PluS4PiGs: Cell tools for pig genetics

PluS4PiGs – Protocols

PluS4PiGs

Getting true Pluripotent Stem Cells in Pigs: a key step for large scale ex-vivo "Genotype to Phenotype" studies

Protocol 5
Derivation of pESC lines, dissociation to produce a single cell suspension and nuclei preparation

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https://api.faang.org/files/protocols/samples/INRAE_SOP_PLUS4PIGS_pESCs_DISSOCIATION_PROTO5_20240710.pdf

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1 Summary

To address the current challenges of animal breeding, a better knowledge of the link between genotype and phenotype is necessary. The use of pluripotent stem cells is an interesting solution because these cells can be driven in vitro to differentiate towards all cell lineages and their genome can be easily manipulated. These characteristics make PSCs powerful tools to assess the causality of genetic variants associated with cellular intermediate phenotypes. We propose, within the framework of the PluS4PiGs project, to use the numerical dimension from multi-omics data at the single-cell and tissue scale to predict the molecules necessary and sufficient to maintain porcine pluripotency and transfer this knowledge for the production and use of porcine pluripotent embryonic stem cell lines (pESCs) for animal and human health applications.

We generated single-cell expression (scRNA-seq) and chromatin accessibility (scATAC-seq) data from two pESC lines produced in our laboratory. All of these data will be used to identify and characterize pESC lines and to compare them with the embryonic pluripotent epiblast.

To perform this project, we first derived pESCs from pig embryos at day 7 and day 9 after insemination with associated metadata describing accurately the samples and the sampling process.

We describe here the procedures used to produce pESCs, and to produce a single-nuclei suspension ready to load for scRNA-seq and scATAC-seq assays.

2 Protocol description

2.1 Required reagents and instruments

- A fully equipped cell culture room
- Sterile disposable cell culture dishes
- 4-well culture plates (NUNC)
- 1.5 mL and 2 mL Eppendorf tubes
- 15 mL Falcon tubes
- Latex gloves
- A dissection stereomicroscope with epi-illumination
- An inverted cell culture microscope
- Dulbecco's Phosphate-Buffered Saline (DPBS)
- DMEM/F12
- Glutamax
- Penicillin-Streptomycin
- Non Essential Amino Acids 100X
- 2-Mercaptoethanol 50mM
- Plasmocin (25mg/ml)
- Vitamin C (ascorbic acid) 300mg/ml
- CD Lipids concentrate
- KOSR
- N2-Plus supplement
- B27 supplement
- XAV-939 25mM
- Activin A (100ug/ml)
- bFGF 250ng/ml

- Embryo holding media (IMV Technologies)
- Accutase (Thermo)
- TrypLE (Thermo)
- P1000, P200 and P20 pipettes with tips
- Neubauer or Malassez cell counting chamber
- Cell strainer (70 µM)
- Labtop minifuge (TOMY)
- Nuclease free water
- Tris HCl pH7.4 (Sigma T2194)
- NaCl (Sigma 59222C)
- MgCl2 (Sigma M1028)
- Tween20 (Thermo 28320)
- Nonidet P40 (Sigma 74385)
- BSA 0.5% (Thermo AM2614)
- DTT (Sigma 646563)
- RNase inhibitor (RNAsin Promega)
- Digitonin (Thermo BN2006)
- Diluted Nuclei Buffer (10X genomics 2000153/2000207)

- AFX medium: qsp DMEM/F12 500ml > 1% L-Glutamine, 1% Penicillin-Streptomycin, 1% non-essential amino acids, 100 µM 2-Mercaptoethanol, 5 µg/mL Plasmocin, 65 µg/mL Vitamin C, 1% CD Lipids concentrate, 2% KOSR, 0.5% N2-Plus supplement, 1% B27 supplement, 2 µM XAV939, 20 ng/mL Activin A, 10ng/ml bFGF

2.2 Reception of embryos

Embryos have been recovered following the protocol:

https://api.faang.org/files/protocols/samples/INRAE_SOP_PLUS4PIGS_EMBRYOS_SAMPLING_PROTO2_20230131.pdf

At arrival, embryos are transferred to AFX medium and individually distributed into a 4-well dish. The embryonic disc is then dissected under a stereomicroscope, and the resulting pieces are seeded into a 4-well coated with a mix of 1:1 Gelatin 0.1% and Matrigel (1/50 in DMEM/F12) in AFX medium.

2.3 ESCs amplification and characterization

The medium is changed daily. 4 days after seeding, the cells are split using Accutase and seeded into 24-well plates coated with a mix of 1:1 Gelatin 0.1% and Matrigel (1/50 in DMEM/F12) in AFX medium + Y27632 10uM (ROCK inhibitor). The cells are then amplified during several weeks and cryobanked.

pESCs have been validated for the expression of bona fide pluripotency markers (NANOG, OCT4, SOX2, ZIC3), their transcriptomic profiles and their ability to differentiate in vitro to various cell types.

2.4 Cell dissociation

At passage 10, for each cell line, around 3 millions of pESCs are recovered from a 60mm dish using Accutase. Cells are washed twice with PBS.

2.5 Nuclei isolation of cells

We kept 500,000 cells for each cell line. Nuclei have been isolated following the protocol from 10X Genomics: CG000365 • Rev A Nuclei Isolation for Single Cell Multiome

Each single cell suspension is washed twice with 100 ul of ice cold PBS+0.04% BSA and placed in a 0.2ml tube. Cells are centrifugated 1minute at full speed using the labtop minifuge. After the second wash, the supernatant is removed and 100ul of ice-cold lysis buffer is used (Tris HCl pH7.4 10mM; NaCl 10mM; MgCl2 3mM; Tween20 0.1%; Nonidet P40 0.1%; BSA 0.5%; DTT 1mM; RNase inhibitor 0.5U/ul). Cells are resuspended by pipetting up and down 10 times and are incubated on ice for exactly 7'. Then 1ml of ice-cold Wash Buffer (Tris HCl pH7.4 10mM; NaCl 10mM; MgCl2 3mM; Tween20 0.1%; BSA 0.5%; DTT 1mM; RNase inhibitor 0.5U/ul) is added to the tube, and cells are resuspended by pipetting up and down 5 times. Cells are centrifugated 1minute at full speed using the labtop minifuge. Then the supernatant is removed without disrupting the nuclei pellet and the washes are repeated 3 times. Finally the nuclei are resuspended in 50 µl chilled Diluted Nuclei Buffer.

2 µl nuclei suspension mixed with 8 µl Diluted Nuclei Buffer and 10 µl Trypan Blue is used to determine the cell concentration using a Malassez cell counting chamber. Around 5000 nuclei/ul have been recovered from each prep.

25,000 nuclei resuspended in 5ul of Nuclei Dilution Buffer are processed for transposition according to : ChromiumNextGEM_Multiome_ATAC_GEX_User_Guide_RevB.

3 Annexe : 10X nuclei isolation protocol

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. Optimization of some protocol steps (e.g. lysis time, centrifugation speed/time and filtration steps) may be needed based on cell type.

For optimal assay performance, nuclei isolation should be performed using this protocol and not the 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

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

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
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

Preparation - Buffers

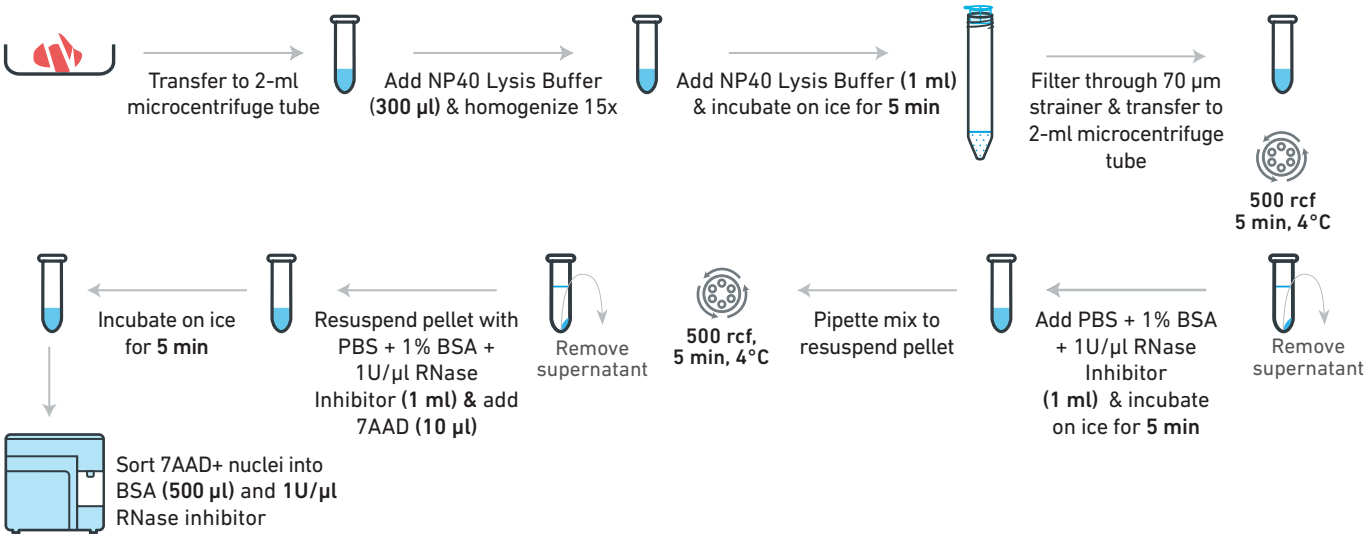
Lysis Dilution Buffer May be prepared ahead	Stock	Final	2 ml
Tris-HCl (pH 7.4)	1 M	10 mM	20 µl
NaCl	5 M	10 mM	4 µl
$MgCl_2$	1 M	3 mM	6 µl
BSA	10%	1%	200 µl
DTT	1000 mM	1 mM	2 µl
RNase inhibitor	40 U/µl	1 U/µl	50 µl
Nuclease-free Water	-	-	1.718 ml
0.1X Lysis Buffer Prepare fresh, maintain at 4°C	**Stock**	**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 Inhibitor BSA Stock Solution			

Specific Reagents & Consumables

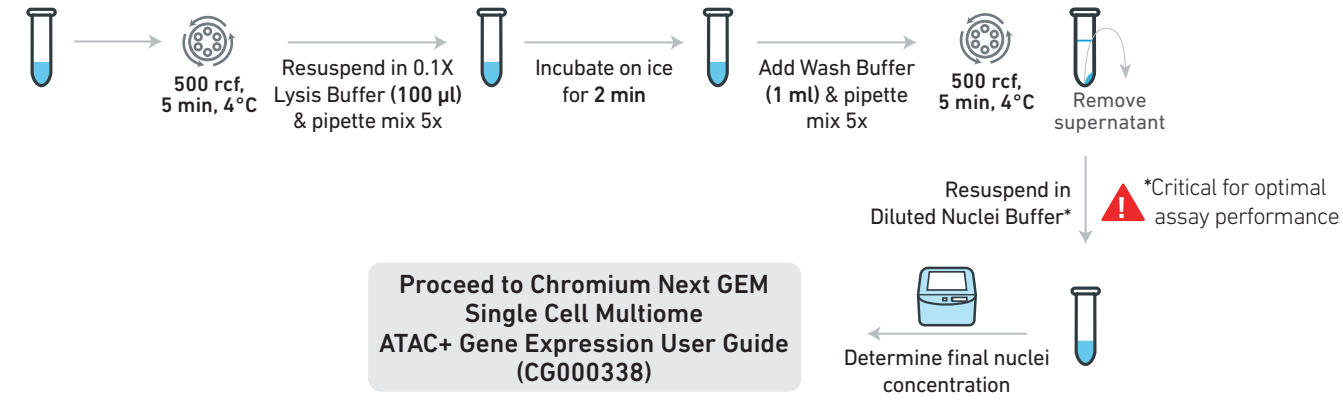
Vendor	Item	Part Number
10x Genomics	Nuclei Buffer* (20X)	2000153/ 2000207
Thermo Fisher	Digitonin	BN2006
	Tween 20 Surfact-Amps Det. Sol.	28320
Sigma-Aldrich	Trizma Hydrochloride Solution, pH 7.4	T2194
	Sodium Chloride Solution, 5 M	59222C
	Magnesium Chloride Solution, 1M	M1028
	Nonidet P40 Substitute	74385
	Sigma Protector RNase inhibitor† (if using an alternative PN, check vendor for equivalent inhibitor activity)	3335402001
	DTT	646563
	7-AAD Ready Made Solution	SML1633-1ML
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 Multiome ATAC Kit A
†Two of this part number are required

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 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).

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.

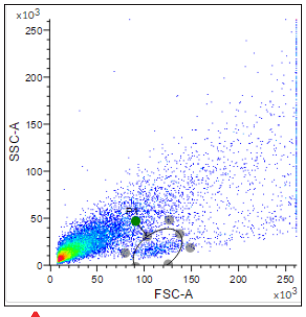
n. Resuspend with **1ml** PBS + 1% BSA + 1U/µl RNase Inhibitor (volume may be adjusted as needed for nuclei sorting).

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

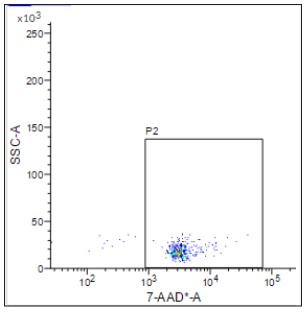
p. Incubate for **5 min** on ice.

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. After sorting, add enough RNase inhibitor to achieve a final concentration of 1U/ul. Ex. If sorting yields 5 ml of volume, add **125 µl** RNase inhibitor.

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.

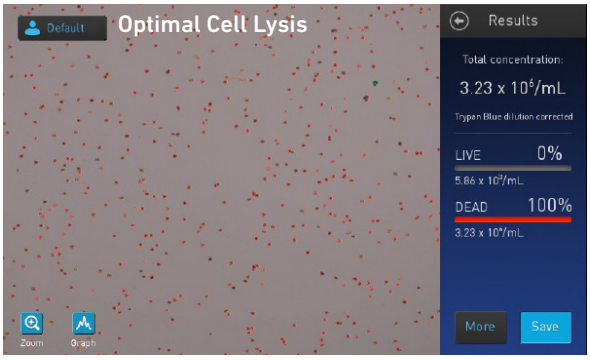
g. Based on the nuclei concentration estimated by the cell sorter and count post-sorting, resuspend in chilled Diluted Nuclei Buffer. See Nuclei Stock Concentration Table and Example Calculation in Appendix. Maintain on ice.

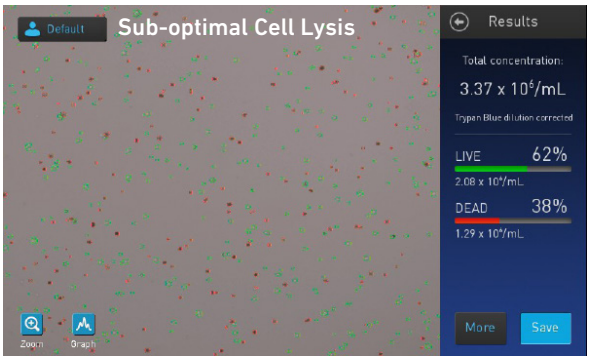
The resuspension in Diluted Nuclei Buffer is critical for optimal Single Cell ATAC assay performance. The composition of the Tris-based Diluted Nuclei Buffer, including Magnesium concentration, has been optimized for the Transposition and Barcoding steps in the Single Cell Multiome ATAC + GEX protocol. 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

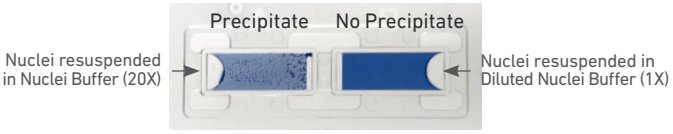




Troubleshooting

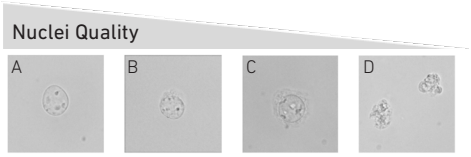
Problem	Possible Solution
Difficult to count nuclei/excess debris	Use a fluorescent dye (ethidium-homodimer-1) and fluorescence compatible cell counter or microscope
Low nuclei recovery	Use a swing-bucket rotor for centrifugation steps

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

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 1,610-4,030 nuclei/µl (see Table above)

References

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

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