DEMONSTRATED PROTOCOL

Nuclei Isolation from Embryonic Mouse Brain for Single Cell Multiome ATAC + Gene Expression Sequencing

Overview

This protocol outlines how to isolate, wash, and count single nuclei from fresh, cryopreserved, and flash frozen mouse brain tissue samples for use with the Chromium Next GEM Single Cell Multiome ATAC + Gene Expression (GEX) protocol (CG000338). Tissue triturated into a nearly single cell suspension and subsequently frozen in media containing 10% DMSO produced metrics comparable to fresh tissue. High quality data can also be obtained using flash frozen tissue.

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 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
E18 Mouse Combined Cortex Hippocampus & Ventricular Zone (comes in NbActiv-1)	Mouse	BrainBits, LLC (Catalog number: C57EHCV)

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

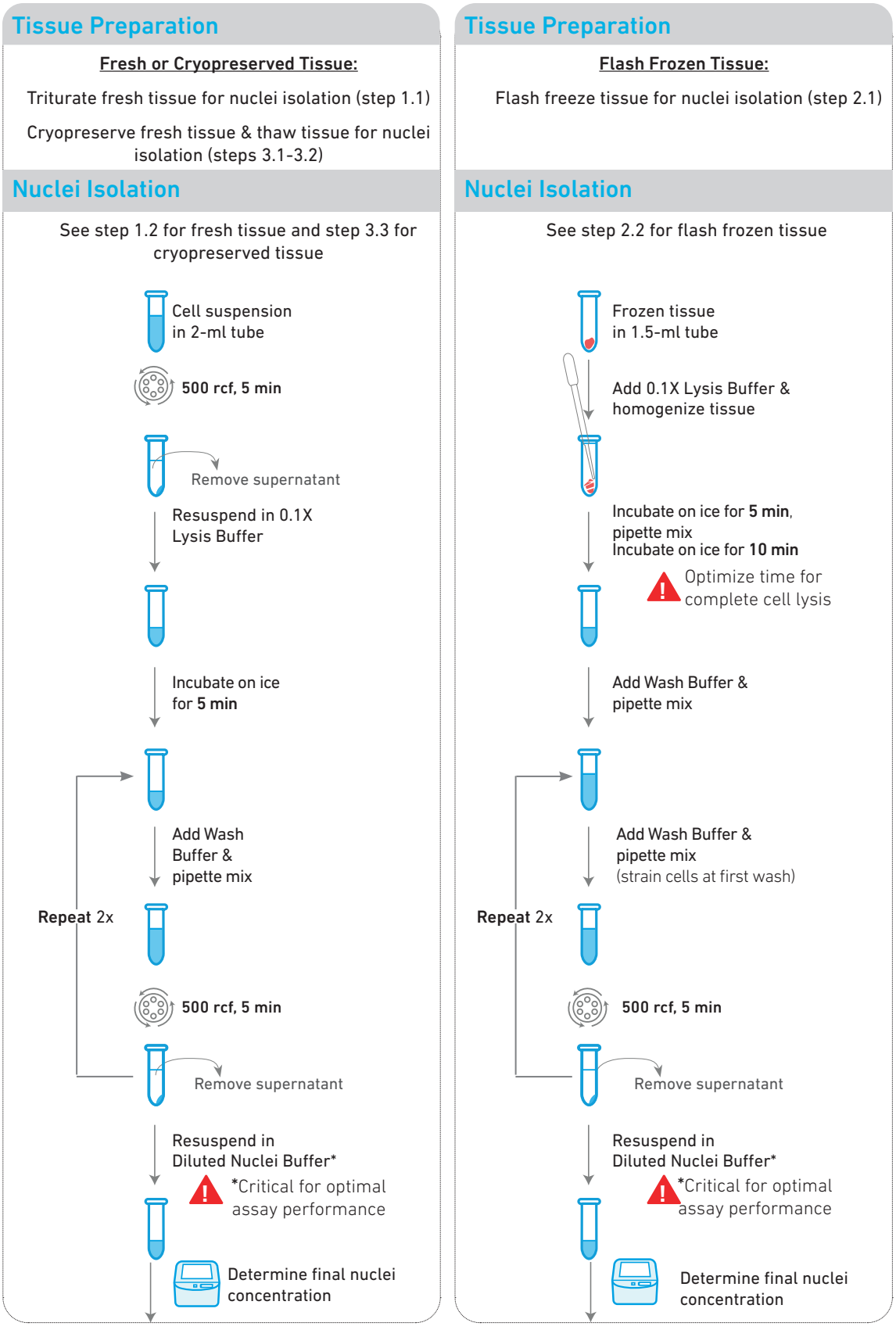
Diluted Nuclei Buffer Prepare fresh, maintain at 4°C	Stock	Final	1 ml
Nuclei Buffer* (20X)	20X	1X	50 µl
DTT	1000 mM	1mM	1 µl
RNase inhibitor (check vendor-specific stock concentration)	40 U/µl	1 U/µl	25 µl
Nuclease-free Water	-	-	924 µl
Wash Buffer **Prepare fresh, maintain at 4°C**	**Stock**	**Final**	**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
Lysis Dilution 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
DTT	1000 mM	1 mM	2 µl
RNase inhibitor	40 U/µl	1 U/µl	50 µl
Nuclease-free Water	-	-	1.72 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			
NbActiv-1			
NbActiv-1 + 10% DMSO			

Specific Reagents & Consumables

Vendor	Item	Part Number
10x Genomics	Nuclei Buffer* (20X)	2000153/2000207
Thermo Fisher Scientific	Digitonin LIVE/DEAD Viability/Cytotoxicity Kit	BN2006 L3224
VWR	Air-Tite All-Plastic Norm-Ject Syringes	53548-003
Miltenyi Biotec	MACS SmartStrainers (30 µm) MACS BSA Stock Solution	130-098-458 130-091-376
Sigma-Aldrich	Nonidet P40 (NP40) Substitute (alternatively, use IGEPAL CA-630)	74385 i8896
	Trizma Hydrochloride Solution, pH 7.4 Sodium Chloride Solution, 5 M Magnesium Chloride Solution, 1 M Sigma Protector RNase inhibitor (substitution is not recommended) DTT	T2194 59222C M1028 3335402001 646563
Fisher Scientific	RNase-Free Disposable Pellet Pestles	12-141-368
Bel-Art	Flowmi Cell Strainer, 40 µm Flowmi Cell Strainer, 70 µm	H13680-0040 H13680-0070
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

Protocol Overview



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

Protocol 1:
Nuclei Isolation from Fresh Tissue

1.1 Tissue Preparation

Triturate Tissue:

a. Triturate the tissue in **1 ml** NbActiv1 with a wide-bore pipette tip to break the tissue into small pieces. If using embryonic brain, triturate with a regular-bore pipette tip.

b. Continue triturating with a regular-bore pipette tip until the tissue is completely broken up.

c. Pass the triturated tissue through a 30 µm MACS SmartStrainer into a 15-ml conical tube.

d. Use the back of a syringe to gently press the tissue through the strainer. DO NOT grind the tissue when passing through the strainer as this may disrupt the chromatin structure.

e. Pass **1 ml** NbActiv1through the strainer and collect the flowthrough in the same conical tube.

f. Determine the concentration using a Countess II FL Automated Cell Counter (see Appendix).

1.2 Nuclei Isolation

Nuclei may be isolated from 100,000-1,000,000 cells using this protocol.

a. Add cells to a 2-ml microcentrifuge tube and centrifuge at **500 rcf** for **5 min** at **4°C**.

b. Remove the supernatant without disrupting the cell pellet.

c. Add **100 µl** chilled 0.1X Lysis Buffer. Pipette mix 5x.

d. Incubate for **5 min** on ice.
OPTIMIZE based on cell type

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

f. Centrifuge cells 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 the cell concentration at step 1.1f and assuming ~50% nuclei loss during cell lysis, resuspend in chilled Diluted Nuclei Buffer. See Nuclei Stock Concentration Table and Example Calculation in the User Guide. Maintain on ice.

The resuspension in Diluted Nuclei Buffer is critical for optimal Single Cell Multiome ATAC + GEX 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).

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

Protocol 2: Nuclei Isolation from Flash Frozen Tissue

2.1 Tissue Preparation

Freeze Tissue:

a. Cut the tissue into small pieces (size of rice grain).

b. Flash freeze the tissue pieces in liquid nitrogen and transfer to a cryovial.

c. Transfer the cryovials to vapor-phase nitrogen for long-term storage.

2.2 Nuclei Isolation

DO NOT thaw the tissue prior to lysis.

a. Using forceps, transfer the frozen tissue to a 1.5-ml microcentrifuge tube.

b. Add **500 µl** chilled 0.1X Lysis Buffer. **Immediately** homogenize 15x using a Pellet Pestle.

c. Incubate for **5 min** on ice.
OPTIMIZE based on cell type

d. Pipette mix 10x with a wide-bore pipette tip (regular-bore pipette tip may be used if tissue disintegrates easily).

e. Incubate for **10 min** on ice.
OPTIMIZE based on cell type

f. Using a regular-bore pipette tip, add **500 µl** chilled Wash Buffer to the lysed cells. Pipette mix 5x.

g. Pass the suspension through a 70 µm Flowmi Cell Strainer into a 2-ml tube. Filter ~**300 µl** suspension at a time, each time through a new 70 µm strainer.

h. Pass the collected suspension through a 40 µm Flowmi Cell Strainer.

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

j. Remove the supernatant without disrupting the nuclei pellet.

k. Add **1 ml** chilled Wash Buffer. Pipette mix 5x.

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

m. Remove the supernatant without disrupting the nuclei pellet.

n. Add **1 ml** chilled Wash Buffer. Pipette mix 5x.

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

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

q. Remove the supernatant without disrupting the nuclei pellet.

r. Based on the cell concentration at step 2.2o and assuming ~50% nuclei loss during cell lysis, resuspend in chilled Diluted Nuclei Buffer. See Nuclei Stock Concentration Table and Example Calculation in the User Guide. Maintain on ice.

The resuspension in Diluted Nuclei Buffer is critical for optimal Single Cell Multiome ATAC + GEX 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.

s. **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.

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

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

Protocol 3: Nuclei Isolation from Cryopreserved Tissue

3.1 Tissue Freezing

a. Triturate the tissue in at least **1 ml** NbActiv1 with a wide-bore pipette tip to break the tissue into small pieces. If using embryonic brain, triturate with a regular-bore pipette tip.

b. Continue triturating with a regular-bore pipette tip until the tissue is completely broken up.

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

d. Remove the supernatant and resuspend the pellet in **1 ml** NbActiv1 media.

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

f. Remove the supernatant and resuspend the pellet in **1 ml** chilled NbActiv1 containing 10% DMSO.

g. Dispense **1 ml** cell suspension into pre-cooled cryovials and place the cryovials inside a pre-cooled cell freezing container e.g., CoolCell FTS30.

h. Place the cell freezing container in a **–80°C** freezer for **≥4 h**. Transfer the cryovials to vapor-phase nitrogen for long-term storage.

3.2 Tissue Thawing

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

b. Add **1 ml** warm NbActiv1. Pipette mix 3x.

c. Transfer the suspension to a 15-ml conical tube containing **10 ml** warm NbActiv1.

d. Place a 30 µm MACS SmartStrainers over a new 15-ml conical and pass the entire suspension through it.

e. Pass **1-2 ml** NbActiv1through the strainer and collect the flowthrough in the same conical tube.

f. Centrifuge at **500 rcf** for **5 min**.

g. Resuspend in **1-2 ml** NbActiv1.

h. Determine the concentration using a Countess II FL Automated Cell Counter (see Appendix for Nuclei Count).

3.3 Nuclei Isolation

Nuclei may be isolated from 100,000-1,000,000 cells using this protocol.

a. Add cells to a 2-ml microcentrifuge tube and centrifuge at **500 rcf** for **5 min** at **4°C**.

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

c. Add **100 µl** chilled 0.1X Lysis Buffer. Pipette mix 5x.

d. Incubate for **5 min** on **4°C**.

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 the cell concentration at step 3.2h and assuming ~50% nuclei loss during cell lysis, resuspend in chilled Diluted Nuclei Buffer. See Nuclei Stock Concentration Table and Example Calculation in the User Guide. Maintain on ice.

The resuspension in Diluted Nuclei Buffer is critical for optimal Single Cell Multiome ATAC + GEX 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.

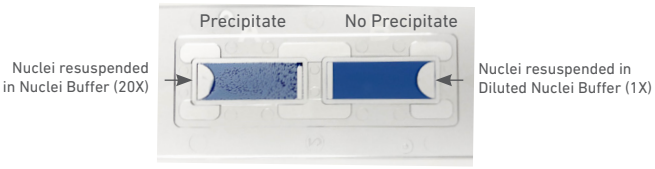
k. Determine the nuclei concentration using a Countess II FL Automated Cell Counter (see Appendix for Nuclei Count).

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

Troubleshooting

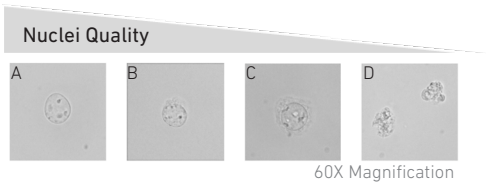
Problem	Possible Solution
High fraction of viable cells post cell lysis	Incrementally increase the lysis time and monitor lysis efficacy microscopically
Difficult to count nuclei/excess debris	Use a fluorescent dye (ethidium-homodimer-1) and fluorescence compatible cell counter or microscope
Excessive debris	Sample may be cleaned by extra washes/ filtering/density centrifugation/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 Count

This protocol provides instructions for counting nuclei using a fluorescent dye – Ethidium Homodimer-1 (part of LIVE/DEAD Viability/Cytotoxicity Kit) and the Countess II FL Automated Cell Counter (with RFP light cube) to enable accurate quantification even in the presence of sub-cellular debris. The optimal cell concentration for the Cell Counter is **1,000-4,000 cells/µl**. Refer to manufacturer's instructions for details on operations.

a. Vortex Ethidium Homodimer-1, centrifuge briefly, and dilute the concentrated stock as per manufacturer's instructions (~1:100 dilution) and aliquot **10 µl** Nuclear Stain in each tube.

b. Using a **regular-bore** pipette tip, gently mix the nuclei suspension. I**mmediately** add **10 µl** nuclei suspension to **10 µl** Nuclear Stain aliquot. Gently pipette mix 10x.

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

d. Insert the slide into the Countess II FL Cell Counter. Image the nuclei using the RFP setting for fluorescent illumination and filtering. Optimize focus and exposure settings. Confirm the absence of large clumps using the bright-field mode.
Note the RFP-positive concentration. Multiply by dilution factor 2 (from step b) to determine nuclei concentration.

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

Example Calculation

Cell number for lysis in step 1.2a: **200,000**
Estimated total nuclei recovered based on count at step 1.2j (assuming ~50% loss): **100,000**
If targeting 5,000 Nuclei Recovery, nuclei pellet at step 1.2h may be resuspended in **30 µl** Diluted Nuclei Buffer for Nuclei Stock Concentration of 1,610-4,030 nuclei/µl (see Table above)

References

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

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