10X Genomics Single Cell Sample Submission Guidelines

The quality of the cell or nuclei suspension to be used with the single cell methods from 10X Genomics play a crucial role in obtaining high quality sequencing data of captured cells. Please visit 10X Genomics webpage for "Demonstrated Protocols" on how to obtain high quality single cell/nuclei suspensions based on your starting material (https://support.10xgenomics.com/single-cell-gene-expression/index), and we also recommend you to watch a video from 10X Genomics discussing sample preparation and clean-up steps, including tips if some of the standard steps do not work for your specific sample (https://www.10xgenomics.com/solutions/single-cell/#)

ESCG recommends that you test the protocol to generate your single cell/nuclei suspension before scheduling an actual run with the 10X methods. It is important that you show ESCG the quality of the suspension by either sending a picture representative of the whole suspension (several cells in the image field) by email, or that you 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:

Been prepared in low-binding tubes
A viability of >90% when cells are derived from fresh tissue / cultures
No aggregates (cell clumps)
No observable debris
Less than 5% of red blood cells
A concentration of 700-1,200 cells/µl (1,300-1,600 for high cell targets)
No inhibitors of reverse transcription or GEM generation
Enough extra cells for counting them, to perform accurate cell loading
Smallest possible volume for suspensions coming from FACS

Please see below for further explanations.

Important

- Please check the quality of your samples, be sure that there are no aggregates
- Please count your samples with an accurate automated cell counter or manually
  before submission to the facility.
- 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.

Prior to loading your sample onto the 10X Chromium chip, we inspect it under the microscope. We request that you stay with us during these steps in case we see a suboptimal suspension and need to discuss it with you before starting the experiment. In the end, ESCG always advises the best possible outcome based on our experience, but it is the user's decision to proceed or not with a non-optimal sample.

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

Explanations to High Quality Cell/Nuclei Suspension Requirements
Cell viability:
Ideally, the cell suspension should contain **more than 90% viable cells** or **more than 90% intact nuclei**. This is estimated on a cell counter at the facility. To minimize physical damage to cells/nuclei from shearing forces, it is critical to pipette gently and slowly during cell resuspensions. Depending on cell type, viability may significantly decrease when cells are kept in suspension for a prolonged period, and some cell types such as peripheral blood mononuclear cells (PBMCs) can even form clumps when kept in PBS for extended periods of time. Cell suspensions should therefore be loaded on the Chromium chip as soon as possible after preparation, **ideally within 30 min.**

If your cell suspension contains many non-viable cells, please use the **Dead Cell Removal kit** (Miltenyi Biotec) and follow 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.

If you are FAC-sorting your sample prior to submitting it to us at ESCG, it is advisable to include a viability marker. There are a variety of markers to use, for example PI, 7- AAD and DAPI. More information can be found here. Cell (or nuclei) concentration and viability in suspension are determined, using Acridine Orange/Propidium Iodine (AO/PI) staining during sample delivery. AO/PI stain 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 sorting process (if possible), to not interfere with viability measurement strategy in the facility. Please let us know if you are planning to use green or red labelled tags in your cell sorting strategy (or your cells express a reporter). We can devise the viability counting, using an additional Trypan Blue staining and manual counting. Cell (or nuclei) concentration in suspension will still be performed using AO/PI stain.

Cellular/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 your cells should be performed in 1X PBS (calcium and magnesium free) + 0.04% BSA (BSA is added primarily to minimize cell losses and aggregation), but if you see cell clumps in your suspension, then **higher BSA concentrations** (up to 2% BSA) in the buffer might contribute sterically to prevent the clumping and will not have any adverse effects on workflow or data.

If the clumping is not helped by a higher BSA-concentration, then you can pass your suspension through a **cell strainer** prior to the loading on the 10X chip here in the facility. For low volume suspensions use a **Flowmi™ Cell Strainer** (Merck), for low cell number suspensions use a **MACS SmartStrainer** (Miltenyi Biotec).

Debris:
Tissue/cell debris in your suspension can cause clogging of the 10X microfluidic chip, contamination of your single cells with ambient RNA, and inaccurate cell counting. If you see debris in your cell suspension, please use a cell strainer.
For larger debris, the **MACS SmartFilter** (Miltenyi Biotec) is recommended, for low cell suspension volumes, the **Flowmi™ Cell Strainer** (Merck) or the **pluriStrainer® Mini** (pluriSelect) are recommended.

For smaller debris, the **Debris Removal Solution** (Miltenyi Biotec) allows for debris removal through centrifugation.

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 you do not, or cannot, remove them during sample prep.
If you want to remove RBCs before loading your sample, 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.

Cell/nuclei number and concentration:
If possible, we request you to bring at least roughly **three times the number of cells/nuclei** you aim for as output, meaning if you aim for having 5,000 cells sequenced, we need you to bring 15,000 cells.
For Targeted Cell Recovery up to 10000 cells, the optimal concentration is in the range of **700 to 1,200 cells or nuclei/µl**.
For Targeted Cell Recovery up to 20000 cells, the optimal concentration is in the range of **1,300 to 1,600 cells or nuclei/µl.** If your suspension is too diluted when arriving at the facility, you can use our centrifuges for concentrating it, please remember to bring extra buffer in case it is needed.

Inhibitors of reverse transcription and GEM generation:
When submitting your sample to ESCG the recommended buffer for the cell 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, so, 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.

Media should not contain excessive amounts of **EDTA** (> 0.1mM) or **magnesium** (> 3mM) as those components will inhibit the reverse transcription reaction. Any **surfactants** (Tween-20, etc) should also be avoided as they may interfere with GEM generation.

Samples for hashing:
For hashing and CITE-seq, it is the responsibility of the User to perform cell surface labelling, as well as any optimisation required.

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, make sure to perform the pooling before bringing them to the facility.

Possible failures during the 10X method run

Clog:
When running samples using the 10X method, occasionally a sample clog may occur. This is generally caused by suboptimal sample preparation (suspension containing clumps or debris), or by loading more than the recommended number of cells/nuclei. If clogging occurs during the generation of the emulsions, these will not be formed properly, and your cells/nuclei will not be encapsulated in single droplets and the processing generally cannot proceed. If the sample suspension was sub-optimal and we had discussed the risk of a clog occurring, then the cost of the run is on the user.

If it is a very precious sample and a re-run cannot be attempted (see below), it may be possible to continue with the protocol and generate cDNA and libraries, although at lower levels and coming from a reduced number of cells.

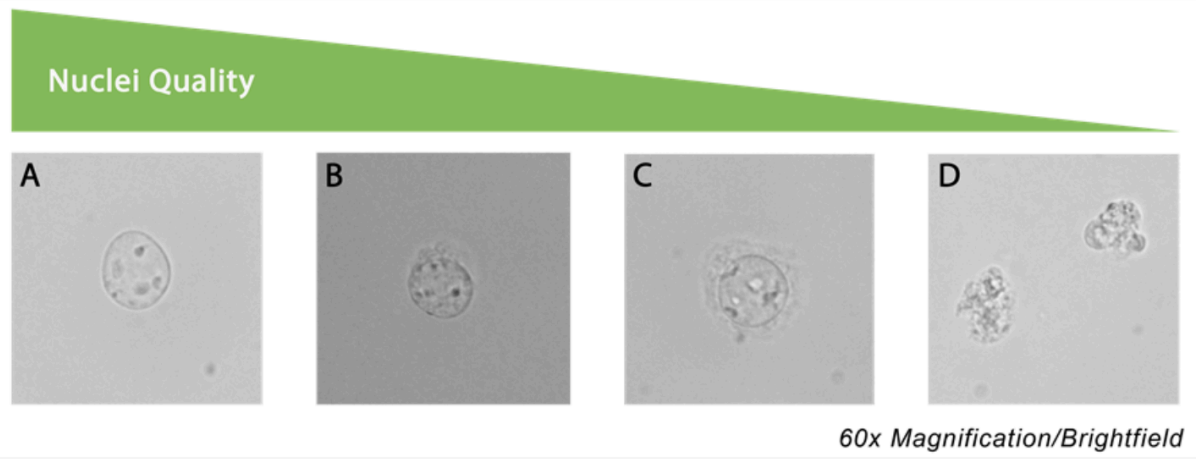
If the quality of the sample suspension was such that no clogging was to be suspected, then we can relate to a run failure, and this will be covered by the 10X Genomics company.

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

In the case of a wetting failure:
If there is a wetting failure or a clog 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.

Examples of nuclei quality below
More information is found at:

https://kb.10xgenomics.com/hc/en-us/articles/360050780051-What-are-the-best-practices-for-working-with-nuclei-samples-for-3-single-cell-gene-expression-



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.***

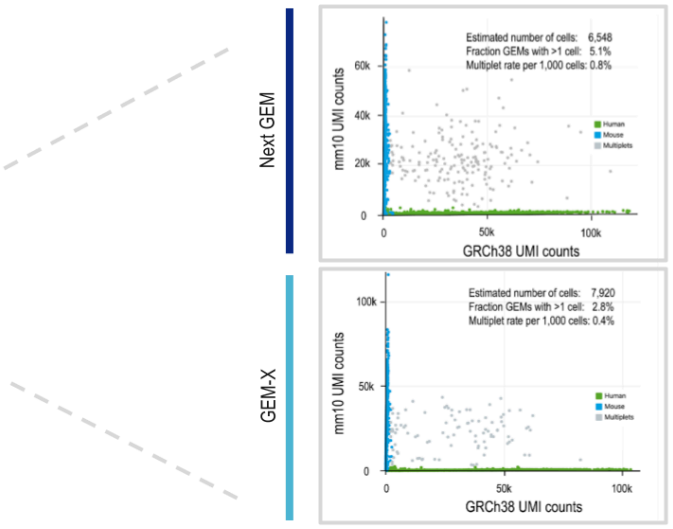
D: Nuclei are no longer intact. ***Do not proceed!***

While working with nuclei samples, it is critical to have RNAse inhibitors in the lysis, wash and resuspension buffer. For the 3'GEX assay, 10X Genomics recommends a concentration of 0.2U/ul of RNAse inhibitor. 10X Genomics recommended RNase Inhibitor is the Protector RNase Inhibitor from Sigma Aldrich (PN-3335399001).

Multiplets

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 cannot 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



10X generally recommends a maximum of 10.000 cells/sample. We recommend aiming for a maximum of 7.500 cells to decrease the risk of overloading the sample. Exceeding 10.000 cells would lead to increased multiplet rates and loss of single cell status of the data. One can expect around +/- 50% load, even more if the sample quality is low. GEM-X technology allows targeted cell recovery up to 20.000 cells/sample.

Information regarding low-quality starting material at ESCG

We have been informed by the staff at the ESCG 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	ESCG Sample ID

_____ _____ _____

Date Name Signature