

Mar 06,
2020

Protocol for nuclei isolation from fresh and frozen tissues for snRNA-Seq and snATAC-Seq on 10x Chromium™ platform using the same nuclei preparation V.1

Luciano Martelotto¹

¹Single Cell Innovation Lab (SCIL), University of Melbourne Centre of Cancer Research (UMCCR)

1 Works for me dx.doi.org/10.17504/protocols.io.9p8h5rw



Luciano Martelotto
University of Melbourne



ABSTRACT

This protocol is an adaptation and extension of the 'Frankenstein' protocol – originally developed for nuclei isolation from fresh and frozen tissue for snRNA-Seq – in order to perform snATAC-Seq on the same nuclei prep.

It has been successfully applied to fresh, snap/flash and cryopreserved frozen cell lines as well as to tissue derived from solid tumours and other tissues such as pancreas adenocarcinoma (PDAC), breast cancers, pheochromocytomas/paragangliomas, normal paraganglia, brain organoids, PDAC organoids, ovary, fallopian tube, mouse brain and sperm using the Chromium Platform (10x Genomics).

ATTACHMENTS

[Protocol_draft10.pdf](#)

GUIDELINES

Protocol Overview



This protocol requires access to a cell sorter and familiarity with sorting cells/nuclei into 96-well plates.



SUCCESS of this protocol heavily rely on **SAMPLE QUALITY**. Below are some of the steps I follow:

-For new sample types, and when possible (with minute samples is hard to do it) I evaluate the lysis efficacy by assessing under light or fluorescent microscope after ⌚ 00:03:00 - ⌚ 00:05:00 for single cell suspensions (cell lines) and ⌚ 00:05:00 - ⌚ 00:10:00 for tissues. This will ensure you avoid over- or under-lysis the cells.

-The cell lysis and washes are always carried out on wet-ice (i.e. ⚡ 4 °C) and in the presence of RNase inhibitor 0.2-0.5 U/uL.

-Centrifugation, in my hands, works well at 🌀 500 x g, 4°C 00:05:00 and this is enough for most of the tissues types I worked on. However, some optimization might be needed for specific tissues.

-I always use LoBind nucleases free tubes.

-I always inspect nuclei under microscope using Trypan Blue to give more contrast and also to count.

-When debris and clump are an issue and I solved this issue I use sorting as explained in the protocols.

-Nuclei sizes and shapes under microscope varies from sample to sample so shape or roundness is not the only feature to check. Also look for signs of disorganisation of chromatin, this is usually quite visible as if burst nucleus.

-In good quality nuclei nucleoli may be visible.

-I use Flowmi 40 µm filters before FACS sorting or before loading onto chip.

-For additional tips on sample prep please check <https://support.10xgenomics.com/single-cell-gene-expression/sample-prep>.

1. Use a plastic pestle to mechanically homogenize tissue and release nuclei
2. Separate the nuclei from debris using a cell sorter (if not, then see note at the end)
3. Collect a specific number of nuclei in a 96-well plate containing 10x RT Buffer* or Wash Buffer**
4. Immediately load the sample into a Single Cell Chip for processing according the Single Cell 3' v3 Reagents User Guide or Single Cell V(D)J 5' Reagents User Guide.
5. In our hands the use of DAPI, 7-AAD and DRAQ-7 dyes show very little or no effect on ATAC data metrics.

*Consider the event overestimation of some sorters (Check step 19). Also, assume that nuclei recovery is ~ 57 %; use this to determine the number of nuclei to collect for each of your samples. [This value is derived from the Cell Suspension Volume Calculator Table in the Single Cell 3' and v3 - Reagents User Guide or Single Cell V(D)J 5' v1 Reagents User Guide]

**Assume that nuclei loss can be up to ~ 50 – 60 % due to loss during washing and counting; use this to determine the number of nuclei to collect for each of your samples.



IMPORTANT NOTE:

If you are NOT sorting nuclei that is in Nuclei Wash and Resuspension Buffer directly into ATAC Wash Buffer-Dig, avoid resuspending pelleted nuclei that was in Nuclei Wash and Resuspension Buffer (PBS-based) directly in Diluted Nuclei Buffer or ATAC Wash Buffer-Dig as nuclei tend to clump.

Instead, you need to do a *buffer exchange* (from PBS to Tris) by adding at least 2x the volume of ATAC Wash Buffer-Dig [Tris-HCl 10 mM (pH 7.4), NaCl 10 mM, MgCl₂ 3 mM, BSA 1%, Tween-20 0.1 %, Digitonin 0.01%] to the nuclei that is **in suspension** in the Nuclei Wash and Resuspension Buffer (PBS-based).

Let equilibrate for 🕒 **00:05:00** (buffer exchange). After this, pellet and do all washes in ATAC Wash Buffer-Dig before resuspending nuclei in Diluted Nuclei Buffer.

Below are examples of nuclei before and after sorting, bioanalyzer traces of 5' nuclei cDNA and snATAC library as well as representative metrics obtained with Cell Ranger for this example.

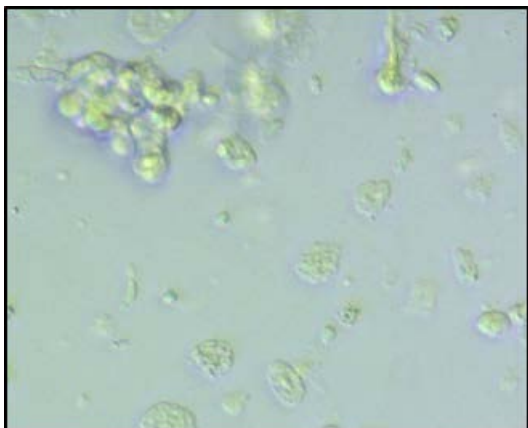


Fig 1. Nuclei before sorting.

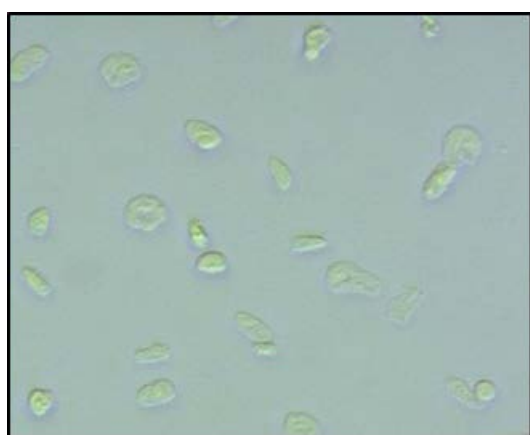


Fig 2. Nuclei after sorting.

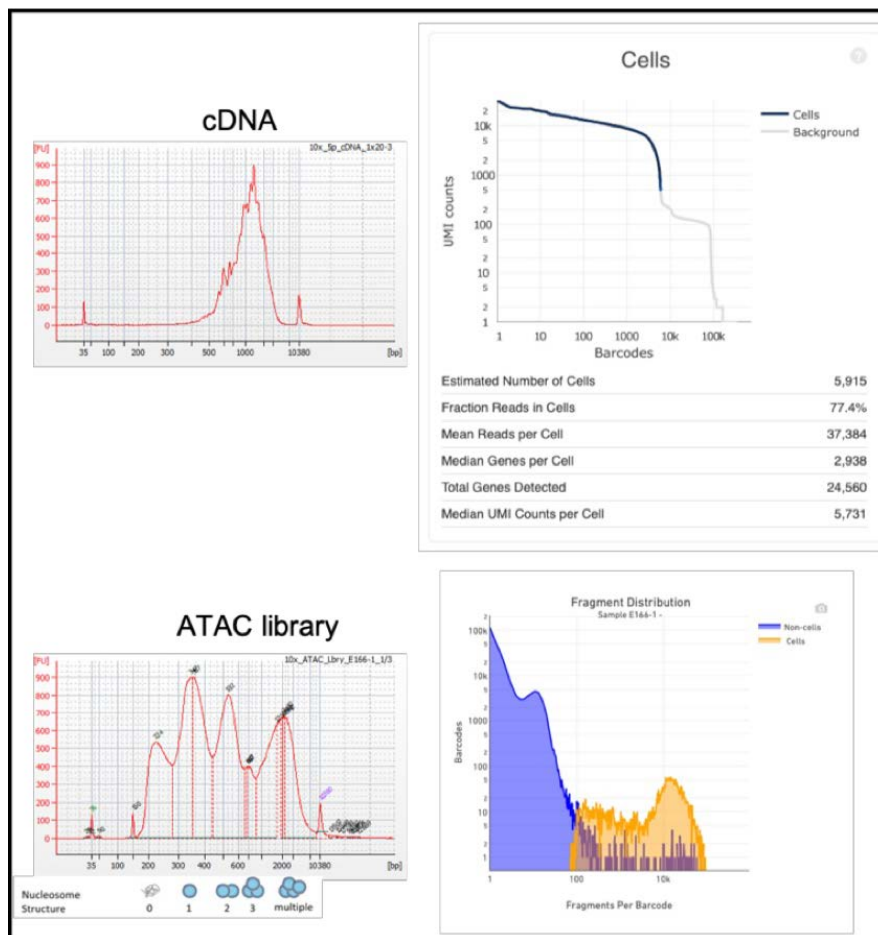


Fig 3. cDNA and ATAC library traces. Metrics obtained with Cell Ranger

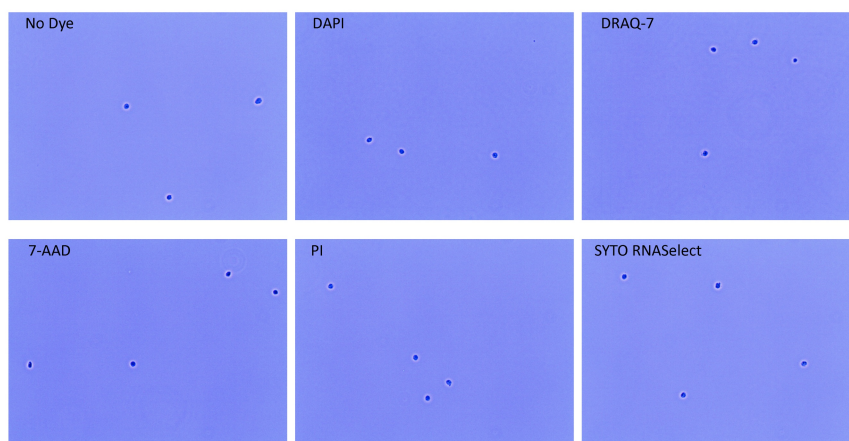


Fig 4. Representative photos of how clean the nuclei looks after sorting and right before loading on the Chromium.

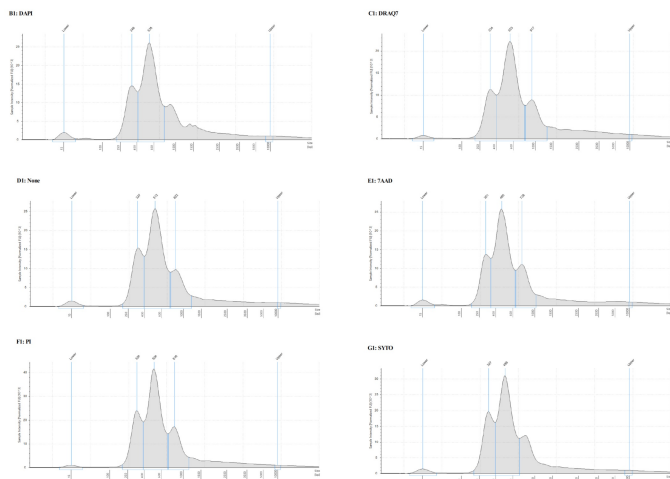


Fig 5. TapeStation traces of ATAC libraries.

No dye

Sequencing

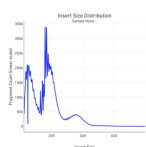
Total number of read pairs	73,865,478
Fraction of read pairs with a valid barcode	97.9%
G30 bases in Read 1	93.4%
G30 bases in Read 2	92.5%
G30 bases in Barcode	92.4%
G30 bases in Sample Index	None

Cells

Estimated number of cells	783
Lower threshold on the number of fragments overlapping peaks per barcode to annotate barcode as cell	83.88
Median fragments per cell	43,893
Median fragments per non-cell barcode	1

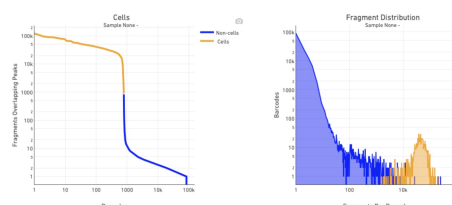
Insert Sizes

Fragments in nucleosome-free regions	39.3%
Fragments flanking a single nucleosome	48.4%



69.5%
Fraction of fragments overlapping any targeted region

57.7%
Fraction of transposition events in peaks in cell barcodes



Targeting

Enrichment score of transcription start sites	3.17	Fraction of total read pairs mapped confidently to genome (≥30 bp)	79.3%
Fraction of fragments overlapping TSS	38.7%	Fraction of total read pairs that are unmapped and in cell barcodes	8.9%
Fraction of fragments overlapping called peaks	59.5%	Fraction of total read pairs in mitochondria and in cell barcodes	8.8%
Fraction of transposition events in peaks in cell barcodes	57.7%		
Fraction of fragments overlapping any targeted region	69.5%		

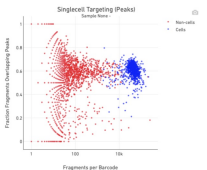
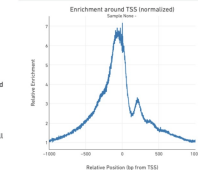


Fig 6. No Dye: Representative ATAC QC metrics from Cell Ranger.

DAPI

Sequencing

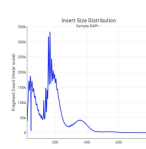
Total number of read pairs	83,819,812
Fraction of read pairs with a valid barcode	97.8%
G30 bases in Read 1	93.2%
G30 bases in Read 2	92.4%
G30 bases in Barcode	92.2%
G30 bases in Sample Index	None

Cells

Estimated number of cells	628
Lower threshold on the number of fragments overlapping peaks per barcode to annotate barcode as cell	2,269.88
Median fragments per cell	49,746
Median fragments per non-cell barcode	1

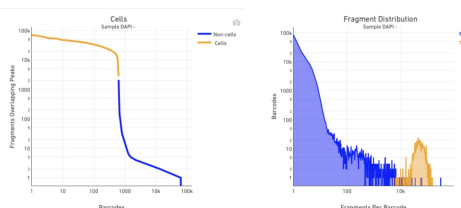
Insert Sizes

Fragments in nucleosome-free regions	36.1%
Fragments flanking a single nucleosome	58.8%



60.0%
Fraction of fragments overlapping any targeted region

42.5%
Fraction of transposition events in peaks in cell barcodes



Targeting

Enrichment score of transcription start sites	6.46	Fraction of total read pairs mapped confidently to genome (≥30 bp)	70.8%
Fraction of fragments overlapping TSS	25.4%	Fraction of total read pairs that are unmapped and in cell barcodes	8.8%
Fraction of fragments overlapping called peaks	44.2%	Fraction of total read pairs in mitochondria and in cell barcodes	18.3%
Fraction of transposition events in peaks in cell barcodes	42.5%		
Fraction of fragments overlapping any targeted region	60.0%		

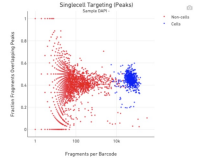
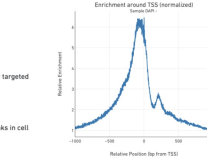


Fig 7. DAPI: Representative ATAC QC metrics from Cell Ranger.

SYTO

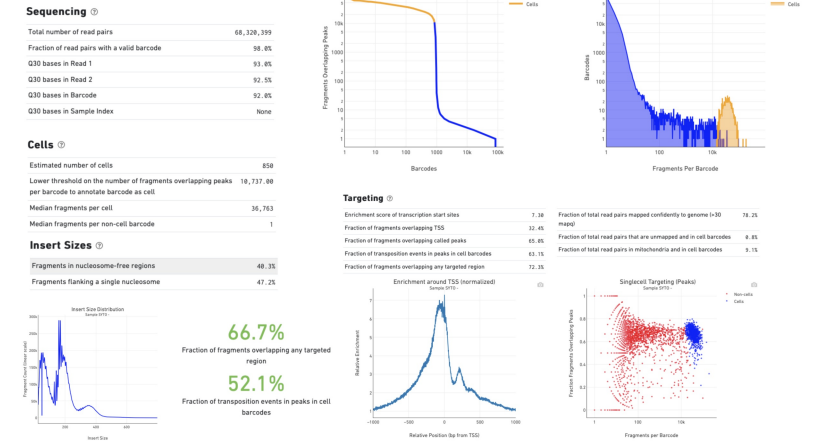


Fig 8. SYTO RNaselect: Representative ATAC QC metrics from Cell Ranger.

PI

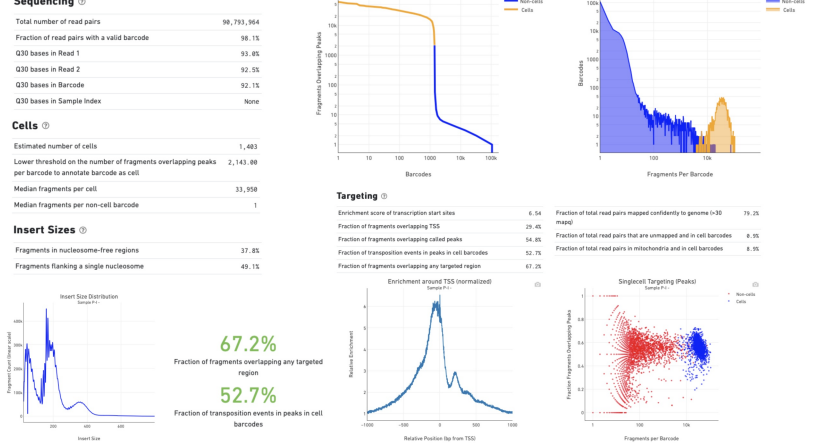


Fig 9. PI: Representative ATAC QC metrics from Cell Ranger.

7-AAD

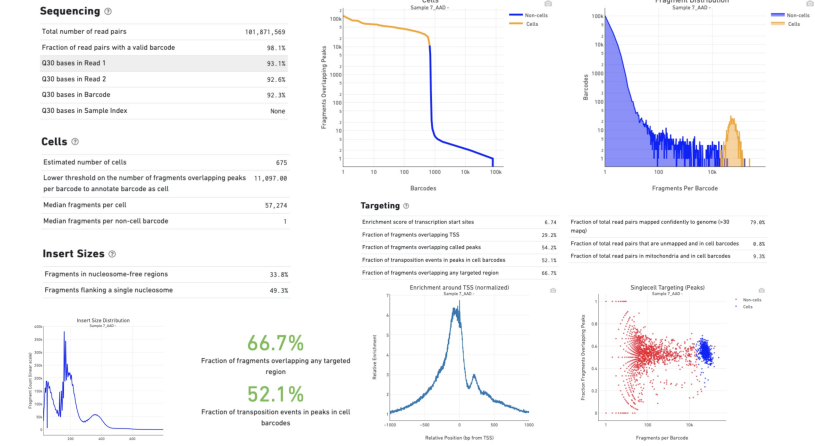


Fig 10. 7-AAD: Representative ATAC QC metrics from Cell Ranger.

DRAQ-7

Sequencing

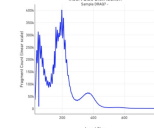
Total number of read pairs	101,843,519
Fraction of read pairs with a valid barcode	98.4%
Q30 bases in Read 1	93.6%
Q30 bases in Read 2	93.2%
Q30 bases in Barcode	92.5%
Q30 bases in Sample Index	None

Cells

Estimated number of cells	850
Lower threshold on the number of fragments overlapping peaks per barcode to associate barcode as cell	6,531.89
Median fragments per cell	60,174
Median fragments per non-cell barcode	1

Insert Sizes

Fragments in nucleosome-free regions	35.5%
Fragments flanking a single nucleosome	51.1%

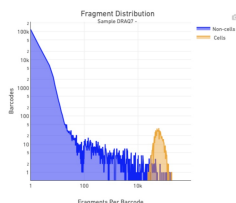
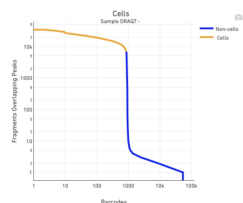


45.8%

Fraction of fragments overlapping any targeted region

20.0%

Fraction of transcription events in peaks in cell barcodes



Targeting

Enrichment score of transcription start sites	6.34	Fraction of total read pairs mapped confidently to genome (≥30 mapp)	81.8%
Fraction of fragments overlapping TSS	15.1%	Fraction of total read pairs that are unmapped and in cell barcodes	8.7%
Fraction of fragments overlapping called peaks	21.8%	Fraction of total read pairs in mitochondria and in cell barcodes	6.5%
Fraction of transcription events in peaks in cell barcodes	28.8%		
Fraction of fragments overlapping any targeted region	40.8%		

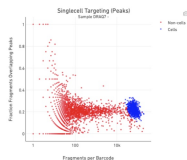
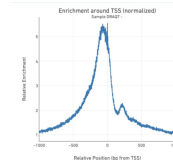


Fig 1. DRAQ-7: Representative ATAC QC metrics from Cell Ranger. **NOTE:** this dye works very well, but for this particular experiment the sorter was a bit jittery and some bad quality nuclei were sorted, so we suspect the metrics were affected by it.

MATERIALS TEXT

Required Buffers and Reagents

1. *Nuclei EZ Lysis Buffer* (Millipore Sigma) (chilled, $\delta 4^{\circ}\text{C}$)

2. *Nuclei Wash and Resuspension Buffer* (prepare chilled, $\delta 4^{\circ}\text{C}$)

1x PBS (No Ca^{2+} / Mg^{2+})

[M]1.0 % volume BSA

[M]0.2 U/ μl – [M]0.5 U/ μl RNase Inhibitor

3. *Nuclei Wash and Resuspension Buffer with DNA binding dye* (prepare chilled, $\delta 4^{\circ}\text{C}$)

1x PBS (No Ca^{2+} / Mg^{2+})

[M]1.0 % volume BSA

[M]0.2 U/ μl – [M]0.5 U/ μl RNase Inhibitor

[M]10 $\mu\text{g/ml}$ DAPI or [M]1 $\mu\text{g/ml}$ 7-AAD or [M]3 Micromolar (μM) DRAQ-7 (see notes)

4. *RT Buffer* for Single Cell Gene Expression 3' v3 reagents* (DO NOT add RT Enzyme C)

RT Reagent: $\square 20 \mu\text{l}$

Template Switch Oligo: $\square 3.1 \mu\text{l}$

Reducing Agent B: $\square 2 \mu\text{l}$

H_2O : (33.4 – X – Y) μl

5. *RT Buffer* for Single Cell Immune Profiling 5' reagents* (DO NOT add RT Enzyme Mix B)

RT Reagent Mix: $\square 50 \mu\text{l}$

RT primer: $\square 5.9 \mu\text{l}$

Additive A: $\square 2.4 \mu\text{l}$

H_2O : (31.7 – X – Y) μl

6. *Diluted Nuclei Buffer* (chilled, $\delta 4^{\circ}\text{C}$)

20x Nuclei Buffer (ATAC kit, part 2000153): $\square 50 \mu\text{l}$

H₂O: 950 µl

7. ATAC Wash Buffer-Dig (chilled, 4 °C)

Tris-HCl (pH 7.4): 10 Millimolar (mM)

NaCl: 10 Millimolar (mM)

MgCl₂: 3 Millimolar (mM)

BSA: 1.0 %

Tween-20: 0.1 %

Digitonin: 0.01 %

* RT Buffer Notes

- X ('sorting volume'): In the cytometric analysis setup described in this protocol, each droplet is 1 nl. Example: 10,000 nuclei = 10,000 nl = 10 µl 'sorting volume'.
- Y ('additional volume'): This accounts for any additional volume deposited by the flow cytometer nozzle. In the cytometric analysis setup described in this protocol (i.e. 70 µm nozzle) there is no additional volume deposited by the nozzle, so Y = 0. If in doubt, or to be on the safe side, just make Y = 5 – 10 µl.
- The 1 nuclei/nl assumption was corroborated empirically by sorting 10,000 nuclei in ten wells containing 70 µl PBS and then measuring the final volume post sorting. It is highly recommended to determine X empirically as value may vary depending on different sorters/nozzle combinations. It is recommended to determine it at least once.
- **Always measure the volume after sorting** and top up to 90 µl with PBS or H₂O if required.
- After adding the RT Enzyme Mix the final volume should be ~ 100 µl.
- It is crucial to **work as fast as possible**. Do not leave nuclei sitting on ice for too long (e.g. 30' is too long).
- **Reduce as much as possible the time from sorting-to-controller run**, ideally keep it under 40'. The longer the time the higher the background will be.
- The sorting and/or resuspension of nuclei in Diluted Nuclei Buffer is critical for optimal snATAC-Seq assay performance.
- Use DNA LoBind tubes for all steps (when possible)!
- Since this protocol does not use nor need Digitonin during the initial cell lysis, it is important to **include Tween-20 and Digitonin 0.01% in the ATAC Wash Buffer-Dig** as it helps in nucleus envelope permeabilisation. Note, Digitonin is optional but recommended.

Additional equipment required

- Protector RNase Inhibitor (Cat. Number: RNAINH-RO, Merck/Roche)
- Nuclei EZ Lysis Buffer (sold as *Nuclei Isolation Kit: Nuclei EZ Prep* by Merck/Sigma, Cat. Number: NUC101)
- 1.5 ml DNA LoBind Eppendorf tubes
- 0.2 µl PCR-tubes/strips (LoBind)
- 15 ml Falcon tubes
- 70 µm-strainer mesh to fit a 15 mL Falcon tube (e.g. pluriStrainer Mini 70 µm, Cell Strainer or Flowmi® Cell Strainer)
- 40-µm cell strainer (e.g. Falcon® RoundBottom Tubes with Cell Strainer or Flowmi® Cell Strainer)
- Round-bottom 96-well plate
- Light or fluorescent microscope
- FACS instrument (i.e. BD FACSAria™ Fusion, SONY SH800S), ideally with 70 µm nozzle.
- Thermocycler
- TapeStation or Bioanalyzer plus consumables
- Cell counter/hemocytometer
- Douncer/pestle
- Refrigerated centrifuge
- Razor blades

SAFETY WARNINGS

Please see SDS (Safety Data Sheet) for hazards and safety warnings.

BEFORE STARTING



All samples and reagents are kept **On ice** or at **4 °C** .

Nuclei Prep and snRNA-Seq

- 1 Mince/chop tissue with a razor blade to small pieces. The tissue may be as small as 2 – 3 grains of (cooked) rice so long it can accommodate the sorting of sufficient nuclei for both snRNA-Seq and snATAC-Seq.



Note that nuclei yield largely depends on tissue cellularity.

2



Add **300 µl** – **500 µl** of chilled Nuclei EZ Lysis Buffer (+ RNase inhibitor) to the tissue in 1.5 ml DNA LoBind tube (for small pieces use **300 µl**).

- 3 Homogenise the sample using a douncer/pestle (gently stroking ~ 10 – 15 times).



For mincing the tissue, you may take the DNA LoBind tube out of the ice, however, be quick and return to ice.

- 4

Add more lysis buffer to **1 ml** , mix gently (bore tips preferred) and incubate **On ice** for **at least** **00:05:00** .



Lysis time of 5 min has been enough for most tissues we tested, but you may need some optimization.

- 5 Filter homogenate using a 70 µm-strainer mesh to fit a 15 ml Falcon tube (e.g. pluriStrainer Mini 70 µm, Cell Strainer. My preferred one is 70-um Flowmi® Cell Strainer, in which case you would collect directly in 1.5 mL DNA LoBind tube).



pluriStrainer Mini 70 µm (Cell Strainer)
Cell Strainer

pluriSelect 43-10070-40

- 6

Collect flow through in a 15 ml Falcon tube and transfer volume back into a new 1.5 ml DNA LoBind tube.

- 7

Centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** .

- 8

Remove supernatant **leaving behind** ~ **50 µl** .

- 9

Add **1 ml** of Nuclei Wash and Resuspension Buffer and gently resuspend the pellet (~1-2 pippete strokes).

- 10

Centrifuge at **500 x g** for **00:05:00** at **4 °C** .

- 11



Remove supernatant **leaving behind** ~ **50 µl** .

- 12 Add  **1 ml** of Nuclei Wash and Resuspension Buffer. **DO NOT** resuspend the pellet.



Additional washes are possible but may incur in nuclei loss. If doing so, I recommend **not** to resuspend nuclei in between washes.

- 13 Centrifuge at  **500 x g** for  **00:05:00** at  **4 °C**.

- 14 Resuspend the nuclei in  **200 µl** -  **400 µl** Nuclei Wash and Resuspension Buffer supplemented with DAPI/7-AAD/DRAQ-7.



Resuspension volume can vary to achieve ~ 150 – 200 events/second during cyrtometric analysis (see step 18).

- 15 Collect all nuclei by washing off nuclei from the wall of centrifuge DNA LoBind tube.



IMPORTANT: Protect from light from here forward.

- 16 Filter nuclei (at least once) with a 40-µm cell strainer (e.g. Falcon® Round-Bottom Tubes with Cell Strainer or Flowmi® Cell Strainer or 40-um Flowmi® Cell Strainer) **before** sorting.

- 17 

Visually inspect nuclei integrity under a microscope and (optionally) count the number of nuclei with a cell counter (Countess II FL Automated Cell Counter) or hemacytometer.

- 18 Prior sorting, you may want to dilute sample to have ~ 150 – 200 events/second to get better defined peaks in cytometric analysis.

- 19 Perform cytometric analysis. Identify single nuclei and sub-populations based on DNA content, gate and sort directly into a round-bottom 96-well plate well containing the respective RT Buffer prepared without the RT Enzyme.



IMPORTANT: for snRNA-Seq, we have seen that FACS sorters tend to overestimate the number of nuclei sorted in about $\geq 40\%$ depending on instrument, so we usually sort 35 – 40 % more nuclei than aimed (e.g. for ~ 5000 nuclei recovery, you would need ~ 8,700 but we sort ~ 12,000 nuclei).

- 20 Proceed immediately with the 10x Genomics Single Cell 3' v3 or 5' protocol (Standard or NextGEM), minimising the time between nuclei preparation/sorting and chip loading.

21 

Add the corresponding volume of RT Enzyme (depending on the kit, **10 µl** for 5', and **8.3 µl** for 3' v3) to the sorted nuclei in RT buffer.

22 Mix well but gently and load chip as per the Single Cell 3' v3 Reagents User Guide or Single Cell V(D)J 5' Reagents User Guide.

snATAC-Seq

23



Ideally, **perform the following steps while the RT reaction is running**.
The protocol below assumes the nuclei input is low.

Sort as many nuclei as possible into a round-bottom 96-well plate well containing **100 µl** of ice-cold ATAC Wash Buffer-Dig.



DO NOT, I repeat DO NOT sort into Diluted Nuclei Buffer



We have successfully sorted as little as ~7000 nuclei and recovered ~3500+ profiled nuclei (~50 % recovery as expected, 1.53 recovery efficiency factor).



Note there will be significant nuclei loss during washes and nuclei counting, so you may want to make sure the washing steps are done carefully. Take into account this loss when deciding aimed nuclei. To reduce loss, follow the tips below (**bold**).

24 

Transfer to 0.2 ml PCR tube (LoBind!).

25 

Add **50 µl** of ATAC Wash Buffer-Dig to the well.

26 

Transfer any remanent nuclei to the 0.2 ml PCR tube (~ **150 µl**).

27 

Centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C**.

28 


Remove supernatant **leaving behind** ~ **10 µl** .

29 

Gently add **100 µl** ice-cold Diluted Nuclei Buffer.



DO NOT resuspend nuclei.

30 

Centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** .

31 


Remove **100 µl** of the supernatant **in two steps**, namely, remove **90 µl** first and then **10 µl** (pellet may not be visible!).

32 

Gently add **100 µl** ice-cold Diluted Nuclei Buffer.



DO NOT resuspend nuclei.

33 

Centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** .

34 

Remove the supernatant (~ **100 µl**) **in two steps**, namely, remove **90 µl** first and then as much volume as possible to leave **leaving behind** ~ **7 µl** – **10 µl** (avoid disturbing pellet).



If you manage to remove the whole volume without disturbing the pellet, then add **7 µl** of Diluted Nuclei Buffer and proceed to step 35.

35 Resuspend nuclei in the ~ **7 µl** – **10 µl** of ice-cold Diluted Nuclei Buffer, **carefully washing walls of the tube** to ensure all nuclei are in solution.

36 



Take **1 µl** – **2 µl** and dilute 1:5 with Diluted Nuclei Buffer.

37 

Mix 1:1 with Trypan Blue and count the number of nuclei with a cell counter (Countess II FL Automated Cell Counter) or hemacytometer (the counting is to have an idea of how many nuclei to expect based on the recovery factor).

38 

Inspect under the microscope.

39 Take  **5 µl** of nuclei in Diluted Nuclei Buffer and proceed directly to Chromium Single Cell ATAC Reagent Kits protocol (CG000168 Rev A). The volume added to the Transposition reaction will vary; **for low input samples we usually use all  5 µl of nuclei prep.**

Alternatively, follow recommendations of the User Guide to estimate volume of nuclei to add to recover a determined targeted nuclei recovery (Page 20, CG000168 Rev A).

To *estimate* the Number of Recovered Nuclei, do the following calculation:

$[\text{Nuclei Concentration (from step 35)} \times \text{Volume of Nuclei (up to 5 µl)}] / 1.53$ (recovery efficiency factor)



This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited