protocol vA.07

Samples preparation guidelines for 10X Genomics FLEX (GEMX)

Contents

Interactions with GECF

IN SHORT: Discuss ahead with us: 1) **fixation/storage** conditions; 2) **When** to bring the cells; 3) the **minimum number of fixed cells** needed relative to your targeted cells number.

Then bring your **cells/nuclei fixed** in **1.5 ml tubes, quenched and stored** according to these guidelines and to the official ones of 10XG. In parallel, send us the **submission form** and **pictures of cells/nuclei before fixation** (if applicable).

Disclaimer

10XG runs work in general well but are still at the technological frontier, and a small % of samples fail for purely technical reasons. We don't charge for these unless we can spot a critical error from the user side (e.g. detrimental deviation from cell prep guidelines/buffers), or if users gave us their green light to carry on despite poor cell quality. If a putative failure represents a major issue for your project (e.g. months-long mice generation), tell us in advance and we'll discuss the risks and means to minimize them (pilot, freezing a fraction of cells…).

General information about FLEX

- It is a **probe-based method**, covering whole coding transcriptome (only mRNAs).
- The probe set covers the whole transcriptome unless a few hundred genes for which probes could not be designed without off-targets. These can still be analyzed on demand. More info here.
- Detection of transgenes, reporters and viral genes requires designing **custom probes** before the experiment, with modest extra-costs. (Caution: transgenes with high similarity to endogenous genes come with complication for the design of custom probes, and with risks of cross-species probes binding)
- **No information is obtained regarding SNPs or isoforms**, since probe-based.
- **Only human, mouse and rat** probes are available.
- FLEX involves multiplexing samples before running them in the 10XG instrument.
- Prior to fixation, **single cells can be labeled** using a specific antibody conjugated to a **Feature Barcode oligonucleotide** (TotalSeq-B, Totalseq-C or Proteintech Genomics antibodies) to quantify protein expression.

- The table below summarizes the different fixation/storage workflows. More details in the text below.

Sample type		Fixation	Storage after cell/nuclei isolation	10XG protocol
Single cells/nuclei	**All cells apart leukocytes and bone marrow mononuclear cells**	4°C - 16-24 h	-80°C for up to 12 months	CG000782
	Leukocytes or bone marrow mononuclear cells	20°C - 16-24 h	-80°C for up to 12 months	
Blood	**PBMCs**	4°C - 20–24 h or up to 1 week	-80°C for up to 12 months	CG000785
	Leukocytes	• 20° - 20–24 h • 4°C - 1 week	-80°C for up to 12 months	
FFPE tissues		no fixation	no storage possible	CG000784
OCT-embedded fresh frozen tissues		Limited testing - consult this 10x Genomics webpage		

Design

Cell size

- **Max cell diameter that can be processed is 30um**, as the workflow contains a 30um filtration step. This diameter is calculated for cells in suspension, not flattened on a petri dish. Also to be taken into account that fixed cells shrinks. If cells are significantly > 30 µm, nuclei isolation should be performed (see below).
- 10XG observe **no differential recovery for small vs large cells** when running a sample composed of cells of different sizes (for cells that are equal to or smaller than 30 µm).
- **Number of genes detected directly depends on the cell size** (=amount of mRNA molecules per cell). Sequencing depth chosen may thus be modulated according to cell size.

Targeted cell number

- Define the number of cells you want data for ("targeted cell number"). Recovery rate is uncertain and depends on physical characteristics of the cells/nuclei, as well as on experiment-specific factors, such as viability, % of debris, etc. therefore **targeted cells number is only indicative**.
- **Maximum targeted cells** per sample: **20'000**.
- If you absolutely want a given minimum number of cells, **consider adding 20% safety margin** to compensate for putative low capture efficacy. Also indicate it in the submission file.

- The **ideal number of targeted cells** depends on the biological question. For comparing two populations, 2'000 cells may be enough. At the other extreme, for identifying new rare subpopulations of cells (<1%), 10'000 or more cells may be needed. If unsure, we recommend 7'000 cells as a good starting point.
- **The rate of doublets increases with targeted cells number**, up to 8% at 20'000 cells. We recommend not targeting > 13'000 cells per sample unless necessary, to keep multiplet rate < 5%.

Multiplexing strategies

The distribution of reads across samples in a pool is determined by the composition of the pool. It is not possible to add reads to only specific samples in the pool. The only option to increase the reads/cell of a specific sample is to sequence at higher depth the whole pool.

Two factors affect the final distribution of reads across samples in the same pool:

- **Cells capture rate may be different among samples in a pool**, depending on cell size, shape, viability
- Sequencing reads will be distributed to different samples of the pool in proportion to their RNA content. So, **cells with higher RNA content will receive more reads than cells with lower RNA content.**

The recommendation therefore is to:

- Ask us to pool samples according to a putative distinct sequencing depth requirement.
- If big differences are expected in RNA content between multiplexed samples, exert caution when designing the composition of the project.

Number of reads/cell

Probe-based approaches such as FLEX require fewer reads than the classical 3' method.

The choice of number of reads/cell impacts costs. These factors can influence this choice:

- the **size of the cells**. For very small cells (5-10um), mRNA levels are low and sequencing saturates fast, thus 25-50k reads/cell capture most of the available information. In contrast big cells (>25um) contain a lot of mRNA can be sequenced up to 100k reads/cell and above and still capture new information.
- the **biological question**: for clustering cells, or to delineate broad transcriptional profiles, 25k reads/cells is enough. If you want to zoom on specific genes, or if you want to delineate new cell type with subtle differences, the more reads the better (up to full sequencing saturation, see above).

If you are not sure, we recommend 50k as a starting point.

Cells preparation guidelines

The main workflow involves dissociation followed by fixation. Yet, when no good dissociation protocol exists, it is possible to try to fix the tissue directly and then dissociate it. Inquire if needed.
IMPORTANT: *A pilot experiment is recommended to test the dissociation/fixation procedure.*

Sample submission

- The samples must be brought to GECF already dissociated, fixed, quenched, and properly stored.
- Measuring cells concentration of the fixed samples is required, and results must be included in the submission form. It is strongly recommended that, for this count, samples are stained with a fluorescent nucleic acid dye.
- Fixed samples stored at -80° according to guidelines must be brought to GECF on dry ice.

Fixation and cell/nuclei isolation

The **fixation and cell/nuclei isolation are performed by the users**.
Very important: many reagents in the fixation protocol need to be **molecular biology grade** (e.g. formaldehyde, glycerol…). It is always better to **order the exact reagents recommended in the protocol**.

- If fixing **dissociated cells**:

- Refer to the latest revision of 10XG protocol CG000782 - Fixation of Cells & Nuclei for GEM-X Flex Gene Expression. This protocol describes in detail fixation, quenching and storing procedures.
- The **recommended minimum number of cells/nuclei** is ≥ 25k cells or nuclei according to the protocol. However, tech support advises to use ≥ 75k cells (< 75k only works with perfect cells/nuclei). *If starting fixation < 75k, we cannot guarantee success. Importantly the minimum number of cells given above are*

true for low number of targeted cells, if one wants to target 20k cells, more are needed.

- If starting from **blood**:
  - Refer to the latest version of 10XG protocol CG000785 - Blood Fixation and Cell Isolation for GEM-X Flex Gene Expression.
  - Pay particular attention that red blood cells are not present in the final isolated population
- **FFPE tissues**:
  - refer to the latest version of 10XG protocol CG000784 - Sample Preparation from FFPE Tissue Sections for GEM-X Flex Gene Expression
  - once nuclei are isolated, no storage is possible
- **OCT embedded fresh frozen tissues**: limited testing - consult this 10x Genomics webpage.

- We provide the **10XG fixation reagents** (kit ref 1000781, included in service price). Contact us/collect these reagents well in advance.
- It is recommended to:
  - perform a fixation test before the real experiment (fixation reagents for the test are billed).
  - perform a 16-24 h incubation at 4°C and store the fixed samples at -80°C for best results.
  - when cell number allow, freeze a separate small aliquot of cells/nuclei to check quantity/quality after post-fixation storage.
- Samples can be FACS-sorted post-fixation, if needed (e.g. to remove debris)
- When working with low cell numbers (<300k cells), complete removal of the supernatant is not required. Up to 30 µl supernatant may be left behind to optimize cell recovery without significantly impacting performance.

Tissue dissociation

- A list of **tested tissue** can be found here.
- **Avoid using plastic petris or dishes for mincing/crushing tissues**, as this can create plastic debris that can clog the capillaries. Only use glass containers.
- Dissociating tissue **prior to fixation**:
  - Only possible on fresh tissue, not on frozen tissue (unless you isolate nuclei).
  - Established tissue dissociation protocols that work for 3' GEX are expected to work with FLEX too.
  - LIVE/DEAD Fixable Stains from Thermo can be used to determine the viability of fixed cells -> this dye allows to **stain unfixed cells**, fix cells, and then **sort the labeled fixed cells**. For more info consult here
- Additional info can be found here

RNase inhibitor

- The addition of **RNase inhibitors is recommended** during sample preparation of **RNase-rich tissues** (spleen, pancreas, lung) or **if isolating nuclei**. Supplementing RNase inhibitors into the wash and resuspension buffers may also help preserve your RNA before sample fixation. Up to 1U/ul may be used for single cell assays.
- CAUTION: **some RNAse inhibitors impact negatively performances** (see here). Use the **recommended ones**:

  - Protector (Sigma 3335399001)
  - RiboLock (Thermo EO0382)
  - Ambion RNAse inhibitor (Thermo AM2684)
  - RNaseOUT (Thermo 10777019)

Final cells suspension preparation

- Absolutely **avoid aggregates or clumps** as they may clog the capillaries and lead to run failure. To avoid these,

cells can be passed through a cell strainer. This is usually not necessary if cells are FACS-sorted.
Some examples of filters:

- Flowmi pipette cell strainer of 40um.
- Miltenyi Biotec 30um PreSeparation Filter, cat#130041407….

- Samples should have **minimal debris** to minimize background. FACS sorting can be performed to remove cellular debris from cell or nuclei suspensions using a nuclear stain.
- If working with **organoids**, find some best practices at this link.

Nuclei suspension preparation

- Nuclei protocols that have been previously tested for compatibility with the Single Cell Gene Expression assay (i.e., 3' GEX) are expected to perform similarly in FLEX.
- If starting from **fresh tissue**, refer to the 10XG protocol CG000124 for Isolation of Nuclei for Single Cell RNA Sequencing & Tissues for Single Cell RNA Sequencing.
- If starting from **frozen tissue**, nuclei can be isolated using the Chromium Nuclei Isolation Kit. Please refer to the latest version of the user guide 10XG CG000505. Isolating nuclei from frozen tissues can be technically challenging and may require customization for different tissue and tumor types.
- For nuclei, it is **critical to have RNAse inhibitors** in the lysis, wash, and resuspension buffers. A concentration of 0.2U/ul of RNAse inhibitor is recommended. See above for commercial references.
- Clumping is an important consideration for the use of nuclei in FLEX. To minimize effects on downstream assay performance, 10XG recommend filtering nuclei samples with **30 µm filters** (Miltenyi Pre-Separation Filters or Sysmex CellTrics Filters) post-fixation to help reduce nuclei clumping/aggregates.
- Nuclei can be **FACS**-sorted to remove debris, aggregates/clumps. Sorted single-nuclei should be used immediately as input to Sample Fixation. Extra care should be taken to avoid nuclei damage as sorting can be stressful and compromise the nuclear membranes without obvious sign, leading to leakage of nuclear content.
- **Density gradients** using OptiPrep or a modified sucrose gradient can also be used for cleaning up a nuclei prep.
- We can provide the Chromium Nuclei isolation kit (required for protocol CG000505) if needed, at list price (10x Genomics PN-1000494). Contact us/collect these reagents well in advance to avoid bad surprises.
- Additional information can be found at this link (recommendations are the same for 3' GEX and FLEX).

Cell number requirements post dissociation/fixation

- After fixation, for probes hybridization, we need
  - **Minimum: 25'000 fixed cells/nuclei** per sample.
  - **Maximum: 500'000 fixed cells/nuclei** per sample**.**
  - **Recommended: 300'000 fixed cells/nuclei** per sample. However, with leukocytes isolated from fixed blood (CG000785), splenocytes (CG000782), and cells from fixed & dissociated spleens and pancreas (CG000783), it is recommended to use **≤100'000 cells**, as more may lead to a decrease in data quality
- The cell/nuclei number required depends also on the number of targeted cells/nuclei.
- The recommended minimum number of cells/nuclei was set to ensure assay success as recovery can vary greatly between sample types. The lower number of cells/nuclei submitted, the higher the risk of not having enough cells/nuclei for the downstream assay. We cannot guarantee success if starting from low number of cells/nuclei.

Viability and clean-up

- **Viability has to be assessed by the user**, prior to cell fixation or nuclei isolation. % of viability and pictures of cells/nuclei before fixation are to be included in the submission to GECF.
- Highly viable single cell or nuclei suspensions (>80%) will have the greatest sensitivity and cell recovery. Even though FLEX is relatively robust with samples at lower viability (down to 50%), 10XG recommends **cleaning up dead cells** if you have cell viability <80%.
- **Cell debris/dead cells cleanup methods** are compatible with FLEX:
  - Miltenyi "Dead Cell Removal kit", 130-090-101, which works at least on mammalian cells (probably also

on insect cells but to be confirmed). To be used on cells before fixation (it doesn't work on fixed cells).
- FACS sorting can be performed to remove dead cells and debris, both before and after fixation (for sorting post-fixation, more info here).

- **Samples with lower viabilities exhibit signs of stress and higher expression of mitochondrial genes**. **Dead cells can thus generally be excluded bioinformatically**. The percentage of dead cells that is considered acceptable 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% may be considered acceptable. Further information can be found here.

Enrichment of specific populations
For population enrichment, cells can be labeled with fluorescent antibodies before or after fixation & storage.

- For highest quality labeling, cells should be labeled **prior to fixation** following protocol CG000781. Following cell surface protein labeling, cells should be immediately fixed following protocol CG000782.
- Labeling cells with fluorescent antibodies **after fixation** may still be possible, but the compatibility of an antibody with fixed samples needs to be tested

More info can be found here.

Random notes

- A general overview of FLEX: https://www.10xgenomics.com/products/single-cell-gene-expression-flex
- 10XG have an online database for publications, to find protocols for specific organisms and tissues.
- 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 ahead and we'll discuss what can be done.
- When processing PBMC/blood cells, absence of left-over/contaminating erythrocytes is important to avoid losing reads to their ultra-abundant globin mRNA. In the same manner, if the isolation protocol includes an erythrocytes lysis step, it is critical to perform very careful washes afterwards to remove their floating mRNAs.
- An alternative to storing fixed samples at -80° is to store them at 4°C for up to 1 week. 10XG rather recommend storage at -80°, and didn't spot any advantage to the 4° storage. It can still be kept in mind as an alternative workflow in case issues arise with a particular cell type.

Versions log

- vA.02-04: some genes are left out when probes have off target. Storage at -80 does not impact cells composition, even after 6 months. Contrary to non-FLEX 10XG, the 30um max diameter limit is a strong one for FLEX, as the FLEX workflow does include a 30um filtration step. FLEX is better than non-FLEX methods for neutrophils. New GEM-X version. The dissociation → fixation workflow is now the recommended one. Added info about 1) staining unfixed cells for viability, fix and then check for viability after fixation 2) Dead cell removal kit not working on fixed cells 3) FACS possible post-fixation 4) Population enrichment 5) Using RNase inhibitor when isolating nuclei. Added link to probe set info. Submit only 1.5 ml tubes.
- vA.05 (19.06.2025): added new fixation temperature for leukocytes or bone marrow mononuclear cells
- vA.06 (28.08.2025): added suggestion to order the exact reagents recommended in the protocol
- vA.07 (09.06.2026): Separated the "fixation-first" protocol as an appendix only. Extra-costs associated with custom probes. Transgenes with high similarity to endogenous genes come with complication for the design of custom probes, and with risks of cross-species probes binding. 7'000 cells as a good starting point for targeted cells if unsure. Rat probes added.