snATAC-seq Protocol

-- Pengxin (Penny) Yang

-- 09.21.2020

-- PI: Dr. Christopher K Tuggle

This protocol is modified from DEMONSTRATED PROTOCOL Nuclei Isolation for Single Cell ATAC Sequencing CG000169 • Rev D of 10x Genomics.

Note:

- Solutions should not be more than 1 week old prior to use.
- 10x Genomics suggest optimize lysis time, lysis buffer, centrifugation time and removing background DNA (https://kb.10xgenomics.com/hc/en-us/articles/360020145652-How-can-I-optimize-my-nuclei-prep-for-Single-Cell-ATAC-sequencing-). Since we will use the lysis buffer recommended by 10x genomics and DNase treatment is included in the protocol below. The main purpose of our optimization is to optimize lysis time. Centrifugation will be the same with the recommended one unless undesired results are obtained.

1. Thaw Cells

1million cells/tube

Before starting, make sure media is pre-warmed in water-bath at 37°C, centrifuge at 4°C

a. Remove cryovial from storage, thaw in the water bath at 37°C for 1-2 min. Swirl gently to mix during thawing. Remove from the water bath when a tiny ice crystal remains. Dry tube.

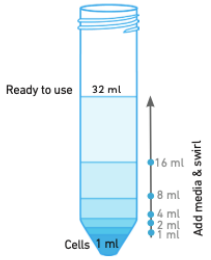
b. Pipette the thawed cells using wide-bore pipette tip to a 50-ml conical tube. Rinse the cryovial with 1 ml pre-warmed media (RPMI + 10% FBS) and add the rinse **drop-wise** to the 50-ml conical tube while gently swirling the tube.

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. (1ml which is added at 1b, +1ml +2ml +4ml + 8ml + 16ml = 32ml total)

Appendix

Illustrative Overview of Incremental1:1 Volume Additions

Incrementally add media volumes at the indicated points & swirl



d. Centrifuge at 300 rcf for 5 min, at 4ºC.
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 the cells to a 2-ml microcentrifuge tube using wide-bore pipette tip. 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.

2. DNase treatment:

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 (see apendix).
c. Pipette mix 5x using wide-bore pipette tip 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 cell concentration (live and death cells) using a Countess II FL Automated Cell Counter (see Appendix) or a hemocytometer. (go to 5.1.2 step)

3. Reduce fraction of dead cells

Note:

- This protocol is not recommended on freshly thawed cells as stressed cells will present phosphatidylserine on their cell surfaces and therefore become bound to and retained in the column. This will result in an even greater initial cell loss during the thawing and clean-up steps.
- The Dead Cell Removal protocol is performed at room temperature
- This protocol is only recommended when starting cell viability is <70%.

This protocol was demonstrated using sample sizes compatible with Miltenyi Biotech MS columns (≤2×10^8 total cells). If using sample sizes compatible with Miltenyi Biotec LS columns (≤2×10^9 total cells), consult the manufacturer's instructions.

a. Centrifuge the cell sample at 300 rcf for 5 min (PBMCs) at room temperature.
b. Remove the supernatant without disturbing pellet.
c. Add 100 µl Dead Cell Removal MicroBeads and resuspend cell pellet using a wide-bore pipette tip.
d. Incubate for 15 min at room temperature.
e. During incubation, prepare MS column by rinsing with 500 µl 1X Binding Buffer.
f. After incubation is complete, dilute the cell suspension (containing Dead Cell Removal MicroBeads) with 500 µl 1X Binding Buffer.
g. Apply cell suspension to the prepared column. The positively selected dead cells will be retained on the column while the negatively selected live cells pass through the column.
h. Collect the effluent (effluent 1) containing the live cell fraction in a 15-ml centrifuge tube.
i. Rinse the column with 2 ml 1X Binding Buffer and collect the effluent (effluent 2). Combine effluents 1 and 2.
j. Centrifuge cells at 300 rcf for 5 min at room temperature.
k. Remove the supernatant without disturbing the pellet.
l. Using a wide-bore pipette tip, add 1 ml 1X PBS + 0.04 % BSA to each tube and gently pipette mix 5x to resuspend cell pellet.
m. Transfer the cell suspension to a 2-ml tube.
n. Centrifuge cells at 300 rcf for 5 min at room temperature.
o. Remove the supernatant without disturbing the pellet.
p. Resuspend pellet in 1 ml 1X PBS + 0.04 % BSA.
q. Repeat n-o for a total of two washes.
r. Resuspend pellet in 1X PBS + 0.04 % BSA using a regular- bore pipette tip to achieve a cell concentration of 700-1,200 cells/µl (calculate the volume of PBS+BSA using the last live cells count in the step 2h). Gently pipette mix 10x or until cells are completely suspended.
s. Determine the cell concentration (live and death cells) using a Countess II FL Automated Cell Counter. If necessary, dilute the cells with additional 1X PBS + 0.04% BSA until the target cell concentration is reached. (go to 5.1.2 step)
t. Once the target cell concentration is achieved, place the cells on ice.
u. Proceed immediately to the 10x Genomics Single Cell protocols.

4. Nuclei Isolation

Nuclei may be isolated from 100,000-1,000,000 cells using this protocol. If cell count is <100,000, nuclei may be isolated using the Low Cell Input Nuclei Isolation protocol (see Appendix).

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

Note:
100,000-1,000,000 refer to the number of live, viable cells after cell removal kit in the initial cell suspension

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.

Note:
*Cryopreserved (human) PBMCs were incubated for 3 min
Optimize the incubation time: 1min 3min 9min→ choose the good one→ 30sec interval test around that time.

The incubation time at which <5% of input cells are viable is chosen as optimal time.

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 a 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.

Note:
For practice, resuspend lysed cells in the Wash buffer;
(suggestion from DNA facility: for practice, you should be able to suspend in what your cells are in.)
For final two whole PBMC samples, resuspend lysed cells in the chilled Diluted Nuclei Buffer provided by DNA facility.

The targeted nuclei recovery would be 10000 which means the nuclei stock concentration should be 3,080-7,700 (nuclei/ul). The volume of added Diluted Nuclei Buffer depends on the total number of nuclei.
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.
Determine the nuclei concentration using a Countess II FL Automated Cell Counter (see Appendix) or a hemocytometer:

5. Nuclei quality and concentration using cell counter/hemocytometer

5.1 Cell Viability Measurements:

Trypan blue along with an automated counter or a hemocytometer can be used to measure the sample's viability. Unlysed live cells will not stain (remain white) while nuclei will stain blue as dead cells do. Measurements of <5% live input cells indicate proper cell lysis. Example cell viability measurements and images are available in the Demonstrated Protocols 'Nuclei Isolation for Single Cell ATAC Sequencing' and 'Nuclei Isolation from Mouse Brain Tissue for Single Cell ATAC Sequencing'

Commented [CKT1]: How many cells do you plan to use? Will you be able to see a pellet of 200,000 to 500,000 cells?

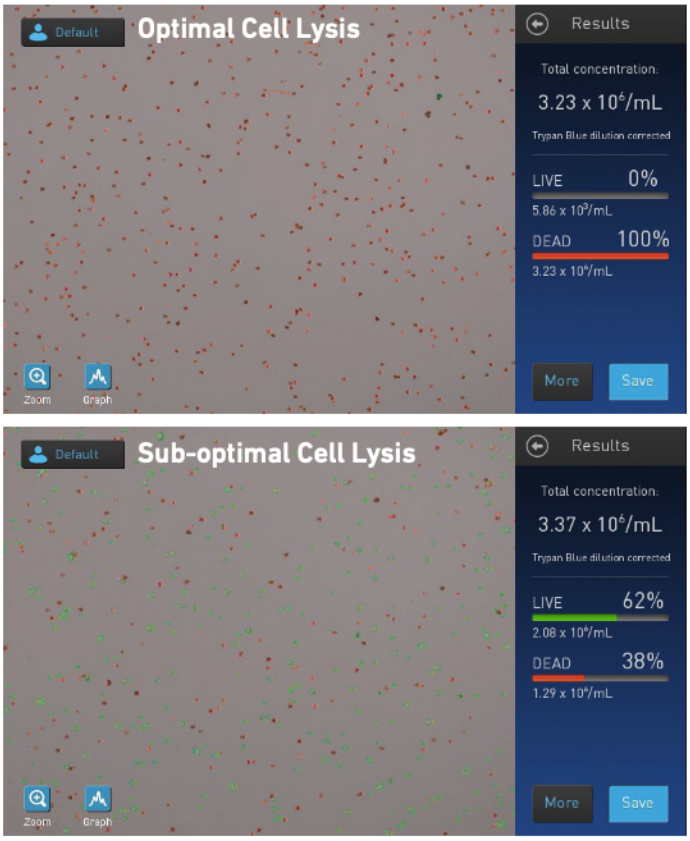
Commented [JHU2R1]: She will try to get the range of 100K-1M cells at the beginning of the nuclei isolation steps. It's possible see the pellet of 500K cells.

Note: For samples with debris, ethidium homodimer-1 or other fluorescent dyes may help distinguish nuclei from debris for accurate quantitation. In our case, we found it's better to use Ethidium homodimer-1 to stain nuclei and measure its concentration of using Countess II FL Automated Cell Counter.

5.1.1 If use Countess II FL Automated Cell Counter:

The optimal range of cell concentration for Cell Counter is 1,000-10,000 cells/µl.

1. Vortex 0.4% trypan blue stain, centrifuge briefly and aliquot 10 µl per tube.
2. 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.
3. Transfer 10 µl trypan blue stained nuclei to a Countess II Cell Counting Slide chamber.
4. 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.



5.1.2 If use the hemocytometer:

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 the space between the coverslip and the chamber

d. Use the 40x magnification to focus on the grid, and determine the nuclei concentration and viability. <5% of input cells should be viable. Optimize focusing and light exposure.

e. Count nuclei that don't touch the top or left lines in the four squares (Figure 1).

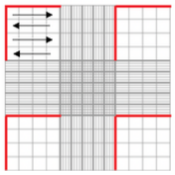


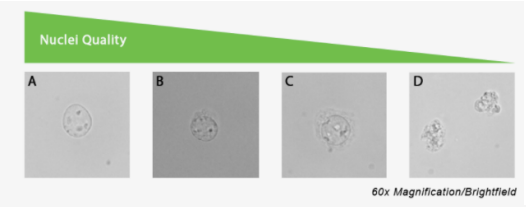
Figure 1. Hemocytometer. Count cells in each square following the lines.

f. Calculate the number of nuclei (nuclei/mL):

$$\text{Nuclei density} = \frac{(\text{Total cells} / 4 \text{ squares}) \times \text{dilution factor (1:10)}}{\text{Square volume (0.0001 mL)}}$$

5.2 Visualization with Microscopy:

The nuclei may also be assessed by visualization under a microscope. A good nuclei suspension will show clump-free, debris-free nuclei. Using a high-powered microscope (at least 40x), it may be possible to visualize individual nuclei to assess the membrane quality. A nucleus with an intact membrane should appear round and smooth (Panel A). A nucleus with a compromised membrane will appear "ruffled" and show evidence of blebbing (Panels B-D). Blebbing is the loss of coherence of the nuclear membrane with the nucleoplasm and is normally caused by overlysis. In general, users need to optimize the lysis protocol.



A: High-quality nuclei have well-resolved edges. Optimal quality for single cell ATAC libraries.
B: Mostly intact nuclei with minor evidence of blebbing. Quality single cell ATAC libraries can still be produced.
C: Nuclei with strong evidence of blebbing. Proceed at your own risk.
D: Nuclei are no longer intact. Do not proceed!

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

6. Making Buffers:

(1) RPMI + 10% FBS. (41 ml / million cells)
50ml = 45ml RPMI + 5ml FBS

(2) PBS + 0.04% BSA (~6.5 ml / million cells)
10ml = 10ml PBS + 10ml * 0.04/100 BSA = 10ml PBS + 0.004ml BSA
(3) DNase Solution (300ul/million cells)
(4) 0.4% trypan blue stain (Juber can provide)
(5) 1x Binding Buffer
provided with MACS Dead Cell Removal Kit. Dilute 20x with Nuclease-free Water (Nuclease-free Water is suggested by 10x genomics; ddH2O is suggested by the datasheet of this dead cell removal kit) to get 1x
(5) Dead Cell Removal MicroBeads (provided with MACS Dead Cell Removal Kit)
(6)
Wash Buffer (1ml/million cells)

Prepare fresh,maintain at 4^0C	Stock	final	2ml
Tris-HCl (pH 7.4)	1M	10mM	20ul
NaCl	5M	10mM	4ul
$MgCl_2$	1M	3mM	6ul
BSA	10%	1%	200ul
Tween-20	10%	0.1%	20ul
Nuclease-free Water			1.75ml

1M Tris-HCl (pH 7.4): ordered.
5M NaCl:
25ml = 5mol/L * 58.44 g/mol * 0.025L = 7.305g NaCl + 25ml H2O (Nuclease-free Water)
1M MgCl2:
25ml = 1mol/L * 95.211 g/mol * 0.025L = 2.380g MgCl2 + 25ml H2O

10% BSA: 1000ul = 100ul BSA + 900ul H2O
10% Tween-20: 1000ul = 100ul + 900ul H2O
(7)
Lysis Buffer(100ul/million cells)

Prepare fresh,maintain at 4^0C	Stock	final	2ml
Tris-HCl (pH 7.4)	1M	10mM	20ul
NaCl	5M	10mM	4ul
$MgCl_2$	1M	3mM	6ul
Tween-20	10%	0.1%	20ul
Nonidet P40 Substitute	10%	0.1%	20ul
Digitonin	5%	0.01%	4ul
BSA	10%	1%	200ul
Nuclease-free Water			1.726ml

10% Nonidet P40 Substitute: 20ml = 2ml Nonidet P40 Substitute + 18ml H2O

5% Digitonin: 50ul = 2.5ul Digitonin + 47.5ul H2O

10X TBS	Stock	final	5ml
Tris-HCl (pH 7.4)	1M	200mM	1ml
NaCl	5M	1.5M	1.5ml
Nuclease-free Water			2.5ml

DNase solution	Stock	final	1ml
TBS	10X	1X	100ul
10x Reaction Buffer with $MgCl_2$	10X	1X	100ul
DNase I	1U/ul	0.1U/ul	100ul
Nuclease-free Water			700ul