10X Genomics GEM-X Multiome Sample Submission Guidelines

This document details the sample submission requirements and recommendations for 10X Genomics Epi Multiome GEM-X experiments at NGI Stockholm. For information regarding ATAC or Multiome with the previous chemistry (Next-GEM), please see “Sample Submission Guidelines for ATAC & Next-GEM Multiome”, available on the NGI website.

GEM-X Epi Multiome supports experiments of 500-20000 nuclei per reaction.

Practical Information

Before the run: The responsible lab staff will reach out the days before a scheduled run to make practical arrangements, including picking up reagents for the **Diluted Nuclei Buffer**. The deadline for delivering samples on the day of the run is 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 notify us 30 minutes before arrival, to allow for preparation of sensitive reagents.

Sample delivery & QC: Upon 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 anything unexpected is observed in the QC and discussion is needed before starting the experiment. For suboptimal samples, NGI staff can offer opinions based on our experience, but it is the user's decision to proceed or not, since the consequences for 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 sample submission on the run day is **11:30 pm**. Samples will not be accepted after this time.
- Nuclei must be delivered in the correct Diluted Nuclei Buffer. **Extra buffer MUST be delivered** with the samples. (see below)
- Nuclei must meet the facility's quality criteria (see below) .
- Count samples with an accurate automated cell counter or manually before submission to the facility. Whenever possible, use fluorescent dyes for the highest accuracy.
- In case of suboptimal samples: If the user insists on going forward with the protocol even though the nuclei suspensions are suboptimal, 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.

Individual vs Scalable transposition

The GEM-X Epi Multiome workflow can target 500-20,000 nuclei per reaction but has two options for the initial transposition step of the protocol.

1. Individual Transposition: ≤30,000 nuclei to target 500-20,000 nuclei. All the nuclei added to the transposition step are used in the same 10X reaction in downstream steps. Starting with ≤30,000 nuclei to target 500-20,000 nuclei. No counting after transposition.
2. Scalable Transposition: Starting with ≥30,000 nuclei. Possibility to count nuclei after transposition and split into two or more reactions.

Nuclei amount and concentration - Individual transposition

If possible, we request that you to bring at least roughly **three times the number of nuclei** you aim for as output. If you aim to sequence 5,000 nuclei, please bring 15,000 nuclei. See table below for permissible nuclei concentrations based on nuclei target number.

Examples of permissible nuclei concentrations

Nuclei Recovery Target (nuclei)	Concentration Range (nuclei/ul)
500-2000	400-1,000
1000-10,000	1000-6000
10,000-20,000	2000-10000

- Please bring samples in 1.5ml low binding tubes.
- If samples are too dilute, you may use the centrifuge in the NGI lab to concentrate them.
    - NB! Samples must be counted and ready to start processing latest 12:30, in order to reach a safe stopping point by the end of the day.. If extensive adjustments are needed, make sure to arrive so you have enough time for preparation.

Buffer for GEM-X Multiome sample submission

Nuclei must be delivered in **Diluted Nuclei Buffer** (see below). Please note that this is different from the buffer for Next-GEM Multiome.

Concentrated Nuclei Buffer, Reducing Agent B and RNase inhibitor is included in the Multiome reagent kit and can be picked up at NGI in advance of your run on an agreed upon date and time.

Extra Diluted nuclei buffer MUST be brought together with the samples, as it is needed for the transposition reaction.

Preparation of Diluted Nuclei Buffer

Prepare Diluted Nuclei Buffer *Maintain at 4°C*

The use of the Tris-based Diluted Nuclei Buffer for nuclei suspension is critical for optimal assay performance. The composition of the Diluted Nuclei Buffer, including Magnesium concentration, has been optimized for the Transposition and Barcoding steps. Suspension of nuclei in a different buffer may not be compatible with the downstream protocol steps.

	Stock	Final	Volume (µl)
20X Nuclei Buffer (PN-2000207; equilibrated to room temp.)	20X	1X	50 µl
Reducing Agent B (PN-2000087; equilibrated to room temp.)	1,000 mM	1 mM	1 µl
RNase Inhibitor (Use PN-2001488 or equivalent. Confirm vendor-specific stock concentration)	40 U/µl	1 U/µl	25 µl
Nuclease-free Water	-	-	924 µl
Total	**-**	**-**	**1000 µl**

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 1 U/ul for Epi Multiome Gene Expression.

Multiplet rates and nuclei 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 Nuclei Loaded	# of Nuclei Recovered
~0.20%	~725	~500
~0.40%	~1,450	~1,000
~0.80%	~2,900	~2,000
~1.20%	~4,350	~3,000
~1.60%	~5,800	~4,000
~2.00%	~7,250	~5,000
~2.40%	~8,700	~6,000
~2.80%	~10,150	~7,000
~3.20%	~11,600	~8,000
~3.60%	~13,050	~9,000
~4.00%	~14,500	~10,000
~4.40%	~15,950	~11,000
~4.80%	~17,400	~12,000
~5.20%	~18,850	~13,000
~5.60%	~20,300	~14,000
~6.00%	~21,750	~15,000
~6.40%	~23,200	~16,000
~6.80%	~24,650	~17,000
~7.20%	~26,100	~18,000
~7.60%	~27,550	~19,000
~8.00%	~29,000	~20,000

Capture rates and cell number variation

While the expected capture rate for Epi Multiome is about 60% for Next-GEM and 70% for GEM-X with the individual transposition protocol, there are multiple sources of variation that influence the final number of nuclei captured. Factors such as capture rates in

GEM-generation, sample sensitivity, cell counting, sample-dependent loss in the post-transposition wash and pipetting can contribute to variability in the final nuclei capture.

Variations of +/- 50% between target and result are common and can be higher if the sample quality is low. The target nuclei number given to the facility should only be viewed as

indicative. For this reason we caution that aiming for the maximum cell number may cause the reaction to be overloaded, resulting in more multiplets.

Sample Quality Requirements

The quality of the nuclei suspension for a 10X Genomics Epi Multiome project is crucial for obtaining high quality data. 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).

10X nuclei preparation guidelines

For preparation of nuclei for Multiome or ATAC experiments, please refer to the following protocols from 10X Genomics:

- Nuclei Isolation for Single Cell Multiome ATAC + Gene Expression Sequencing
- Nuclei Isolation from Complex Tissues for Single Cell Multiome ATAC + Gene Expression Sequencing

10X Genomics also offer a compatible Chromium Nuclei Isolation Kit for use with tissues.

Adhering to the protocols above is, according to 10X genomics, critical for optimal assay performance.

In addition, it is 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.

NGI strongly recommends that you test your nuclei isolation protocol before scheduling a run. It is important that you evaluate the quality of the suspension and preferably supply a representative picture.

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

- *High quality nuclei with well-resolved edges*
- *High viability of cell suspension, >90%, before nuclei isolation*
- *Low amounts of intact cells (<5%)*
- *Minimal amount of aggregates/debris*
- *Been prepared using RNAse inhibitor recommended by 10X*

- *No inhibitors of reverse transcription or GEM generation in buffers*
- *Concentrations appropriate for the intended nuclei capture (see table 1 above)*
- *Been prepared/delivered in low-binding tubes*
- *Sufficient volume/amount to also perform counting and QC*

Please see below for further explanations.

Nuclei handling & storage

Nuclei **must be prepared fresh** for Epi Multiome experiments. Ideally nuclei should be loaded on the Chromium chip within 30 minutes of isolation.

Freezing or cryopreservation of nuclei is not recommended by 10X as it can damage the nuclear membrane and cause nuclear leakage, leading to increased background in the experiment. Cryopreservation of the cells or tissue prior to nuclei isolation is recommended as an alternative solution.

To minimize physical damage to nuclei from shearing forces, it is critical to pipette gently and slowly during nuclei resuspension. Deliver nuclei in low binding 1,5 eppendorf tubes.

Nuclei Quality assessment

It is the user's responsibility to ensure high quality of the isolated nuclei and perform the necessary quality checks. While the facility will count the nuclei, there is limited room for additional quality assessment. There may be time to check one sample with bright field microscopy. Otherwise the on site QC will be performed using a cell counter with which nuclei integrity can not be assessed.

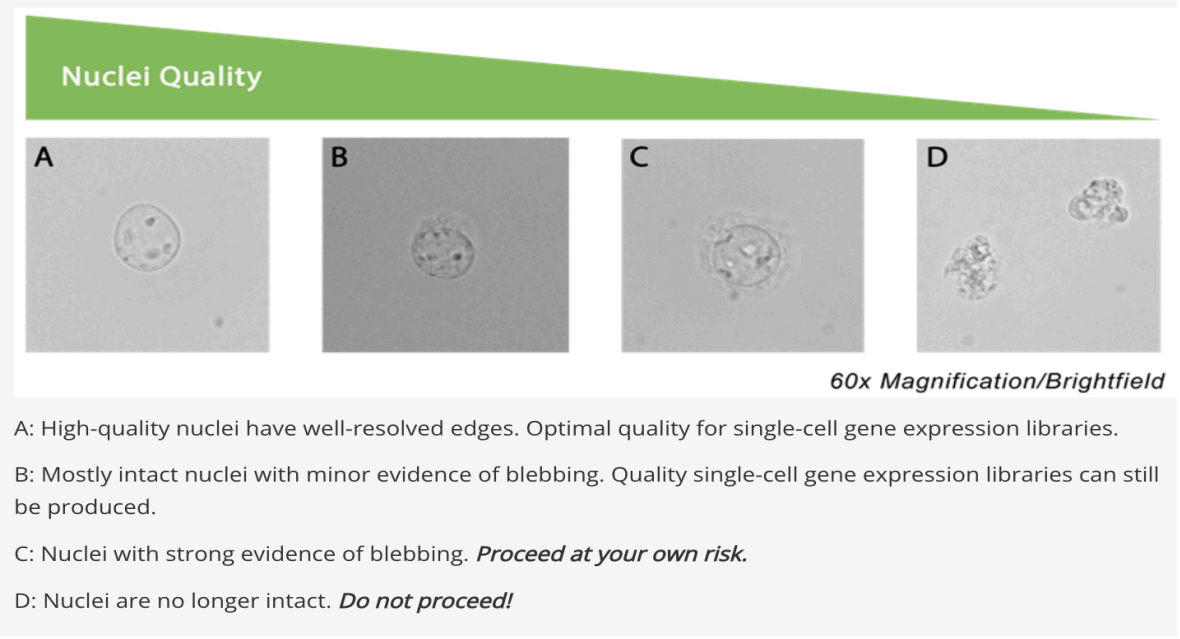
After nuclei isolation we recommend the following quality control evaluation:

1. Evaluate the nuclear membrane integrity under a microscope at 40x or 60x magnification to ensure no blebbing of the nuclear membrane. High-quality nuclei have well-resolved edges. See below for examples.

2. Perform a nuclear chromatin staining (HOEST, DAPI, PI etc.) and evaluate the chromatin status, using a fluorescent or confocal microscope. Uniform chromatin staining indicates good quality of isolated nuclei. Irregular borders and intranuclear condensed staining areas ("dotty" staining) indicate nuclei undergoing apoptosis (https://www.pnas.org/doi/10.1073/pnas.0405374102).

We encourage users to provide the quality control evaluation information (images of nuclear membrane and stained chromatin status) of the extracted nuclei to the facility.

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?*



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!***

Removing dead cells

If the cell suspension prior to nuclei isolation 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 for ATAC/Multiome analysis it is important not to use intercalating/minor groove binding dyes. More information can be found here. Take care not to damage the nuclear membrane and take into consideration that the samples should be loaded on the 10 X microfluidic chip as soon as possible.

Upon sample submission to NGI, nuclei concentration is determined using Acridine Orange/Propidium Iodine (AO/PI) staining. AO/PI staining results in green/red fluorescently fluorescently labelled tags on the blue or far-red channel during the sorting process if possible, to avoid interference with quality assessment 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.

Nuclei aggregates

Aggregates can cause clogging of the 10X microfluidic chip and will increase the likelihood of having doublets/multiplets of nuclei. BSA can potentially be added to buffers used in nuclei isolation to decrease aggregation. However, it is recommended to limit BSA to maximum 1%. Higher concentrations may inhibit transposition in Epi Multiome and ATAC experiments. Note that the final nuclei resuspension must be in the **Diluted Nuclei Buffer** (which does not have BSA).

If aggregates are present, samples can be filtered (see below). Please note that nuclei aggregation can be a sign of overlysis indicating that the isolation needs further optimization.

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 smaller debris, centrifugation-based approaches may work. For larger debris, filtering the sample is an option (see below). FACS-sorting is recommended if high amounts of debris and/or dead cells are present in cell samples.

Filtering of samples

If the samples display nuclei aggregates or larger debris, filtering using a cell strainer of appropriate size may be helpful. We recommend either **Flowmi™ Cell Strainer (Merck),** for low volume samples, or a **MACS SmartStrainer** (Miltenyi Biotec) for samples with higher volume but low concentration. Please note that filtering the suspension will lead to some loss of cells and volume.

Technical failures during the 10X method run

The most common technical failures associated with a 10X Chromium run relate to formation of the emulsion in the microfluidics chip. These are more common in the Next-GEM chemistry-based kits (ATAC). With the introduction of the GEM-X Chemistry used in the

Universal 3’ and 5’ Gene expression and the Epi Multiome, the risk of this type of failure 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 suboptimal 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 10X Genomics. If so, there will be no cost for the user for the failed sample.

If there is a wetting failure for a Multiome sample, the facility will default to NOT re-running the sample immediately regardless of if of extra sample and reagents are available. This is due to the leftover nuclei suspension sitting on ice during the transposition and GEM-generation, potentially leading to decreased quality.

Suboptimal sample form

If a user decides to continue with samples that do not meet the quality criteria above, or in 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 at the facility. 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