



Samples preparation guidelines for 10X Genomics FLEX

Interactions with GECF

IN SHORT: Discuss ahead with us **fixation/storage** conditions and when to bring the cells. Then bring your **cells/nuclei fixed, 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**.

Disclaimer

10XG scRNA-seq runs work in general well, but are still at the edge of current technological capacities. There is indeed a non-null percentage of wells/runs that fail for purely technical reasons. Of course, 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 quality cells/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

- Some general information about FLEX (aka Fixed RNA profiling):

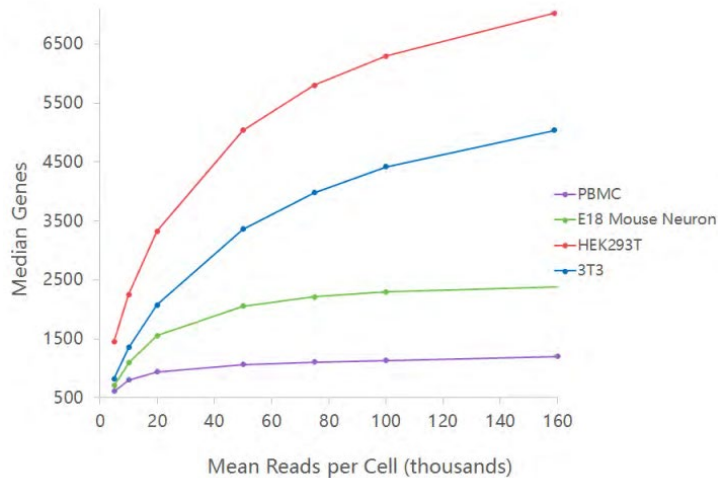
- It is a **probe-based method**, covering whole coding transcriptome (only mRNAs).
- The probeset covers the whole transcriptome unless a few hundreds genes for which probes could not be designed without off-targets. These can still be analyzed on demand, inquire if needed.
- detection of exogenous genes (GFP, reporters, transgenes, viral genes....) requires designing **custom probes** before starting the experiment.
- **no information regarding SNPs or isoforms**, since probe-based.
- **only human and mouse** probes are available.
- Cells are fixed before the experiment in 4% formaldehyde. Two main workflows exist:
 - fixing already **dissociated single cells or nuclei**
 - fixing **tissue** and then dissociating it into fixed single cells;
If starting from tissue (fresh or frozen), fixation prior to dissociation is preferred for optimal results.
- Isolated cells from **FFPE tissue sections** can also be used (not fully supported, inquire in advance).
- The fixed samples can be **stored at -80°C for up to 6 months**, with no impact on performances.
- 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 protein binding molecule, such as an antibody conjugated to a **Feature Barcode oligonucleotide** (like TotalSeq-B antibodies). Please inquire.
- The table below summarizes the different fixation/storage workflows. More details in the text.

Sample type	Fixation	Storage	Additional options	Multiplexing
Single cells/nuclei	- 16-24 h at 4°C	-80°C for up to 6 months	-	yes
			Cell Surface Protein Labeling	no
			Custom Probe Design	if needed, but not supported by 10XG
Tissue (with fixation prior to dissociation)	16-24 h at 4°C	-80°C for up to 6 months	-	yes

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.
- If cells are significantly $> 30 \mu\text{m}$, nuclei isolation should be performed (see below).
- 10XG say that they 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 $30 \mu\text{m}$).
- **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, as well as on experiment-specific factors, such as viability, % of debris, etc. therefore **targeted cells number is only indicative** and number of actual recovered cells can differ significantly.
- 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, therefore calculate the number of cells needed thoughtfully. **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, 1’000 cells may be enough. At the other extreme, for identifying new rare subpopulations of cells ($<1\%$), 5’000 or more cells may be needed. If unsure, we recommend 4’000 cells as a good starting point.
- The maximum recovery is 10’000 cells if using “4 samples” and “8 samples” packages, but only up to 8’000 cells when using “16 samples package”.
- FLEX uses a multiplexing strategy which allows to decrease the undetectable multiplet rate, as compared to classical GEX method. Still, **the rate of doublets increases with targeted cells number**. Therefore, we recommend not targeting more than 6’000 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 decreases if fewer samples are run (as less barcodes are used). Therefore, when a 4 samples package is used with only 3 samples for instance, we rather don’t recommend to target more than 4’500 cells per sample, or we recommend to give us more cells so that we can split samples over several barcodes. Given the complexity of the relationship, we

suggest you inquire in advance in case you won't use all samples 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		Cells Equally Distributed on 16 Probe Barcodes	
			Cells Loaded/ Well	Cells Recovered/ Well	Cells Loaded/ Well	Cells Recovered/ Well
~0.4	825	500	3,300	2,000	13,200	8,000
~0.8	1,650	1,000	6,600	4,000	26,400	16,000
~1.6	3,300	2,000	13,200	8,000	52,800	32,000
~2.4	4,950	3,000	19,800	12,000	79,200	48,000
~3.2	6,600	4,000	26,400	16,000	105,600	64,000
~4.0	8,250	5,000	33,000	20,000	132,000	80,000
~4.8	9,900	6,000	39,600	24,000	158,400	96,000
~5.6	11,550	7,000	46,200	28,000	184,800	112,000
~6.4	13,200	8,000	52,800	32,000	211,200	128,000
~7.2	14,850	9,000	59,400	36,000	n/a*	n/a*
~8.0	16,500	10,000	66,000	40,000	n/a*	n/a*

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 in RNA content between multiplexed samples are expected, it may be beneficial to take some safety margin when designing the composition of the "samples package" or pool.

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 and therefore can influence how many cells you target, it is thus good to have a rough idea already before processing the cells. 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 in groups of known cell types, and/or to delineate their broad transcriptional profile, 25k reads/cells is enough (even for big cells). If you want to zoom on specific genes, then the more reads the better (up to complete sequencing saturation, see above). If you also want to

delineate new cell type with subtle differences, having more reads is also better.

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. The big advantage of the latter is that transcriptional state is fixed and not altered during dissociation. 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.
- After quenching, measuring cells concentration is required, and results must be included in the submission form. It is strongly recommended that, for this count, samples be stained with a fluorescent nucleic acid dye.
- Fixed samples stored at -80° (resuspended in Quenching Buffer supplemented with Enhancer and Glycerol) must be brought to GECF on dry ice.

Fixation

The **fixation is performed by the users**.

- If fixing previously dissociated cells:
 - Refer to the latest version of 10XG [CG000478](#) user guide for [Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling](#). This protocol describes in detail fixation, quenching and storing procedures.
 - This **recommended minimum number of cells/nuclei** is $\geq 300'000$ cells or $\geq 500'000$ nuclei. *This was set to ensure assay success as recovery can vary greatly between sample types. It may be possible to use less, however, there is potential risk of not having enough cells/nuclei for the downstream assay. Therefore, we cannot guarantee success if starting from lower number of cells/nuclei.*
- If fixing directly tissue:
 - Refer to the latest version of 10XG [CG000553](#) user guide for [Tissue Fixation & Dissociation for Chromium Fixed RNA Profiling](#).
 - For further details about downstream tissue dissociation, see the paragraph below.
- We provide the **10XG fixation reagents** (kit ref 1000414, included in service price). Contact us/collect these reagents well in advance. Collect other necessary reagents in advance on your side.
- It is recommended to perform a 16-24 h incubation at 4°C and store the fixed samples at -80°C for best results.
- If <500,000 cells, complete removal of the supernatant is not required. Up to 30 µl supernatant may be left on the pellet to optimize cell recovery without significantly impacting assay performance

Tissue dissociation

- A list of tested tissue can be found here: <https://www.10xgenomics.com/support/single-cell-gene-expression-flex/documentation/steps/sample-prep/single-cell-flex-tested-tissues>

- Several workflows exist:
 - In general the recommended workflow is dissociating tissue **after fixation**:
 - the preferred protocol for this is [CG000553](#), validated by 10XG starting from 25 mg tissue.
 - If starting from **frozen** tissue: this “tissue fixation and then dissociation” workflow is mandatory,

and CG000553 should be followed.

- The fixation of whole tissues for use in the Fixed RNA Profiling assay is not supported. Tissue samples must first be chopped into smaller pieces before fixation.
- Dissociating tissue **prior to fixation**:
 - Only possible on fresh tissue, not on frozen tissue.
 - Previously established tissue dissociation protocols for fresh tissue that yielded good results for 3' GEX are also expected to perform similarly in Fixed RNA Profiling, and can be used with this assay.
 - Alternatively, fresh tissue can be dissociated using the latest version of 10XG [CG000147](#).
- If starting from **FFPE** tissue: Refer to the latest version of 10XG [CG000632](#) protocol.

- **Avoid using plastic petris or dishes for mincing/crushing tissues**, as this was shown to create plastic debris that can clog the capillaries. Only use glass containers.

RNase inhibitor

- The addition of **RNase inhibitors is recommended** during sample preparation of **RNase-rich tissue** (spleen, pancreas, lung). 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**:

Other RNase inhibitors tested and shown to negatively impact data quality:

- SUPERaseIn RNase Inhibitor
- Ribonucleoside Vanadyl Complex

It is strongly recommended to use the RNase Inhibitor part number listed in the Multiome ATAC + Gene Expression nuclei isolation demonstrated protocols (Sigma Protector RNase inhibitor, PN-3335399001).

Ambion™ RNase Inhibitor (AM2684 Thermo Fisher Scientific), RNaseOUT™ Recombinant Ribonuclease Inhibitor (10777019 Thermo Fisher Scientific) and, RiboLock RNase Inhibitor (Thermo Fisher, PN-EO0382) may be used as an alternative when Sigma Protector RNase inhibitor is not available. Note: there was limited testing of these inhibitors for Multiome ATAC + Gene Expression, so use with caution.

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. Avoid doing that if it comes with a risk of skewing your data.

Some examples of filters:

- Flowmi pipette cell strainer of 40um or 70um (we have both at GECF).
- Miltenyi Biotec 30um PreSeparation Filter, cat#130041407....
- Samples should have **minimal debris** for best results; debris can have associated RNA that can contribute to non-cell background. FACS sorting can be performed to remove cellular debris from single-cell or nuclei suspensions using 7-AAD. Sorted samples should be collected in PBS + 1% BSA supplemented with RNase inhibitor (Protector RNase inhibitor from Sigma, PN-3335399001, at a final concentration of 0.2 U/ul).
- **Neutrophils** can be difficult to detect with non-FLEX methods (<https://kb.10xgenomics.com/hc/en-us/articles/360004024032-Can-I-process-neutrophils-using-10x-Single-Cell-applications>), FLEX is better on that matter. Neutrophils should be fixed immediately after collection at 4°C overnight (16-24 hr) to limit degradation after collection. Please, warn us in advance if this cell type is important for you.
- If working with **organoids**, please find some best practices at the link below: <https://kb.10xgenomics.com/hc/en-us/articles/9888301131021-What-are-best-practices-for-working-with-organoids-in-the-Fixed-RNA-Profiling-Assay->

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 the Fixed RNA Profiling assay.
- 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.
- While working with nuclei samples, it is **critical to have RNase inhibitors** in the lysis, wash, and resuspension buffers. Similar to the 3'GEX assay, a concentration of 0.2U/ul of RNase inhibitor (Protector RNase inhibitor: Sigma, PN-3335399001) is recommended.
- Clumping is an important consideration for the use of nuclei in the Fixed RNA Profiling assay. 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 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, the minimum input that we need is
 - **50k cells per sample or 100k nuclei per sample** and **FFPE dissociated suspension**.
 - **200k cells (or 400k nuclei) per pool**.

Therefore, 100k cells/sample for 2 samples, and 66k cells/samples for 3 samples. More is better of course, as it is recommended to start the next reaction with 1mio cells/nuclei per pool, if possible.
- 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...), we recommend that you 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.
- The recommended minimum number of cells/nuclei was set to ensure assay success as recovery can vary greatly between sample types. For fixation and hybridization, it may be possible to use less than the recommended minimum. However, there is potential risk of not having enough cells/nuclei for the downstream assay. Therefore, we cannot guarantee success if starting from lower number of cells/nuclei.

Viability

- **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 the Fixed RNA Profiling assay.
 - Miltenyi offer a kit to remove dead cells if needed (“Dead Cell Removal kit”, 130-090-101), which works at least on mammalian cells (probably also on insect cells but to be confirmed).
 - FACS sorting can also be performed to remove death cells and debris, before fixation.
- However, Chromium Fixed RNA Profiling assay 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.

Random notes

- A general overview of 10XG 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. Yet we recommend to always use the latest version as improvements are often significant.
- 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.

Versions log

- vA.01: first release.
- vA.02 (30.09.2024): some genes are left out when probes have off target. According to 10XG, storage at -80 does not impact cells composition, even after 6 months (on their tested tissues, so cannot be sure for every cell type, but a good rule of thumb). 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. Mentioned that FLEX is better than non-FLEX methods for neutrophils.