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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.3

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bdeai3ae

Human Cell Atlas Method Development Community


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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, $MgCl_2$ 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.

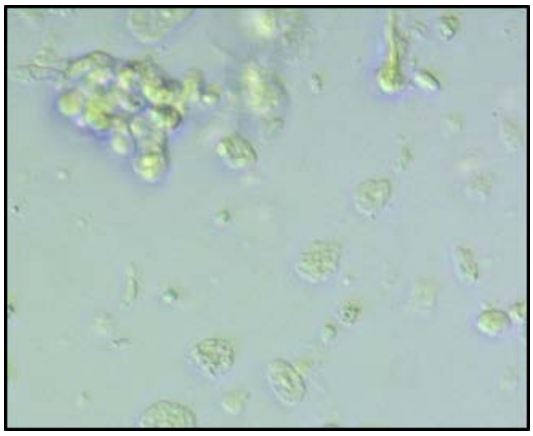

Fig 1. Nuclei before sorting.

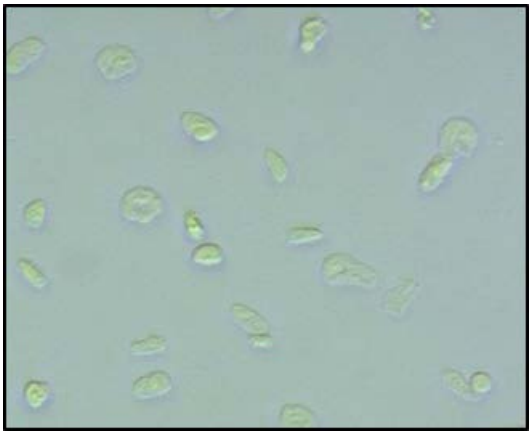

Fig 2. Nuclei after sorting.

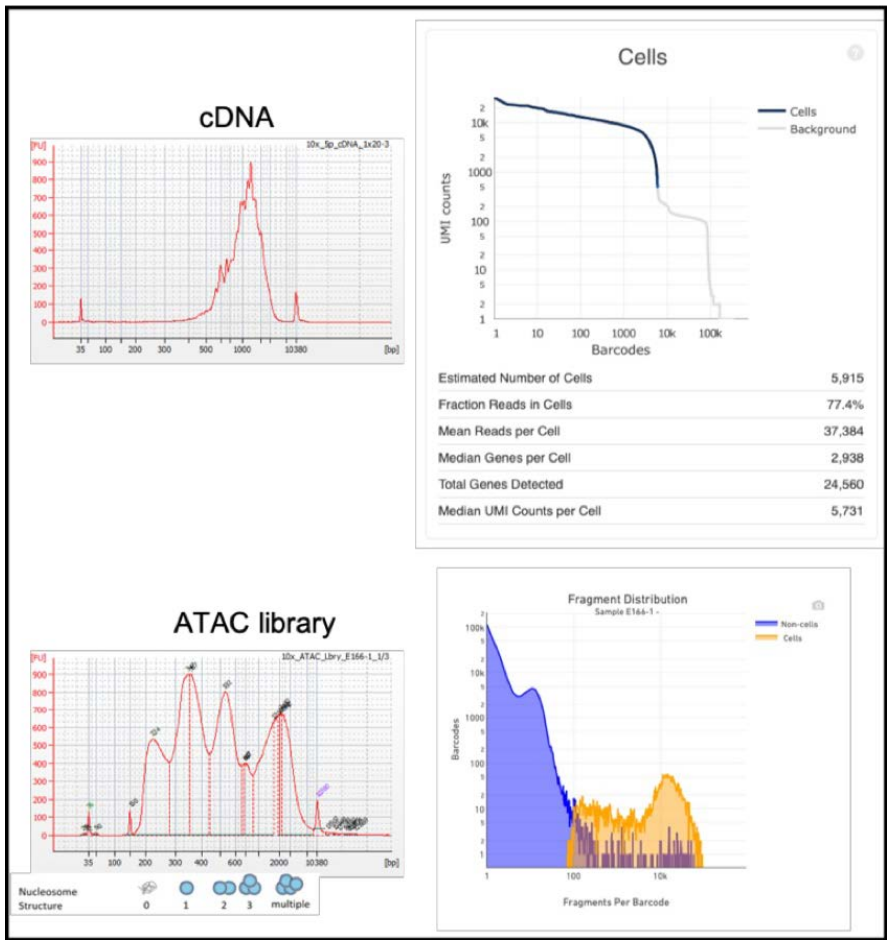

Fig 3. cDNA and ATAC libary traces. Metrics obtained with Cell Ranger

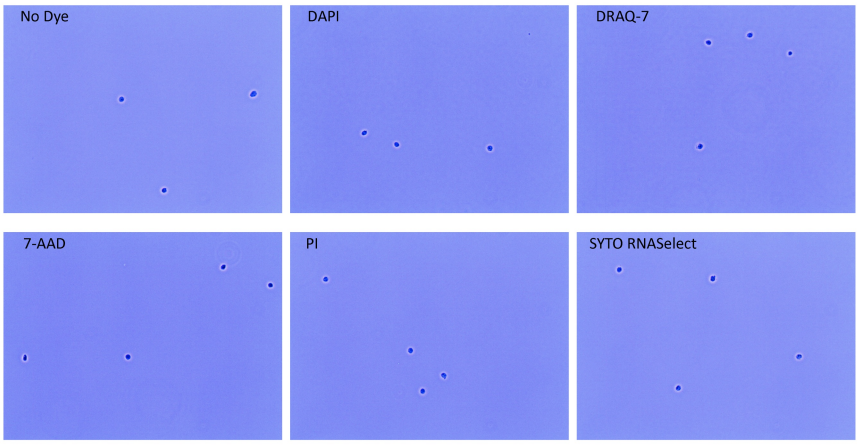

Fig 4. Representative photos of how clean the nuclei looks after sorting and right before loaidng on the Chromium.

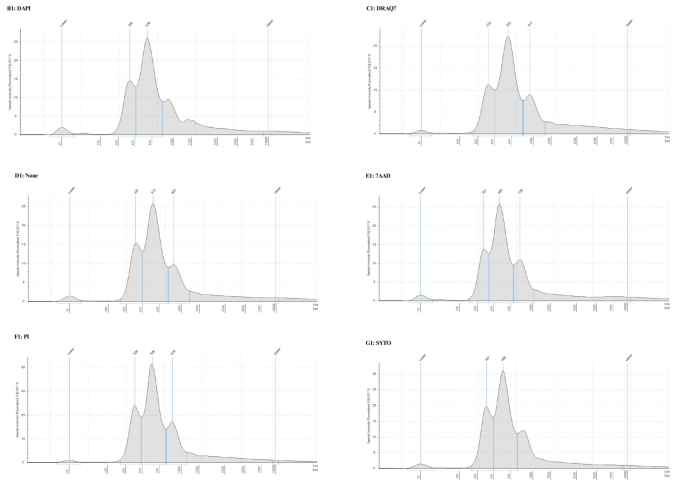

Fig 5. TapeStation traces of ATAC libraries.

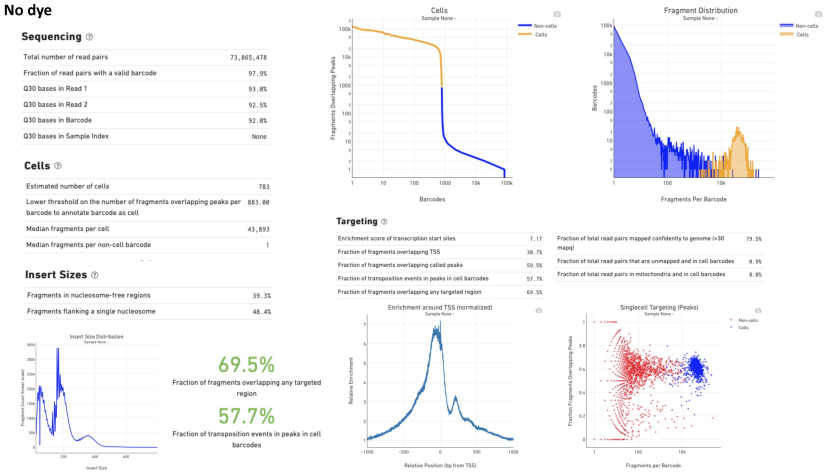

Fig 6. No Dye: Representative ATAC QC metrics from CellRanger.

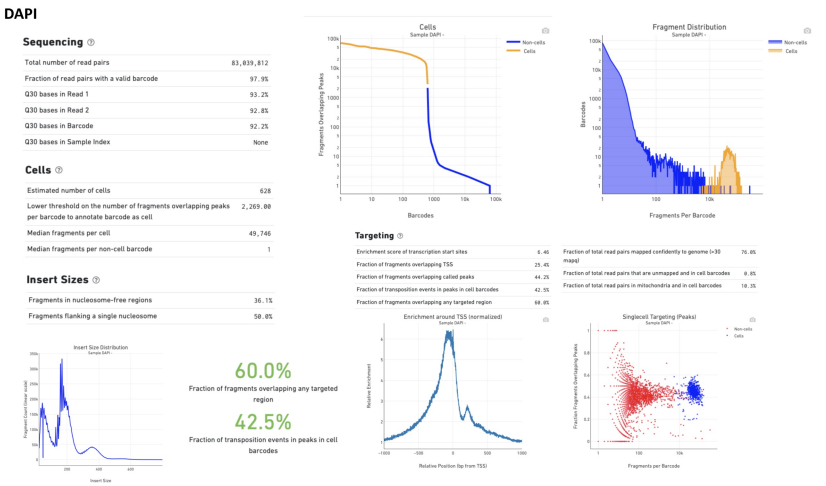

Fig 7. DAPI: Representative ATAC QC metrics from CellRanger.

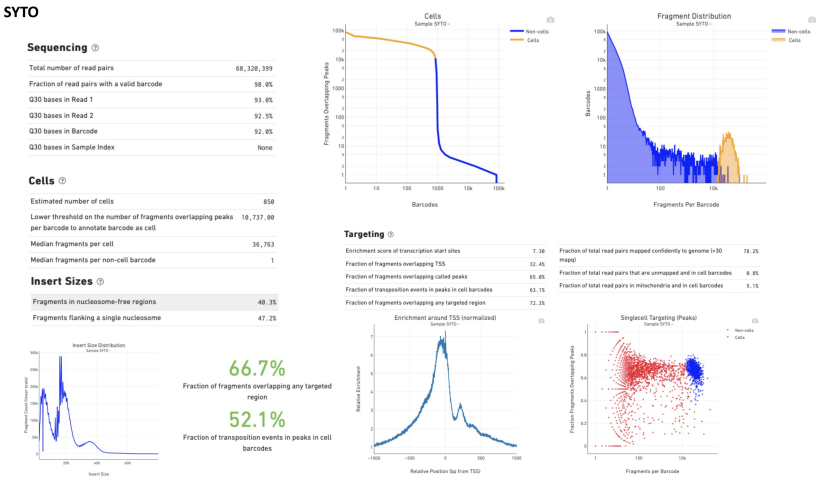

Fig 8. SYTO RNASelect: Representative ATAC QC metrics from CellRanger.

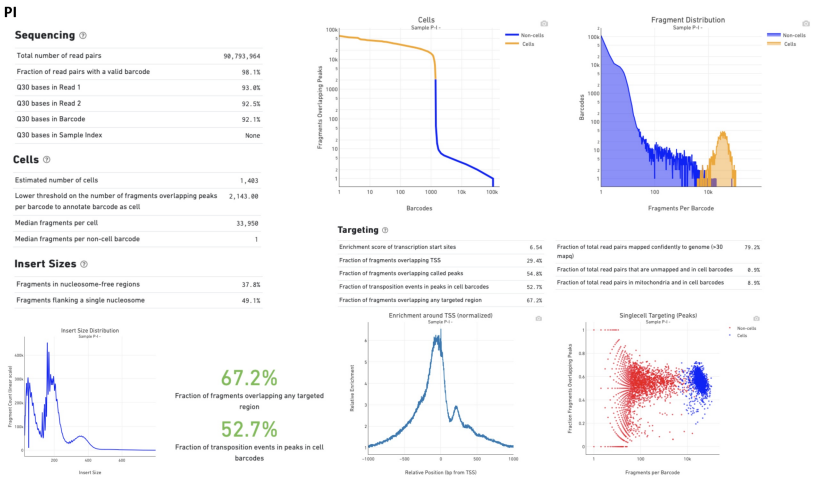

Fig 9. PI: Representative ATAC QC metrics from CellRanger.

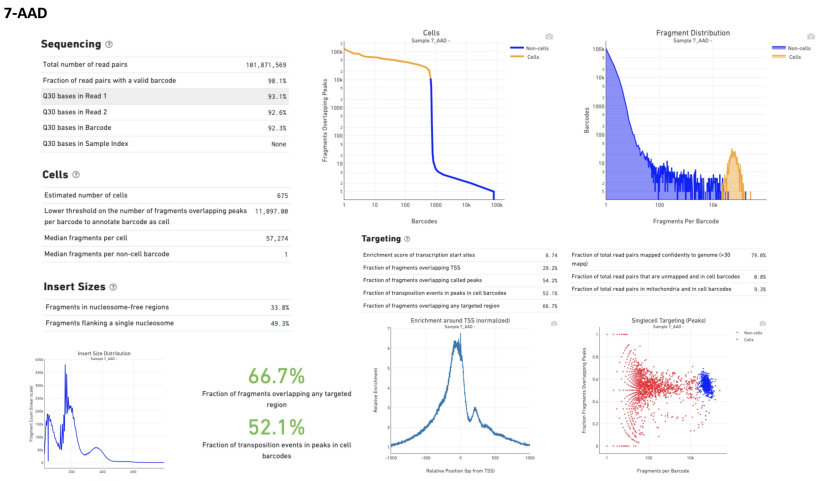

Fig 10. 7-AAD: Representative ATAC QC metrics from CellRanger.

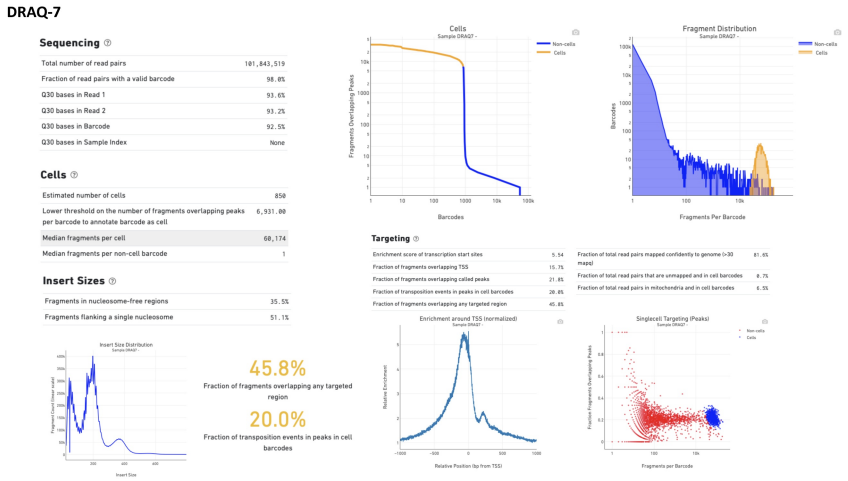

Fig 1. DRAQ-7: Representative ATAC QC metrics from CellRanger. ***NOTE:*** *this dye works very well, but for this particular experiment the sorter was a bit jittery and some bad quality nuclei were sorted, so we suspect the metrics were affected by it.*

MATERIALS TEXT

Required Buffers and Reagents

1. *Nuclei EZ Lysis Buffer* (Millipore Sigma) (chilled, **4 °C**)

2. *Nuclei Wash and Resuspension Buffer* (prepare chilled, **4 °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, **4 °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 µg/ml** DAPI or [M]**1 µg/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: **20 µl**

   Template Switch Oligo: **3.1 µl**

   Reducing Agent B: **2 µl**

   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: **50 µl**

   RT primer: **5.9 µl**

   Additive A: **2.4 µl**

   H_2O: (31.7 – X – Y) µl

6. *Diluted Nuclei Buffer* (chilled, **4 °C**)

   20x Nuclei Buffer (ATAC kit, part 2000153): **50 µl**

H_2O: **950 µl**

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

   Tris-HCl (pH **7.4**): [M] **10 Milimolar (mM)**

   NaCl: [M] **10 Milimolar (mM)**

   $MgCl_2$: [M] **3 Milimolar (mM)**

   BSA: [M] **1.0 %**

   Tween-20: [M] **0.1 %**

   Digitonin: [M] **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 H_2O 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 um, 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/hematocytometer
- 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 hematocytometer.

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 >/= 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.

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 **50 µl** - **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** — **100 µl** of ATAC Wash Buffer-Dig to the well.

26 Transfer any remanent nuclei to the 0.2 ml LoBind PCR tube (~ **150 µl** — **200 µl**).

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

28 Remove the supernatant **in two steps**, that is, remove a **l**arger **v**olume first using a P100/P200 pipette (~ **120 µl** — **170 µl**) and then as much volume as possible with P20 pipette **leaving behind ~ 15 µl . Do not disturb the pellet.**

29 Gently add **200 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 the supernatant **in two steps**, that is, remove a larger volume first using a P100/P200 pipette (~ **190 µl**) and then as much volume as possible with a P20 pipette **leaving behind ~ 7 µl — 10 µl . Do not disturb the pellet.**

Alternatively, if the nuclei are limited, you may resuspend the pellet in **5 µl** of Diluted Nuclei Buffer and proceed directly onto Step 36. Do not count as it'll be innacurate anyways and you'll lose precious nuclei.

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

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

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

35 Use same slide to inspect under the microscope.

36 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 5 µl of the nuclei prep above** (see note in Step 31).

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:
[Nuclei Concentration (from step 35) x Volume of Nuclei (up to 5 µl)] / 1.53 (recovery efficiency factor)