10X Genomics Single Cell Sample Submission Guidelines

Practical Information

The responsible lab staff will reach out the days before a scheduled run to make practical arrangements. The deadline for delivering samples on the day of the run is 13:30 for 3' GE and 5' GE experiments, 12:30 for ATAC and Multiome experiments.

On the day of the run we request that any changes to plans or times are communicated to us. Also, please let us know 30 minutes before arrival, to allow for preparation of sensitive reagents.

At sample delivery we will perform a quality control of the samples using a cell counter and/or microscope. We request that the user stays during these steps in case the sample is of suboptimal quality and discussion is needed before starting the experiment. NGI can offer opinions based on our experience, but it is the user's decision to proceed or not with a non-optimal sample, as in such cases the quality of the resulting data may be hard to predict.

We kindly request that only one person joins in the lab for cell counting.

Important

- Deadline for single cell sample submission on the run day is:
  - 13:30 pm for 3' and 5' gene expression experiments.
  - 12:30 pm for ATAC and Multiome experiments on the day of the booked appointment. Samples will not be accepted after this time.
- Please make sure that the quality of the samples meets the criteria below.
- Please count your samples with an accurate automated cell counter or manually before submission to the facility. Whenever possible use fluorescent dyes, such as AO/PI for the highest accuracy.
- In case of suboptimal samples: If the user insists on going forward with the protocol, even though they have suboptimal cell or nuclei suspensions, the user will be required to sign a form confirming that they have received information about low sample quality and will be invoiced regardless of the resulting library and data quality. The person who brings the sample must have the permission to make the decision of proceeding with samples, or if they need, contact the responsible person to get confirmation.

10X cell preparation handbook

It is highly recommended to consult the 10X handbook on Cell Preparation for Single Cell Protocols when planning a project. It covers best practice advice and general protocols for sample preparation and is a very good starting point for deciding on a sample preparation strategy.

Cell/nuclei amount and concentration

If possible, we request you to bring at least roughly **three times the number of cells/nuclei** you aim for as output. If you aim to sequence 5,000 cells, please bring 15,000 cells.
For Targeted Cell Recovery up to 10,000 cells, the optimal concentration is in the range of **700 to 1,200 cells or nuclei/µl**.
For Targeted Cell Recovery up to 20,000 cells, the optimal concentration is in the range of **1,300 to 1,600 cells or nuclei/µl.**
For OCM Targeted Cell Recovery of up to 5,000 cells, required concentration is in the range of **900 to 1,200 cells or nuclei/µl**

- If your suspension is too diluted when arriving at the facility, you can use our centrifuges to concentrate it.
- Please remember to **always bring extra buffer** in case it is needed for dilution/resuspension.
- Please bring samples in 1.5ml lowbinding tubes.

Buffer for cell suspension/sample submission

For 10X gene expression experiments the recommended buffer for the cell/nuclei suspension is 1X PBS (calcium and magnesium free) containing 0.04% BSA (400 µg/ml). Sensitive cell types may require suspension in alternative buffers to maximize viability. If necessary, PBS can be replaced with the most common cell culture buffers.

If cell viability cannot be maintained in a buffer, it is also possible to resuspend your cells in most common cell culture media, with up to 10% FBS or up to 2% BSA.

Importantly, **the media/buffer must not contain inhibitors of reverse transcription** such as excessive amounts of **EDTA** (> 0.1mM) or **magnesium** (> 3mM). Any **surfactants** (Tween-20, etc) should also be avoided as they may interfere with GEM generation.

For 10X Epi protocols, such as ATAC or Multiome, the nuclei should be delivered in the 10X Nuclei Buffer associated with the respective kit, which can be collected from the facility prior to your run.

Please remember to always bring extra buffer/media to the facility when delivering the samples, in case the sample needs to be diluted.

RNase inhibitors

While working with nuclei samples for gene expression assays, it is critical to have RNAse inhibitors in the lysis, wash and resuspension buffer. Make sure to use an RNase inhibitor approved by 10X, as some are known to interfere with downstream processing (see here for more information). 10X primarily recommends using either RNase Inhibitor 40x (10x Genomics, PN-1000887) or Protector RNase Inhibitor (Sigma-Aldrich/MilliporeSigma).

If using the 10X Genomics nuclei isolation Demonstrated Protocols or the 10x Genomics Chromium Nuclei Isolation Kit, use the RNase Inhibitor and amount recommended in the respective protocol. For other protocols a starting point for optimization, based on the Demonstrated protocols is 0.2 U/ul for gene expression assays and 1 U/ul for Epi Multiome Gene Expression & ATACs).

For cell samples it is recommended to include RNase inhibitors in the sample preparation of cell types that contain high levels of RNases, such as granulocytes.

Samples for hashing/CITE-seq

For hashing and CITE-seq, it is the responsibility of the user to perform cell surface protein labelling, as well as any required optimisation.

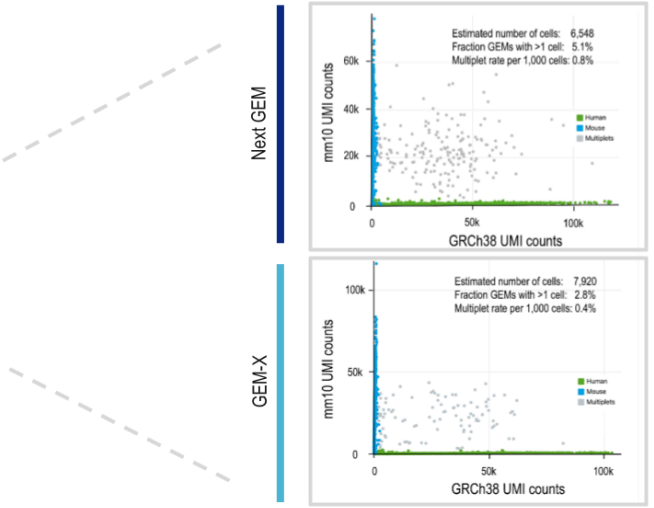
Please see information from 10X Genomics about best practices for cell surface labelling here:
https://cdn.10xgenomics.com/image/upload/v1660261285/support-documents/CG000149_Demonstrated_Protocol_CellSurface_Protein_Labeling_Rev_D.pdf
If cells are to be pooled, perform the pooling before bringing them to the facility.

Please note that presence of plasma cells in the cell suspension could contribute to background noise in the dataset. Increased fraction of plasma cells in the cell suspension could potentially lead to high fraction of antibody reads in aggregate barcodes, which leads to loss of a high fraction of UMI reads.

Multiplet rates and cell target numbers

The multiplet rate is the fraction of cell-associated barcodes estimated to be associated with more than one cell. Please keep in mind that multiplets can not always be removed bioinformatically.

Multiplet Rate	# of Cells Recovered	
	Next GEM	GEM-X
0.8%	1,000	2,000
1.6%	2,000	4,000
2.4%	3,000	6,000
3.2%	4,000	8,000
4.0%	5,000	10,000
4.8%	6,000	12,000
5.6%	7,000	14,000
6.4%	8,000	16,000
7.2%	9,000	18,000
8.0%	10,000	20,000



Multiplets in Singleplex reactions

For the GEM-X singleplex 3’ and 5’ assays, 10X generally recommends a maximum of 20.000 cells/sample, for Next-GEM (including ATAC and Multiome) the maximum is 10.000 cells. We recommend aiming for a maximum of 15000 cells or 7500 cells respectively to decrease the risk of overloading the sample. Exceeding the maximum recommended cell number would lead to increased multiplet rates and loss of single cell status of the data.

Multiplets in the OCM assay

The OCM assay has higher multiplet rates compared to the singleplex 3’ and 5’ assays, with 7,6% of multiplet when targeting 5000 cells per sample.

Multiplet Rate (%) per Sample*	# of Cells Loaded	# of Cells Recovered per sample
~1.5%	~1,650	~1,000
~3.1%	~3,300	~2,000
~4.6%	~4,950	~3,000
~6.1%	~6,600	~4,000
~7.6%	~8,250	~5,000

Capture rates and cell number variation

There are several sources of variation that influence the final number of cells captured in a 10X chromium experiment including capture rates, sample sensitivity, cell counting and pipetting. **Variations of +/- 50% between target and result are common** and can be higher if the sample quality is low.

GEM-X experiments typically have a capture rate of 75-85% of the cells loaded on the chip. For ATAC/Multiome the capture rate is 65% on the chip, but about 60% if dead volumes after tagmentation are taken into account.

Sample Quality Requirements

The quality of the ingoing cell/nuclei suspension for a 10X Genomics single cell project is crucial for obtaining high quality sequencing data of captured cells.

The 10X Genomics webpage contains resources such as demonstrated protocols, FAQs and videos on how to obtain high quality single cell/nuclei suspensions based on your starting material (https://www.10xgenomics.com/support).

NGI recommends that you test your protocol to generate your single cell/nuclei suspension before scheduling a run. It is important that you evaluate the quality of the suspension and preferably supply a representative picture (several cells/nuclei in the image field). It may also be possible to book a time to bring the suspension to the facility for evaluation.

In order to obtain high quality data from your experiment the suspension should have:

- *For cells, a viability of >80% for cells.*
- *For nuclei, >70% intact, high-quality nuclei & <5% intact cells.*
- *No aggregates*
- *No debris*
- *<5% red blood cells*
- *Concentrations of 700-1200 cells/ul if targeting up to 10,000 cells*
- *Concentrations of 1300-1600 cells/ul if targeting up to 10,000-20,000 cells*
- *Extra volume of sample for QC and counting*
- No inhibitors of reverse transcription, such as for example Mg or EDTA, or surfactants, in buffer.

Please see below for further explanations.

Cell viability

Ideally, the cell suspension should contain **more than 80% viable cells**. This will be assessed at the facility before the run.
To minimize physical damage to cells/nuclei from shearing forces, it is critical to pipette gently and slowly during cell resuspension. Depending on cell type, viability may significantly decrease when cells are kept in suspension for a prolonged period, and some cell types may also form aggregates when kept in PBS, such as peripheral blood mononuclear cells (PBMCs). Cell suspensions should therefore be loaded on the Chromium chip as soon as possible after preparation, **ideally within 30 min.**

Removing dead cells
If the cell suspension has low viability, one option is to perform a dead cell removal step. We recommend using the **Dead Cell Removal kit** (Miltenyi Biotec) and the 10X "Demonstrated Protocol Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing". Many of our users have used it with success. FACS is another alternative.

Considerations for FACS

If samples are FACS-sorted prior to submission, it is advisable to include a viability marker to remove dead cells. There are a variety of markers available, for example PI, 7- AAD and DAPI. For ATAC/Multiome it is important not to use intercalating/minor groove binding dyes. More information can be found here

Upon sample submission to NGI, cell/nuclei concentration and viability is determined, using Acridine Orange/Propidium Iodine (AO/PI) staining. AO/PI staining results in green/red fluorescently labelled cells if cells are alive/dead, respectively. Users are advised to use fluorescently labelled tags on the blue or far-red channel during the sorting process if possible, to avoid interference with viability measurement at the facility.

Please let us know if you are planning to use green or red labelled tags in your cell sorting strategy, or if your cells express a reporter. In such cases cell/nuclei viability and counting can be performed using a brightfield-based approach such as Erythrosin B staining. However, this may not be as accurate as AO/PI, especially in the presence of debris.

If sorting nuclei, take care to not damage the nuclear membrane. Also consider that nuclei, in particular, should be loaded on the Chromium chip as soon after isolation as possible.

Nuclei Quality Control

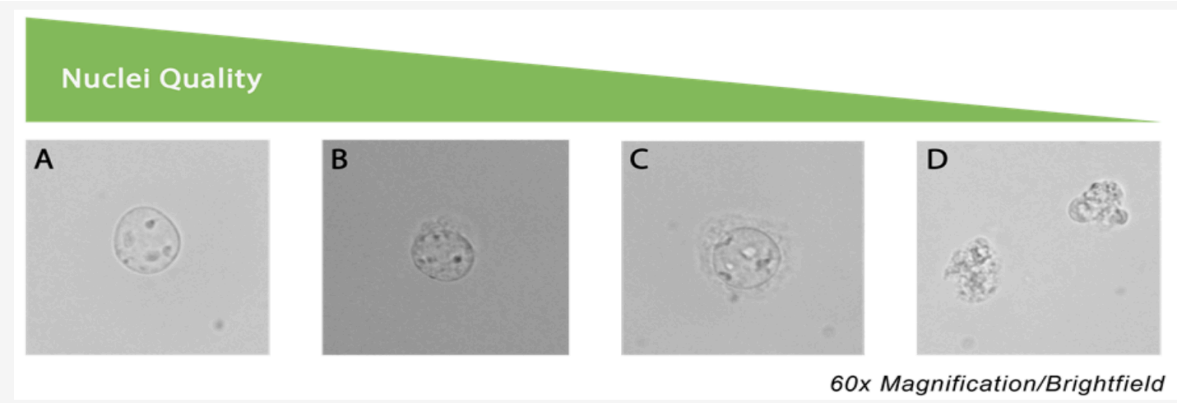
Ideally, the nuclei suspension should contain **more than 70% intact nuclei of high quality (i.e. no blebbing) and less than 5% intact cells.**. It is the responsibility of the user **to ensure high quality of the isolated nuclei and perform the necessary quality control.**

1) Evaluate the nuclear membrane integrity under a microscope preferably at minimum 40X and preferably 60x magnification to ensure intact nuclear membranes without blebbing. High-quality nuclei have well-resolved edges.
2) We recommend performing a nuclear chromatin staining (HOECHST, DAPI, PI etc.) and evaluate the chromatin status, using a fluorescent or confocal microscope. Uniform chromatin staining indicates good quality of isolated nuclei. Observation of irregular borders and intranuclear condensed staining areas ("dotty" staining) indicate nuclei undergoing apoptosis (https://www.pnas.org/doi/10.1073/pnas.0405374102).

The user is encouraged to provide the quality control evaluation information (images of nuclear membrane and stained chromatin status) of the extracted nuclei to the facility.

To minimize physical damage to nuclei from shearing forces, it is critical to pipette gently and slowly during nuclei resuspensions. Nuclei suspensions should be loaded on the Chromium chip as soon as possible after preparation, ideally **within 30 min from isolation**.

Examples of nuclei quality below. For more information about 10X recommendations see this article: What are the best practices for working with nuclei samples for Chromium Universal and Epi assays?s



A: High-quality nuclei have well-resolved edges. Optimal quality for single-cell gene expression libraries.

B: Mostly intact nuclei with minor evidence of blebbing. Quality single-cell gene expression 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!***

Cell/nuclei aggregates

Aggregates can cause clogging of the 10X microfluidic chip and will increase the likelihood of having doublets/multiplets of cells. Washing and resuspension of the cells should be performed in 1X PBS (calcium and magnesium free) +0.04% BSA. BSA is primarily included to minimize cell losses and aggregation. If the cells aggregate, **higher BSA concentrations** (up to 2% BSA) may help prevent the clumping and will not have any adverse effects on workflow or the data.

If aggregates are present despite a higher BSA-concentration, samples can be filtered using a **cell strainer**. We recommend either **Flowmi™ Cell Strainer for low volume samples** (Merck), or a **MACS SmartStrainer** (Miltenyi Biotec) for samples with higher volume but low concentration. Please note that filtering the suspension will likely lead to some loss of cells and volume.

Debris

Tissue/cell debris in the suspension can lead to inaccurate cell counts, clogging of the 10X microfluidic chip, and increased background from ambient RNA.. If debris is present in the sample we recommend filtering the sample using a cell strainer.
For larger debris, the **MACS SmartStrainer** (Miltenyi Biotec) is recommended, for low cell suspension volumes, the **Flowmi™ Cell Strainer** (Merck) or the **pluriStrainer® Mini** (pluriSelect) are recommended. Please note that filtering the suspension will likely lead to some loss of cells and volume. For smaller debris, centrifugation-based approaches may work.

FACS-sorting is recommended if high amounts of debris and/or dead cells are present in cell samples.

Red blood cells

Red blood cells (RBCs) can be seen under the microscope as small cells, very uniform in shape with a yellow coloring and a small black dot in the middle. Removing RBCs may not be necessary before loading your sample, however, if they are not removed, they will contribute to the total number of cells that are loaded and will increase the amount of sequencing you will need to perform to detect the other cell populations of interest.

It is possible to remove the RBC population bioinformatically during Loupe Cell Browser analysis if it is not possible/desirable to remove them during sample prep.

If you want to remove RBCs before loading your sample, the 10X Demonstrated Protocol for tumor dissociation includes an RBC lysis step using a **Red Blood Cell**

Lysis Solution (Miltenyi Biotec). You can also use **Ficoll purification** to remove RBCs from PBMC samples prior to loading the cells. For more information please read this knowledge base article.

Technical failures during the 10X method run

The most common technical failures associated with a 10X chromium run relates to formation of the emulsion in the microfluidics chip. These are more common in the Next-GEM chemistry-based kits (ATAC/Multiome). With the introduction of the GEM-X Chemistry used in the Universal 3' and 5' Gene expression the risk of this type of failures has been drastically reduced.

Clogs

During the generation of the emulsion on the 10X Chromium instrument, one potential cause of failure is the occurrence of a sample clog. This is generally caused by suboptimal sample preparation (suspension containing aggregates, fibers or debris), or by loading more than the recommended number of cells/nuclei.

Clogging is typically associated with a lower volume of the formed emulsion. If not associated with a wetting failure (see below) it may be possible to continue with the protocol and generate cDNA and libraries, although with lower yields and capturing a reduced number of cells. However, it may be preferable to re-run the sample if possible (see below).

If the sample was of high quality, 10X genomics will typically cover the cost. If the sample suspension was sub-optimal the cost of the run is on the user.

Wetting failure

Another type of failure that can occur during a sample run is a wetting failure, where the emulsion fails to form properly. This often has no obvious cause and will therefore typically be reimbursed by the 10X Genomics company. In such cases, there will be no cost for the user for the failed sample.

In case of a clog or wetting failure

If there is a clog or a wetting failure on a clean, high-quality sample, the facility will attempt to re-run the sample **IF** there are enough cells/nuclei and **IF** the facility has reagents in stock. However, we cannot guarantee that a sample will be re-run.

If a sample can not be re-run and the reaction is replaced by 10X it is possible to submit a new sample at a later occasion, or remove the cost from the project. For kits offered as full kits, we can only offer the first option.

Suboptimal sample form

If a user decides to continue with samples that do not meet the quality criteria above, or in some cases of custom project set-ups that do not follow 10X recommendations, we will request that the form on the next page is filled out and signed. This is to ensure and document that the user has received information regarding the sample quality and possible consequences.

Information regarding low-quality starting material at NGI

We have been informed by the staff at the Single Cell Genomic Applications facility that the samples listed below were of suboptimal quality. We chose to continue the 10x genomic sample preparation despite being informed that it may fail or result in low-quality data. If so, we accept that we will still be billed for the run of the sample, as the reagents of the kit will be consumed.

Cell/Nuclei samples with low quality

Sample name	NGI Sample ID

_____ _____ _____

Date Name Signature