10X Genomics GEM-X Flex (Fixed RNA) User guidelines

In short, please adhere to the following guidelines. For more information please see further down.

- **Read and reply to the Policy for 10X FLEX sample handling (page 2-3)**

- **Sample preparation:**
  - Always check 10X Genomics website for the latest protocols for sample preparation.
  - Fixed single cell or nuclei suspensions need to be prepared by the user before submitting samples to NGI. Reagents for preparing and fixing samples are purchased by the user
  - Samples must be fixed by:
    - Using the GEM-X Flex Sample Preparation V2 Kit
    - Following the 10X Genomics long-term storage protocol (overnight fixation is required!).
- **Sample storage & delivery:**
  - Freeze samples according to 10X recommendations for long term storage in Quenching Buffer with Enhancer and glycerol at -80C
  - Samples fixed for long-term storage and correctly stored are stable at -80C for up to 12 months.
  - Samples must be transported to NGI on dry ice.
  - Samples should be delivered in 1,5 ml DNA LoBind tubes with conical bottom.
  - Submit post-fixation cell/nuclei counts and pre-fixation counts/viability if available.
  - Inform NGI if sample centrifugation requires different settings than the ones suggested by 10X Genomics.
- **Sample quality of stating material before fixation:**
  - Start with high quality samples. Sample quality is directly related to data quality.
    - Cells should have >80% viability, free of debris.
    - Nuclei should be of high quality with intact nuclear membranes
    - Tissues should be fixed or snapfrozen promptly.
    - Cell size in suspension should be ≦30 µm. For larger cells, nuclei isolation is recommended to prevent clogging of the 10X microfluidic chips
- **Sample quality assessment post-fixation:**
  - Samples should be free of debris, aggregates or fibers.
  - Post fixed samples should have low amounts of live cells (<10%)
  - Low amounts of red blood cells
- **Required cell and nuclei numbers:**
  - We recommend submitting 300,000 cells/nuclei if possible (fixed and frozen), or at least >100,000:

Please see below for more details on sample quality.

Policy for FLEX sample handling

Due to time restrictions, once the samples have been thawed for processing, it is not possible for NGI staff to contact users regarding sample quality or decisions on whether to proceed with the experiment. The sections below detail the NGI default decisions and lists some alternative decisions that users need to make in advance regarding how they prefer NGI to proceed with their experiment.

1.User Responsibilities

The user is responsible for assessing and approving sample quality before delivery to NGI. Delivered samples will be processed if cell numbers allow, regardless of other quality issues.

The user is economically responsible for the processing of the samples. NGI assumes that users are aware of any quality issues of their samples, and have decided to proceed with processing the samples upon delivery to NGI, provided cell numbers allow.

To ensure high quality data:

- Assess sample quality and adhere to quality standards, as described below and by 10X Genomics.
- Perform a pilot prep, including freezing and thawing, to gain insight on sample behavior, expected loss of cell numbers etc.
- Count cells with an accurate cell counter, preferably using fluorescence.
    - NB! When thawing samples for processing, NGI will perform a cell count. In accordance with 10X recommendations, we will only consider cells labeled with PI (i.e. dead). Remaining viable cells, non-nucleated cells etc will be ignored but may still contribute RNA (see below).

Optional:

If you wish to get input on sample quality from NGI before an experiment, it is possible to:

- Send representative pictures of samples, preferably using both fluorescence and brightfield.
- Send a representative sample to NGI for QC purposes only.
- Send representative aliquots of the samples to be processed, for QC purpose only.

NGI staff will assess the results and give feedback. Please note that if the samples to be analysed are dissimilar to this initial QC, NGI will still default to proceed according to the default criteria below, unless explicitly told otherwise ahead of time.

2.NGI default criteria

NGI will default to process all samples that have >= 100,000 cells/nuclei included as a balanced pool with as many cells as possible up to 300,000. I.e. the number of cells is determined by the lowest sample. If there are enough cells/nuclei, samples will be processed **regardless of other quality parameters.**

NB! Total cell number sequenced and pool balance can never be guaranteed, but chances of reaching target cell number decrease if average input number is lower than 100,000 and if sample quality is suboptimal.

3.User Decision Required

User must decide before sample delivery, according to which of the options below they wish to proceed if samples have limiting cell/nuclei count when counted at NGI. Please indicate the preferred option when returning the NGI sample sheet.

☐ **Option 1: Prioritise total cell numbers, but include samples with low cell numbers.**
Include as many cells from the samples with <100,000 but >25,000 cells/nuclei as possible, and higher number of cells/nuclei for other samples. *Samples with <25,000 cells will be excluded unless explicitly stated otherwise by user*

- **Consequence:** Samples with low cell numbers will have lower representation, but the chance of reaching total cell target increases.

☐ **Option 2: Prioritise balance of samples over cell numbers.**
Include all samples with > 25,000 cells/nuclei; balance the cell number to include equal amounts for all samples. *Samples with <25,000 cells will be excluded unless explicitly stated otherwise by user*

- **Consequence:** Reduces chance of reaching cell target for any sample, but increases chance of balanced cell numbers between samples.

☐ **Option 3: Exclude low-input samples**
Exclude samples <100,000 cells/nuclei - proceed with the remaining samples.

☐ **Option 4**: **Abort experiment if low cell numbers**
Abort experiment if not all samples reach 100,000 cells/nuclei.

- Can only be guaranteed for projects with 1-16 samples, processed the same day

GEM-X Flex - overview

GEM-X Flex is a probe-based, transcriptome-wide method compatible with human and mouse samples. The probe sets include nearly all endogenous protein-coding genes. Genes that are not included in the probe set, including exogenous genes and non-coding genes are not detected unless custom probes are included. Similarly, information on SNVs and transcript isoforms is lost, as only ligated probes are sequenced.

10X GEM-X Flex is currently available in two kit versions; **V1** and **V2**. Please see below for more information about the differences for these two kits.

V1 & V2 (Apex)

The major difference between the two available versions is the multiplexing strategy. In V1 the transcriptome probes used for hybridisation already contain the multiplexing oligos, whereas in V2, also called Apex the multiplexing oligos are decoupled from the transcriptome probes and added in a separate step. The V2 workflow enables higher levels of sample multiplexing capacity, making it possible to target a higher total cell number per reaction. V2 shows higher degree of sample loss during probe hybridisation, compared to V1, but is also more sensitive (see product sheet)

V1 summary:

- Up to 16 samples, can be multiplexed per reaction, each sample with up to 20,000 cells/barcode
- Up to 320,000 cells in total can be targeted per reaction.
- Less sample loss in probe hybridisation compared to V2.

A recent preprint indicated that the pre-barcoded transcriptional probes in V1 introduce reproducible technical variation between samples. This effect was much reduced in V2. (Weir et al., 2026, preprint)

V2 Apex summary:

- Up to 384 samples can be multiplexed per reaction
- Up to 20,000 cells per sample/barcode, if using a maximum of 48 barcodes.
- Up to 1 million cells per reaction.
- 10-30% increased cell loss during hybridisation compared to V1
- Improved sensitivity.

Sample preparation guidelines

10X protocols and resources

The 10X Genomics website offers a vast amount of information and guidance. We have collected links to some that we find particularly relevant, including sample preparation protocols, lists of tested tissues etc together with some general information in the document **Additional Flex information** available on the NGI website. These resources are intended as a helpful starting point. However, we do not guarantee that the linked content is current. It is the user's responsibility to verify that the information in the **Additional Flex information** document is the most up-to-date before use.

Pilot sample preparation

NGI recommends always testing the sample preparation protocol, freezing and thawing according to the 10X guidelines, with QC at each stage to achieve an estimate of expected sample loss and quality.

Cell number requirements

How many cells to deliver to NGI?

NGI recommends delivering >300,000 cells/nuclei whenever possible, and a minimum of >100,000 cells/nuclei, in order to start probe hybridisation with a sufficient amount of sample.

10X recommends starting the probe hybridisation step with 300,000 cells/nuclei for most cell types, although the possible range is 25,000-500,000. For some cell types with high RNase content such as granulocytes, spleen and pancreas, hybridisation should be started with maximum 100,000 cells/nuclei

While it is technically possible to start hybridisation with as little as 25,000 cells, starting with low cell numbers may make it impossible to reach the desired cell target.

Please keep in mind that freezing, thawing and counting will be associated with some sample loss, so include extra material.

How many cells will be sequenced?

The user provided cell target number is only indicative. We can not guarantee that the desired cell target number is met.

When starting with at least 100,000 cells, chances to reach up to 20,000 cells per sample sequenced are good. However, this will vary depending on the sample type and quality. For

example up to 50% cell loss in the hybridisation step is considered normal and the loss may be higher. Processing may also lead to uneven representation of samples within a multiplexed reaction.

Cell Viability

Sample quality will be directly reflected in the quality of the resulting data. High-quality single cell or nuclei suspensions should be used for optimal assay performance. Single cell suspensions with high starting viability (**>80%**) will result in greatest sensitivity and cell recovery. Although there is not a strict cutoff, 10X Genomics recommends cleaning up dead cells if cell viability is <80%.

The Fixed RNA Profiling assay is robust with samples at much lower viability, with successful results demonstrated even with low viability samples (50% or lower). Low viability samples may have more variable cell calling and lower sensitivity. It is important to note that samples with lower viability may exhibit signs of stress or higher expression of MT genes.

Two options for removing dead cells are FACS sorting or by using the Miltenyi Dead Cell Removal Kit although this will lead to longer processing time and some cell loss.

Debris & cell aggregates

Samples should have minimal debris and aggregated cells/nuclei for best results. Debris can have associated RNA that is detected outside cells and can lead to increased background. Aggregated cells lead to higher multiplet rates and both debris and aggregates can potentially cause clogging of the microfluidic chip. While the Flex reagents are typically replaced by 10X Genomics in case of a clog or wetting failure for clean samples, there are no guarantees a replacement would be issued when aggregates/debris are present.

If samples have debris and/or aggregates we recommend trying some method of removal such as use of appropriate filters (for example 30 µm filters such as Miltenyi Pre-Separation Filters or Sysmex CellTrics Filters (see the relevant demonstrated protocol for sample preparation in the document **Additional Flex Information** or on the 10X genomics web page).

Cell sorting can also be used to remove dead cells (prior to sample fixation) and cellular debris or to enrich a specific population of cells.

Red Blood Cells

It is advisable to avoid the presence of red blood cells (RBCs) in the submitted sample. RBCs will contribute RNA and take up reads in the sequencing, potentially necessitating higher sequencing depth. See also Should I deplete red blood cells from my sample before loading.

When counting cells prior to run, NGI will only consider nucleated cells stained with PI, and assume the user finds the level of RBCs in the sample acceptable.

Cell counting recommendations

It is strongly recommended to use a fluorescent dye and cell counter for determining cell/nuclei numbers. Pre-fixation counting can be performed with for example AO/PI for viability assessment.

An aliquot of the fixed cells can be stained with for example an ethidium homodimer-1 or PI staining solution and used for counting and QC. After fixation & permeabilisation, >90% of the cells should stain as dead; a higher viability may indicate issues with the fixation process that can have negative consequences for the outcome of the experiment.

Debris-free samples (cells or nuclei suspensions) can also be counted using trypan blue or erythrosine. We recommend strongly against using these dyes for counting samples with any sub-cellular debris present.

Please refer to the relevant 10X demonstrated fixation protocols for more guidance and recommendations on cell counters.

When counting cells prior to a run at NGI, NGI will only consider nucleated cells stained with PI. Remaining viable cells and non-nucleated cells will be ignored with the assumption the user finds the numbers of such acceptable.