Problem	Possible Solution
High fraction of viable cells post cell lysis	Incrementally increase the lysis time and monitor lysis efficacy microscopically
Difficult to count nuclei/excess debris	Use a fluorescent dye (ethidium-homodimer-1) and fluorescence compatible cell counter or microscope
Excessive 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

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.

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

References

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

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