protocol vA.02

10x Genomics scATAC-seq v2 Guidelines

Workflow of interactions with GECF

- **Schedule the experiment** with us at least **3 weeks in advance** (tell us already nb of samples and bsl2 level). Envision doing a 1-sample pilot, or a bulk ATAC trial, before large critical experiments.

- **Optimize nuclei isolation procedure** (lysis conditions and time). We can check the nuclei.

- Few days in advance, send us the **submission form** (to be found on our website).

- **On experiment day**

  - bring your **nuclei on ice**, without DNA stain (7AAD tolerated)
  - prepared and washed according to the relevant 10XG protocol
  - at the **concentration** relevant for your targeted nuclei number,
  - in ideally **minimum 25ul**, resuspended in the **Diluted Nuclei Buffer** (you need to get it from us).
  - If possible, also bring an aliquot of unlysed cells so we can check their viability, otherwise calculate cell viability yourself and let us know.

This method can give great results but since it is very **difficult to evaluate nuclear integrity** just by looking at nuclei at the microscope, we recommend before any large scale critical experiments:

- A cheaper **pilot experiment** with only one sample
- **test ATAC quality** by performing a bulk ATAC on this sample type with the planned nuclei isolation protocol.

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Targeted nuclei number

- Define the number of nuclei you want data for ("**targeted nuclei number**"). Recovery rate is uncertain and depends on characteristics of the nuclei, as well as on experiment-specific factors, such as debris, etc. therefore **targeted nuclei number is only indicative**.
- If you absolutely want a given minimum number of nuclei, indicate it in the submission form and we will add a safety margin in our calculations.
- Given the tendency of nuclei to aggregate, we encourage not to push the method too close to its upper limit of 10k nuclei/sample unless really necessary.
- The **rate of doublets** increases with targeted nuclei number (see table below), therefore we recommend against targeting more than 5'000 nuclei unless really necessary.

Targeted Nuclei	Multiplet Rate (%)
500	~0.4%
1 000	~0.8%
2 000	~1.6%
3 000	~2.3%
4 000	~3.1%
5 000	~3.9%
6 000	~4.6%
7 000	~5.4%
8 000	~6.2%
9 000	~6.9%
10 000	~7.7%

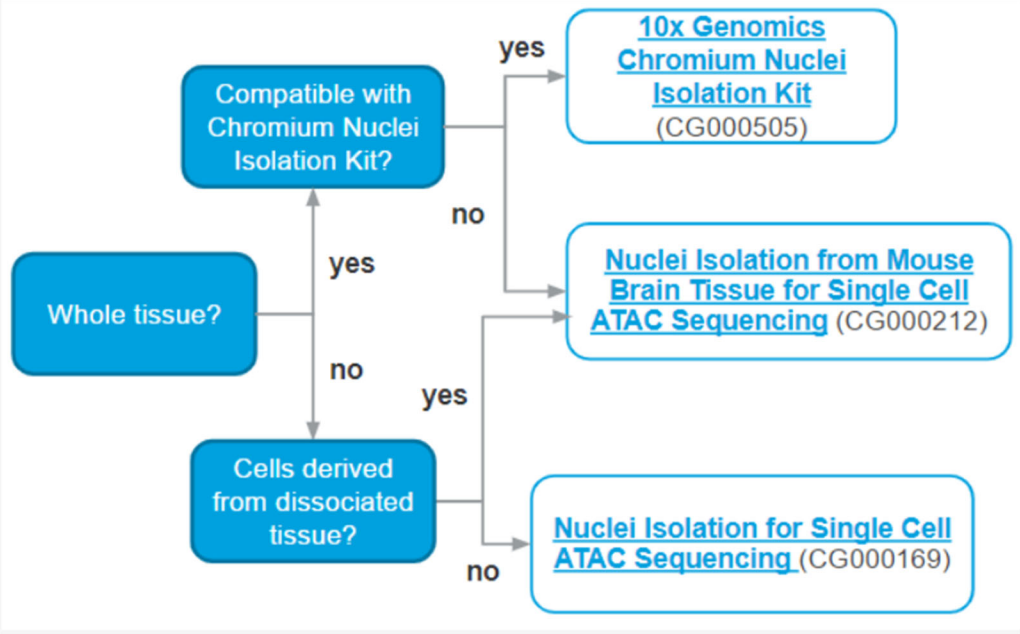
- Each additional nucleus comes with a **sequencing cost**, therefore calculate the number of nuclei needed thoughtfully. **Once the nuclei have been processed, it is <u>not</u> possible to sequence only a fraction of them**.
- The **ideal number of targeted nuclei** depends on the biological question. For comparing two populations, 1'000 nuclei may be enough. At the other extreme, for identifying new rare subpopulations (<1%), 5'000 or more nuclei may be needed. If unsure, we recommend 3'000-4'000 nuclei as a good starting point.

Nuclei preparation

Obtaining nicely dissociated, healthy nuclei with a minimum number of unlysed cells is the most important and often trickier step of the experiment. Therefore, we strongly recommend that you **optimize the nuclei isolation procedure beforehand**, including any putative step where the nuclei will have to wait on ice before

being processed by us (waiting for other samples, trip towards GECF…).

- **10XG recommend** you choose the nuclei isolation protocol among these options:



- We have in house the **10XG Nuclei isolation kit** (**CG000505**) and we can sell aliquots.
- 10XG recommends **specific nuclei isolation protocols** for the different applications (scRNA, scATAC or scMultiome). For optimal assay performance, nuclei isolation should be performed using the protocol suggested for the specific application.
- If none of the above protocols are satisfying, look in the literature for publications performing 10XG ATAC analysis on your exact cell type. If the protocol has not been specifically validated for 10XG ATAC, submit it to us and/or 10XG tech support for review, and test it in advance.
- **Test in advance the chosen nuclei isolation protocol**. It may require an **optimization of both duration of lysis and concentration of lysis buffer**: https://kb.10xgenomics.com/hc/en-us/articles/360027639631-How-do-I-perform-a-lysis-timeline-to-optimize-my-nuclei-isolation-for-Single-Cell-ATAC-sequencing Make sure to dilute the lysis buffer in the specific buffer mentioned. Choose the lowest strength/time that works well, because **overlysed nuclei can have reduced chromatin structure**.
- **Perform the optimization with nuclei concentration that you will use for your experiment**, as this can impact on aggregation behavior.
- Number of **starting cells**:
  - The nuclei isolation procedure will likely lead to ca 50% nuclei compared to starting cell number.
  - In case the starting number of cells is low, the nuclei isolation protocols of 10XG have in general specific guidelines, such as fewer washes. Follow them if relevant.
- To avoid aggregates, don't over-spin the nuclei. Optimize spinning conditions in advance.
- **FACS** may be needed/useful to remove debris after nuclei isolation, but given the fragile nature of nuclei, follow these recommendations:
  - WARNING: FACS should **not** be done **after permeabilization** of nuclei, as they are more fragile after

permeabilization.

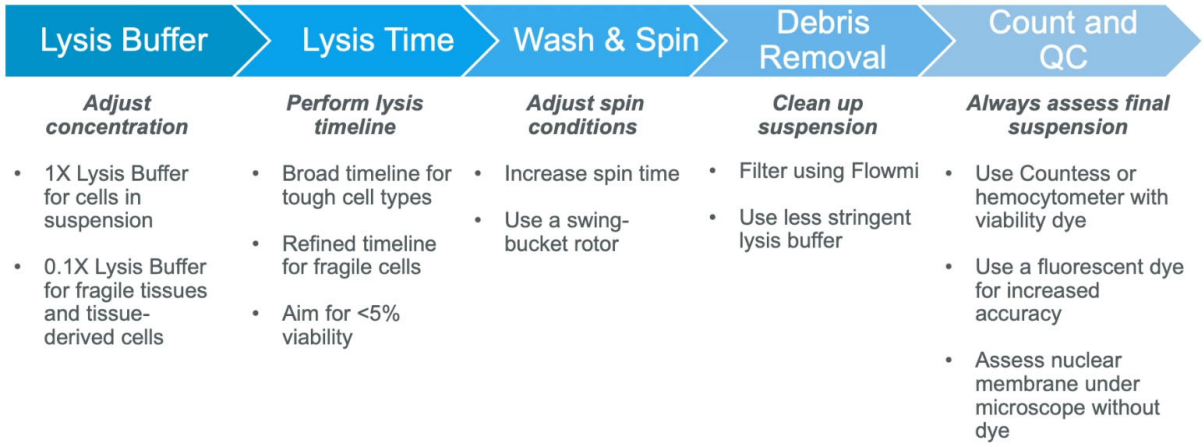
- FACSing guidelines for nuclei can be found here and here

- If the final **resuspension volume** is going to be low and cell pellet is not visible, add some **Diluted Nuclei Buffer** to the nuclei before the last centrifugation. In this way it is possible to leave medium after the centrifugation (decreasing the risk of aspirating away the nuclei), without impacting on the composition of the final resuspension buffer.

Notes:

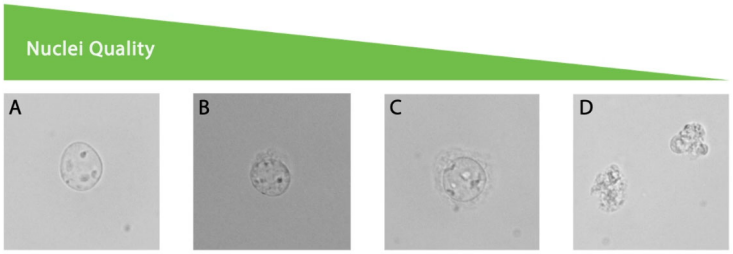
- If isolation of nuclei from your tissue fails, refer to this support page: https://kb.10xgenomics.com/hc/en-us/categories/360001072491
- 10XG support page regarding tissue sample quality and affected metrics can be found here.
- The CryoPrep system from Covaris can be used to isolate nuclei from difficult to dissociate tissues.
- Useful further tips from 10XG: https://kb.10xgenomics.com/hc/en-us/articles/360020145652-How-can-I-optimize-my-nuclei-prep-for-Single-Cell-ATAC-sequencing
- For concentrating/washing cells before nuclei isolation, a good starting point to optimize the conditions could be: spinning at RT° at 300xg for 5min for small cells (5-10um), 250xg for 4min for medium cells (10-17um), and 150xg for 3 min for larger cells (17-25um). Optimizing the conditions is important to ensure efficient pelleting while minimizing carryover of debris or aggregation of cells. When doing it the first time, check also supernatant for absence of cells. If you have or suspect to have a sub-population composed of small cells, prefer small cells settings to avoid losing them.
- Frozen cells can be used as a starting material for nuclei isolation, leading to a modest loss of data quality (10XG FAQ section of website). **Nuclei cannot be frozen**. General info about freezing cells can be found here.
- If it is suspected that the cells suspension contains a lot of ambient DNA, it is possible to treat cells with DNase (at the cells step only, before the nuclei isolation procedure/washes).

Summary slide from 10XG regarding steps that can be tweaked/optimized:



Nuclei quality

- **Isolated nuclei should be intact/healthy**, with clear edges and no significant blebbing:



A & B: OK to proceed. C: “at your own risk”. D: Do not proceed.

- Nuclei should be **well dissociated** from each other, with minimal amounts of doublets. If performing FACS, carefully gate on FACS to avoid doublets.
- The nuclei prep should contain **no more than 5% alive cells**. These will indeed cluster separately in the scRNA-seq data and complicate the analysis.
- **Avoid cell debris** as much as possible, as they are encapsulated along with nuclei and impact data negatively. If assessing nuclei quality is tricky, a fluorescent stain such as DAPI can be used to differentiate them from debris (but only on the aliquot of nuclei for QC).
- Absolutely **avoid aggregates or clumps** as they may clog the capillaries and lead to run failure. To avoid these, optimize spinning conditions, to avoid “over-spinning”. Nuclei can also be passed through a **cell strainer**:
  - Flowmi pipette cell strainer of 40um, cat# BAH136800040-50EA
  - Miltenyi Biotec 30 um PreSeparation Filter, cat# 130041407
  - CellTrics filters 20 um, cat# 04-004-2325
- Unless specifically agreed by 10XG/us, **do not stain the nuclei with a DNA intercalating dye**, as it is likely to impact the ATAC procedure. **The 7AAD dye is the only one that has been approved** for now by 10XG.

Nuclei concentration, resuspension buffer type and volume

- The **concentration** of the nuclei must be in the following acceptable ranges, depending on your targeted nuclei number. The last column on the right shows concentrations to aim for:

Targeted Nuclei	*Compatible Nuclei Stock Conc. (nuclei/ul)*	Recommended Nuclei Stock Conc. (nuclei/ul)
500	*155-390*	350
1,000	*310-780*	700
2,000	*610-1,540*	1000
3,000	*925-2,300*	1400
4,000	*1,230-3,075*	1800
5,000	*1,540-3,850*	2300
6,000	*1,850-4,600*	2600
7,000	*2,150-5,400*	2800
8,000	*2,460-6,150*	3000
9,000	*2,770-6,900*	3300
10,000	*3,080-7,700*	3500

- Ideally we need **>25ul** of nuclei **at the right concentration** to perform the QC and the run itself in good conditions. If these values cannot be reached, contact us in advance (it is possible to submit down to ca 7-8ul but rendering the nuclei QC process much trickier).
- The loading of the nuclei and calculations are very different for ATAC than for other 10XG methods, therefore other guidelines do not apply here.
- If FACS is used, consider that **FACS often overestimate cells/nuclei concentration** and ask the sorting facility to give you a concentration a bit higher than aimed at.
- Make sure your counting device works with small nuclei. If unsure, Neubauer chambers are recommended.
- **Nuclei must be brought diluted in the Diluted Nuclei Buffer** provided by us (we will give it to you in the morning of the experiment).
- If you use custom resuspension buffer (not recommended), it must be devoid of EDTA (it inhibits Tn5) .
- FACS buffer contains 1mM EDTA, so make sure the FACS buffer content in the final cell suspension is minimal.
- Place **nuclei on ice** once prepared.
- If used, **trypsin must be inactivated** after use (serum, BSA…).

Starting cell viability

- **Cell viability must be high** before starting the nuclei isolation. **With nuclei, it is difficult to exclude dying/dead cells bioinformatically** as this relies on mitochondrial RNAs, which are absent from nuclei.
- **The acceptable percentage of dead cells** depends on your experiment. If your cells come from a healthy suspension cell line, anything more than 10% dead cells is probably a bad sign, while if working with primary cells that underwent hours of dissection and sorting, 20-25% may be considered acceptable. When samples contain more dead cells, it is the user/PI decision to either move forward anyway, or perform a dead cells removal procedure (see below), or cancel the experiment.
- If possible, bring an aliquot of unlysed cells so we can check their viability. Or, measure it on your side.
- **MACS-sorting** in general leads to higher viability than **FACS-sorting**, so prefer the former if possible. If FACSing, prefer larger nozzle and lower pressure (discuss ahead with flow facility team)
- If you sort the cells by FACS before nuclei isolation, include a **viability stain** to get rid of dead cells if possible (only **7AAD** is accepted).
- Miltenyi offer a **kit to remove dead cells** (“Dead Cell Removal kit”, 130-090-101), which works at least on mammalian cells (probably also on insect cells but to be confirmed).

Diverse notes

- **Multiplexing** solutions are not compatible with ATAC, even in an unsupported fashion.
- If you have to prepare nuclei in different batches, make sure you **avoid batch effects**. Typically, avoid processing all controls in a batch and “treated” samples in a distinct batch.
- We can split the processing of nuclei in the instrument in several batches if this avoids some samples waiting on ice for very long times. Avoid introducing batch effects by doing that though.
- If you plan a big experiment, it is strongly recommended first running just a single sample (**pilot experiment**) to assess quality of data and nuclei recovery rate.
- 10XG regularly update their reagents and workflows/pipelines. If you absolutely want us to use a specific

version for comparing with a previously generated dataset, tell us well in advance.

- **Biosafety**:
  - We will process bsl2-level samples under a bsl2 hood. **Please warn us before the experiment day**.
  - Primary human cells that have not been screened for absence of HIV, HBV and HCV infection (ideally also hCMV), are considered bsl2 material.
  - To determine the biosafety level of cell lines, this German website is used as a reference by the Biotechnology office of the Confederation: https://zag.bvl.bund.de/zelllinien/index.jsf?dswid=7026&dsrid=373 . A few cell lines are also listed here: https://www.bafu.admin.ch/dam/bafu/en/dokumente/biotechnologie/fachinfo-daten/einstufung_von_organismenzelllinien.pdf.download.pdf/
  - Since nuclei are used, the isolation procedure may be sufficient to declassify your cells from bsl2 to bsl1, investigate with us if relevant.
- If many **granulocytes** are present, it is recommended to sort them out as they may perform netosis which affects ATAC-seq data quality (see here).
- For the **bioinformatics** analysis, when an **ectopic gene** must be added to the reference genome, give us its sequence in advance. If the transgene was inserted from a lentivector, the reads may lie in the 5'UTR/3'UTR therefore include these elements in the sequence. Finally, if the extra gene is already present in the genome, extra care is needed since multimapped reads are discarded by CellRanger.
- **Droplets containing multiple beads** occur at a mean frequency of ca 4%, and fragments from cells captured in these droplets are split into these multiple beads, hence will be detected as multiple cells, each with low UMIs. It is very likely that such "ghost cells" are filtered out during data processing, but to be on the safe side, **new rare cell population composed of cells with low UMI counts should be validated by a different method**.
- The **number of reads/nuclei** you want depends on the biological question: for clustering cells in groups of known cell types, and/or to delineate their broad transcriptional/chromatin profile or activated pathways, 25k reads/nuclei is enough. If you want to zoom and ask whether specific genes are expressed, then the more reads the better (up to complete sequencing saturation, which is likely between 50k and 100k for nuclei).

Disclaimer

10XG runs work in general well, but should still be considered at the edge of current technological capacities. There are indeed a non-null percentage of wells/runs that fail for purely technical reasons. Of course, we don't charge for these unless we can spot a critical error from the user side (e.g. failure to follow cell prep guidelines/buffers), or if users gave us their green light to carry on despite poor nuclei quality. Importantly, if a putative failure would represent a major issue for your project (e.g. months-long mice generation to repeat), tell us in advance and we'll discuss the risks and means to minimize them. As a general rule, if you have extra cells, and if the experiment is critical, freeze them as a backup.

Versions log

- vA.01- vA.02 (07.10.2024): Initial release.