



Samples preparation guidelines for 10X Genomics FLEX (GEMX)

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 non-null % 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 cells quality/low viability. Importantly, 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 (in particular a pilot).

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 exogenous/viral/reporter genes requires pre-designing **custom probes**.
- **No information is obtained regarding SNPs or isoforms**, since probe-based.
- **Only human and mouse** probes are available.
- FLEX involves multiplexing samples before running them in the 10XG instrument. This explains the pricing structure in “samples packages”, and the few subtleties regarding number of samples and multiplet rates.
- Prior to fixation, **single cells can be labeled** using a specific antibody conjugated to a **Feature Barcode oligonucleotide** (like TotalSeq-B or TotalSeq-C antibodies).
- The table below summarizes the different fixation/storage workflows. More details in the text below.

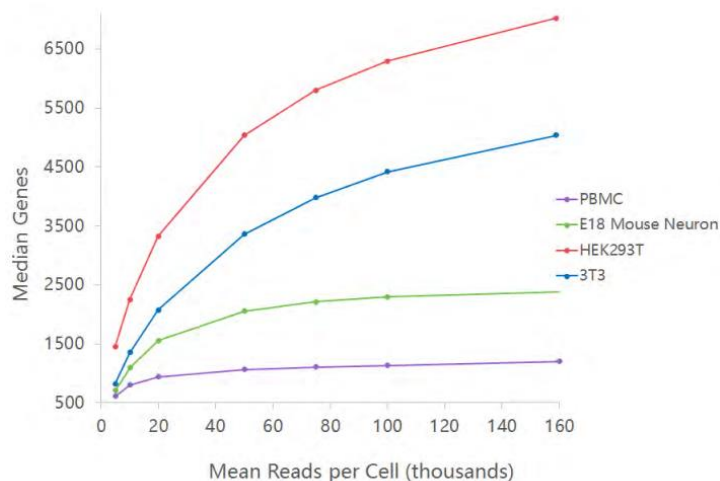
Sample type		Fixation	Storage after cell/nuclei isolation	10XG protocol
Single cells/nuclei	All cells apart leukocytes and bone marrow mononuclear cells	4°C - 16-24 h	-80°C for up to 12 months	CG000782
	Leukocytes or bone marrow mononuclear cells	20°C - 16-24 h	-80°C for up to 12 months	
Tissue (fixation prior to dissociation)		4°C - 16-24 h	-80°C for up to 12 months	CG000783
Blood	PBMCs	4°C - O/N (20–24 h) or up to 1 week	-80°C for up to 12 months	CG000785

	Leukocytes	<ul style="list-style-type: none"> • RT - O/N (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. See plot from 10XG (for an older method):



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.
- Each additional cell comes with a sequencing cost. **Once the cells have been processed, it is not possible to sequence only a fraction of them.**
 - 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 5’000 cells as a good starting point.
 - FLEX uses a multiplexing strategy which allows decreasing the undetectable multiplet rate, as compared to classical 10XG method. Still, **the rate of doublets increases with targeted cells number**. Therefore, we recommend not targeting more than 13’000 cells per sample unless really necessary, as this leads to a multiplet

rate above 5% (see table below).

- The rate of detection (and rescue) of multiplets for a given total number of recovered cells in a pool decreases if fewer samples are run (as less barcodes are used). Therefore, when a given package is used with **less samples than expected**, give us more cells so that we can split samples over several barcodes. Given the complexity of the relationship, inquire in advance and we'll assess the best strategy individually.

Undetectable Multiplet Rate (%)	Cells Loaded/ Probe Barcode	Cells Recovered/ Probe Barcode	Cells Equally Distributed On:					
			4 Probe Barcodes		8 Probe Barcodes		16 Probe Barcodes	
			Cells Loaded/ Well	Cells Recovered/ Well	Cells Loaded/ Well	Cells Recovered/ Well	Cells Loaded/ Well	Cells Recovered/ Well
~0.2	725	500	2,900	2,000	5,800	4,000	11,600	8,000
~0.4	1,450	1,000	5,800	4,000	11,600	8,000	23,200	16,000
~0.8	2,900	2,000	11,600	8,000	23,200	16,000	46,400	32,000
~1.6	5,800	4,000	23,200	16,000	46,400	32,000	92,800	64,000
~2.4	8,700	6,000	34,800	24,000	69,600	48,000	139,200	96,000
~3.2	11,600	8,000	46,400	32,000	92,800	64,000	185,600	128,000
~4.0	14,500	10,000	58,000	40,000	116,000	80,000	232,000	160,000
~5.0	18,125	12,500	72,500	50,000	145,000	100,000	290,000	200,000
~6.0	21,750	15,000	87,000	60,000	174,000	120,000	348,000	240,000
~7.0	25,375	17,500	101,500	70,000	203,000	140,000	406,000	280,000
~8.0	29,000	20,000	116,000	80,000	232,000	160,000	464,000	320,000

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

- **Recovery rate may be different among samples in a pool**, depending on cell size, shape, viability
- All samples in a pool will have the same sequencing depth. This means that sequencing reads will be distributed to different samples of the pool in proportion to their RNA content. So, **samples with higher RNA content per cell will receive more reads/cell than samples with lower RNA content per cell**

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 “samples package”.

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're not sure, we recommend 50k as a starting point.

Practical guidelines

IMPORTANT: *It is possible to either dissociate tissues into single cells or nuclei (as for classical 10XG dissociation) and then fix, or to fix tissues and then dissociate cells. More below.*

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** (recommended):
 - 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 $\geq 25k$ cells or nuclei according to the protocol. However, tech support advises to use $\geq 75k$ 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 fixing directly **tissue** (an option if no validated fresh tissue dissociation protocol exists):
 - Refer to the latest version of 10XG protocol [CG000783 - Tissue Fixation & Dissociation for GEM-X Flex Gene Expression](#).
 - For further details about downstream tissue dissociation, see the paragraph below.
- 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.
 - freeze a separate small aliquot of cells/nuclei to check quantity/quality after post-fixation storage, if cells/nuclei amount is sufficient.
- 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).
 - Previously established tissue dissociation protocols that yielded good results for 3' GEX are also expected to perform similarly in FLEX.
 - 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](#)
- When no validated dissociation protocol for fresh tissue is available, it is possible to dissociate tissue **after fixation** according to the protocol [CG000783](#).
- It works with fresh or flash-frozen tissues.
 - Tissue samples must first be chopped into smaller pieces before fixation.
 - Samples can be stored either at the step of fixed tissue pieces or at the step after cell dissociation. Tech support advise against storing the same sample at both steps. So, since the samples need to be stored to be given to us, **the storage step of fixed tissue pieces should be avoided**.
- 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 assay background. FACS sorting can be performed to remove cellular debris from single-cell or nuclei suspensions using a nuclear stain. Once sorted, the samples need to be collected and stored according to the appropriate protocol.
- If working with **organoids**, please find some best practices at this [link](#).

Nuclei suspension preparation, if dissociating prior to fixation

- 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: <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-> Although this article is relevant for 3' GEX, the recommendations for FLEX are the same.

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.
- If the number of samples you submit is lower than the number of samples in the “samples pack” you purchased (4, 8, 16, 32, 64...), submit more cells accordingly to compensate, when possible. This allows us to split your samples across several barcodes in our probes hybridization step and thereby decrease multiplet rate.

Viability and clean-up if starting from dissociated cells

- **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. Although there is not a strict cutoff, 10XG recommends **cleaning up dead cells** if you have cell viability <80%.

- **Cell debris/dead cells cleanup methods** are compatible with FLEX:

- 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). To be used on cells before fixation (it doesn't work on fixed cells).

- FACS sorting can also be performed to remove dead cells and debris, both before and after fixation (for sorting post-fixation, more info [here](#)).

- However, FLEX is relatively robust with samples at lower viability, with successful results demonstrated even with low viability samples (50%).

- **Samples with lower viabilities may exhibit signs of stress or higher expression of mitochondrial genes.**

- **Dead cells can generally be excluded bioinformatically** as they contain a high percentage of mitochondrial RNAs, but it may not be done easily if cells are in the process of dying. 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 from 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, searchable by organism and tissue, useful to find protocols for specific tissues: <https://www.10xgenomics.com/resources/publications?query=&page=1>

- 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 tech support confirm that they 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.

- Data from Flex are well correlated with data from 3' GEX assay. More information can be found [here](#).

Versions log

- vA.02 (30.09.2024): some genes are left out when probes have off target. Storage at -80 does not impact cells composition, even after 6 months (on their tested tissues, so cannot be sure for every cell type). Clarified that 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.

- vA.03 (07.03.2025): Updated for new GEM-X version. Added note on comparability of Flex and 3'.
- vA.04 (11.04.2025): Added to discuss how many minimum cells must be decided with us ahead of time. 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. Added to 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