

# 10X Genomics GEM-X Flex (Fixed RNA)

## User guidelines

10X GEM-X Flex is only compatible with human and mouse tissue. Since it is a probe-based method (transcriptome-wide), only endogenous genes (which are included in the probe set; non-coding genes are not covered) can be detected. In order to detect any exogenous genes, custom probes have to be designed and included (not supported by 10X Genomics, but guidelines can be found [here](#)). A full list of probes can be found [here](#). Information about SNVs or isoforms is lost during the assay since only ligated probes are sequenced. 10x Genomics GEM-X Flex has a cell-capture rate of up to 80% but the expected capture is ~69% and multiplet rates of 0.4% per 1,000 cells.

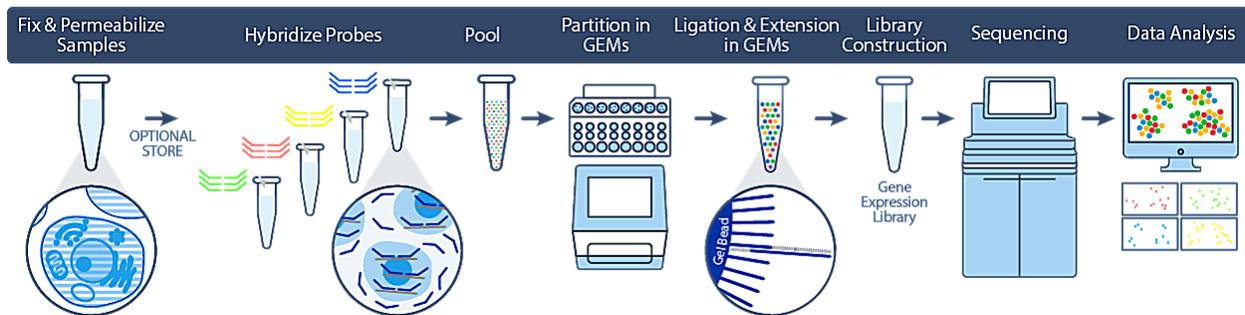


Figure 1. Workflow overview

In short, please adhere to the following guidelines (read the whole document for further guidance!):

- Always check [10X Genomics website](#) for the latest protocols for sample preparation.
- Start with high quality samples (>80% viability, free of debris). The 10X FLEX assay has for some samples been proven to work even with lower viability (50%). However, this is very sample dependent and NGI cannot guarantee that the experiment will yield usable data from low quality samples. Low viability samples may have more variable cell calling and lower sensitivity. Sample quality is directly correlated to data quality.
- The maximum recommended cell size is 30  $\mu\text{m}$ . Larger cells may clog the microfluidic channels of 10x Genomics chips. If cells are > 30  $\mu\text{m}$ , nuclei isolation should be performed.
- Fixed single cell or nuclei suspensions need to be prepared by the user before submitting samples to NGI. Please submit your post-fixation cell counts to NGI before handing in your samples. All reagents necessary for cell/nuclei isolation and fixation must be purchased by the user.
- Samples must be fixed following 10X Genomics long-term storage protocol (overnight fixation is required!). We recommend starting with  $1 \times 10^6$  cells whenever possible. For

sample fixation, the recommended minimum of fresh cells or nuclei suspensions are 25,000 (and up to  $10 \times 10^6$ ) cells or nuclei using the [Fixation of Cells & Nuclei for GEM-X Flex gene expression Demonstrated Protocol](#) (for other input materials, see protocol recommendations further below or check the 10X Genomics' website).

- It is recommended that samples are counted using Propidium iodide (PI). Make sure the majority of cells (<90%) stain with PI as compared to brightfield, to make sure cells are properly fixed and permeabilized.
- Required cell and nuclei numbers (after fixation) for processing at NGI:
  - The recommended minimum input for probe hybridisation (after thawing, washing counting) is 25,000 cells/nuclei.
  - The recommended default for probe-hybridisation for most samples is 300,000 cells/nuclei per barcode.
  - The maximum input for probe hybridization is 500,000 cells/nuclei per barcode.
  - For leukocytes from fixed blood, splenocytes/fixed spleen or pancreas the recommended maximum input is 100,000 cells.
  - **NB!** If delivering low numbers of cells, it may be difficult to reach 20,000 cells/sample loading on the chip as there is always some sample loss associated with thawing, washing, counting etc.
- Fixed single cell/nuclei suspensions resuspended in Quenching Buffer, Enhancer and 50% Glycerol can be stored at  $-80^{\circ}\text{C}$  for up to 6 months post-fixation and should be shipped on dry ice . Shipping at  $4^{\circ}\text{C}$  is not recommended.
- Sequencing costs are dependent on the targeted cell number. It is not possible to only sequence a fraction of the cells.
- Samples should be delivered to NGI in 1,5ml eppendorf tubes with conical bottom. 10X Genomics recommends using the Eppendorf DNA LoBind tubes to minimise sample loss.

Please note that if the requirements above are not met, we can take no responsibility for the outcome of the experiment

## Further information (from 10X Genomics)

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## Sample prep guidelines

### Sample quality recommendations

High-quality single cell or nuclei suspensions should be used for optimal assay performance. Single cell or nuclei suspensions with high 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 you have cell viability <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.

This could be an indicator of:

- Poor sample quality, leading to a high fraction of apoptotic or lysed cells prior to sample fixation.
- The overall biology of the sample, for example tumor biopsies, which may have increased mitochondrial gene expression due to metabolic activity and/or necrosis.

Samples should have minimal debris for best results; debris can have associated RNA that is detected outside cells.

Cell debris and dead cells cleanup methods are compatible with the Fixed RNA Profiling assay. These include the use of appropriate filters and/or other methods (e.g. FACS, Miltenyi Dead Cell Removal). Please note that a considerable % of cells will be lost during the use of the Dead Cell Removal Kit, thus it is recommended to use this kit only if there is a sufficient cell number to begin with. If possible, test it with a relevant and non-precious sample.

Cellular Debris and cell clumps can be removed using 30 µm filters such as Miltenyi Pre-Separation Filters or Sysmex CellTrics Filters. Please also review: [What cell strainers are recommended for use with Fixed RNA Profiling assay?](#)

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

## Demonstrated protocols for fixation

Always prepare samples for long-term storage. This includes an overnight fixation. Refer to the appendix of the following Demonstrated Protocols when preparing fixed sample suspension for storage. Please ensure that you are always following the most up-to-date protocol from 10X Genomics (check [10X Genomics website](#)).

1. **Cells/Nuclei**. Protocol: [Fixation of Cells & Nuclei for GEM-X FLEX gene expression](#)
2. **Tissue samples**. Protocol: [Tissue Fixation & Dissociation for GEM-X FLEX gene expression](#). This protocol is preferred if you do not have an optimised tissue dissociation protocol for single cell suspensions.
3. **FFPE blocks**. Protocol: [Isolation of Cells from FFPE Tissue Sections for GEM-X FLEX gene expression](#)
4. **Blood**. Protocol: [Blood fixation and cell isolation for GEM-X FLEX gene expression](#)

Importantly, 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.

The fixation of whole tissues for use in the Fixed RNA Profiling assay is not supported. Tissues must first be chopped into smaller pieces before fixation for optimal assay performance. This step is required for uniform fixation and permeabilization. Please also see: [Which tissue dissociation protocols are supported for use with the Fixed RNA Profiling assay?](#)

## Can formaldehyde from alternative vendors be used?

10X Genomics performed limited testing to validate a few additional formaldehyde sources for sample fixation including the following:

1. Sigma PN 47608: Formaldehyde solution (for molecular biology, BioReagent, ≥36.0% in H<sub>2</sub>O (T))
2. Sigma PN 252549: Formaldehyde solution (ACS reagent, 37 wt. % in H<sub>2</sub>O, contains 10-15% Methanol as stabilizer (to prevent polymerization))
3. Sigma PN F8775: Formaldehyde solution (for molecular biology, 36.5-38% in H<sub>2</sub>O)

If the formaldehyde validated in the 10x Demonstrated Protocols above is not available in your region, any Molecular Biology Grade stock solution of 37% formaldehyde that is ~10% methanol stabilized (with little to no precipitant) may be substituted.

## Improving cell/nuclei recovery

The following strategies can be implemented to reduce cell/nuclei loss throughout the workflow for the Fixed RNA Profiling assay (i.e., Single Cell Gene Expression Flex):

- Use of low binding microcentrifuge tubes
- Use of a swinging bucket rotor centrifuge - cells will pellet at the bottom of the tube in a tighter pellet thus reducing loss during washes
- Increasing the speed/time of centrifugation to improve pelleting (cell type dependent).
- Use of a transfer pipette to remove supernatant to reduce the risk of aspirating cells
- Avoid pipetting near the cell pellet
- Leaving up to 30µl of supernatant during wash steps so the cell pellet is not disrupted

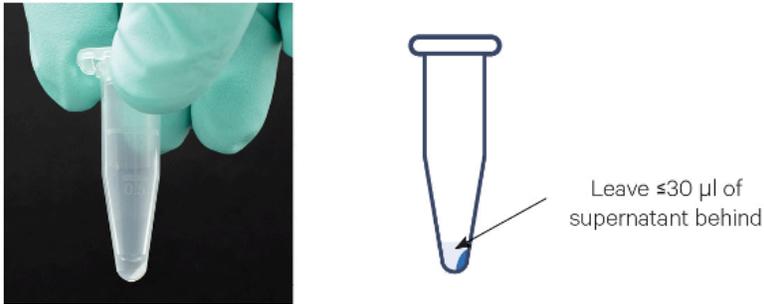


Figure 2. Wash step of cell/nuclei suspension

There is always expected cell/nuclei loss from all workflow steps (i.e. sample fixation through post-hybridization washes/filtration to chip loading). Overall, cell loss will be dependent on many factors including user, cell type, and number of cells in the sample.

If the cell yield post-tissue dissociation is low when following the [Tissue Fixation & Dissociation for Chromium Fixed RNA Profiling Demonstrated Protocol](#), the following strategies can be implemented to increase cell yield:

- Increase the tissue dissociation time.
- If using an Octo Dissociator, after transferring the dissociated cells from the C tube, perform an additional PBS rinse of the C tube and pass the rinse through the 30 µm strainer to collect additional cells.
- If manually dissociating tough tissue, use the back of a 1-ml syringe plunger to push any undissociated tissue pieces through the 70 µm strainer.

We recommend performing a pilot experiment to optimize the dissociation of tissue post-fixation.

## Cell counting recommendations

It is strongly recommended that the sample be stained with a fluorescent dye such as ethidium homodimer-1 or PI staining solution and counted using an automated fluorescent cell counter (Countess II Automated Cell Counter or a Cellaca counter) for accurate sample counting. The use of fluorescent dye during cell counting enables accurate quantification even in the presence of sub-cellular debris. The optimal cell concentration for the automated cell counter is 1,000-4,000 cells/µl.

Please note:

- Countess III is currently incompatible with small cell/nuclei counting due to software limitations which gates out cells/nuclei < 5 microns.
- When counting on automated counters, using suboptimal samples (i.e., with debris and cell clusters) may produce high variations in counts

Debris-free samples (cells or nuclei suspensions) can also be counted using trypan blue. Trypan blue is strongly not recommended for samples with any sub-cellular debris present.

## General guidelines

### Maximum number of cells per barcode

GEM-X Flex allows for 500-20,000 cells per barcode.

When multiplexing using 4-16 barcodes, up to 20,000 cells can be targeted and demultiplexed per probe barcode, yielding a total of 80,000-320,000 cells recovered per GEM reaction. For maximum loading the undetectable multiplet rates (i.e. multiplets that can not be easily filtered out) is 8%.

### Recommended number of cells per barcode

Regardless of the level of multiplexing, the recommended starting point for targeted cells is 4000 cells per Probe Barcode, yielding a multiplet rate of 1,6%.

For each multiplexed sample, assuming 1 unique Probe Barcode is used per sample, the undetected (i.e. with same Probe Barcode) cell multiplet rate for the GEM-X FX chip is approximately 0.4% multiplets per 1,000 cells recovered. Up to 320,000 cells can be recovered per reaction on the GEM-X FX chip with a low multiplet rate - any GEMs with multiplets derived from dissimilar Probe Barcodes can be demultiplexed.

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

## List of tested tissues (by 10X Genomics)

For a list of tested tissues (fresh or frozen) with the 10X Genomics FLEX assay see [here](#).

For a list of tested FFPE tissues, see [here](#).

## Compatibility with protein labeling

GEM-X Flex is compatible with cell-surface and intracellular protein labeling using antibody-oligonucleotide conjugates, for example with TotalSeq-C antibodies and Proteintech Multipro antibodies. Antibody staining must be performed before cell fixation. For intracellular proteins, a brief initial fixation is performed prior to cell permeabilization, staining and final fixation. For guidelines on staining, and also antibody-oligonucleotide conjugation, best practise advice etc., see [Cell Surface & Intracellular Protein Labeling for GEM-X Flex Gene Expression](#)

Staining and any necessary optimisation is performed by the researcher, prior to delivering the samples to NGI.

## Compatibility of cell types with high RNase levels

10X Genomics has performed limited testing in-house on RNase-rich tissues (e.g. spleen). These samples performed similarly to other non-RNase-rich tissues in the Fixed RNA Profiling Assay. Fixation of these samples limits the degradation after collection that is often observed due to high levels of RNases and other inhibitory compounds.

## Can fixed samples be FACS sorted?

Post-fixation samples can be sorted using FACS for advanced sample clean-up, as well as for the enrichment of specific populations in the Fixed RNA Profiling Assay.

Fluorophores that were found to be incompatible with fixation (limited testing by 10X Genomics):

- FITC
- Brilliant Violet 605™
- PE-Cy7 (tandem dye)
- PE-Dazzel 594 (tandem dye)

Best practices for sorting fixed samples:

- Samples should be collected in PBS + 1% nuclease-free BSA supplemented with RNase inhibitor (Protector RNase inhibitor from Sigma, PN-3335399001). We recommend a final concentration of 0.2 U/ul of RNase inhibitor.
- Samples should be spun down and resuspended in PBS + 0.02% nuclease-free BSA before proceeding with probe hybridization.

## Kit options - Greater flexibility with N-Plex

GEM-X Flex is available in several kit versions containing different numbers of barcodes per reaction, with either 1 barcode (single plex), 4 barcodes, 16 barcodes or N-plex. At NGI we mainly offer N-plex and 16 barcodes.

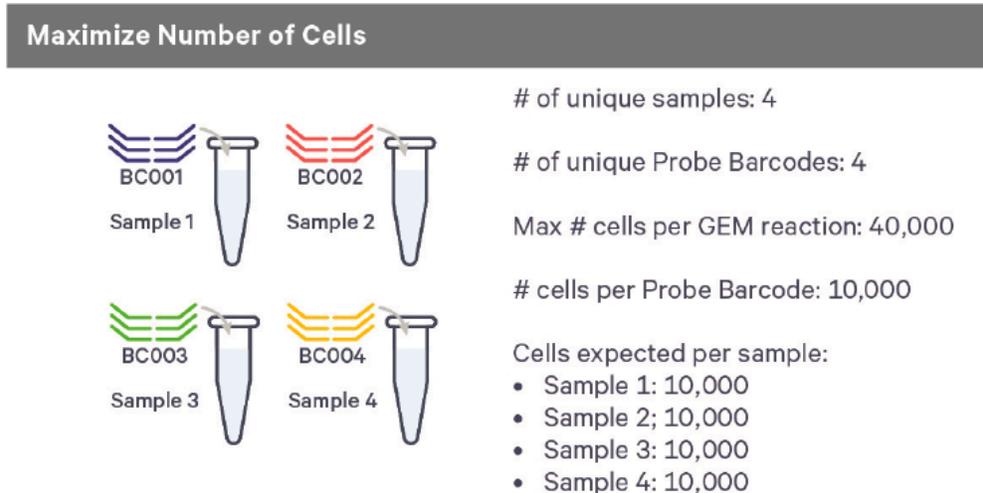
N-plex Flex: This version allows for the greatest flexibility in terms of number of barcodes used per reaction. 1-16 barcodes can be used, but in order to maximize reagent usage, we require an average of 4 samples (or barcodes) per reaction for a project.

16 BC kit: If a project requires 16 barcodes per reaction and at least 4 reactions, the 16BC kit is the most cost efficient option. For this kit, we require researchers to commit to a full kit (4 reactions\*16 BCs)

## Multiplexing strategies

10X Genomics recommends using either 4 or 16 samples for multiplexing with the Fixed RNA Profiling Assay to maximize the multiplexing efficiency of the kits. However, other configurations are possible.

The Flex barcodes can be used either for multiplexing different sample or for increasing the number of cells analysed for samples (subpooling).



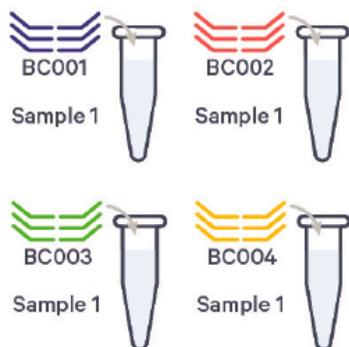
This illustration provides an overview of multiplexing configurations using the Chromium Fixed RNA Kit with 4 barcodes. The same principle can be applied when using up to 16 barcodes; Max # of cells per Probe Barcode is 20,000 cells, but 10X recommends 4000 cells as a starting point

**Please note** that since cell counting is never completely accurate, the balance of samples within a pool can not be guaranteed. Cell counting is made more difficult by the properties of FLEX kit buffers decreasing accuracy of automatic cell counters.

## Subpooling

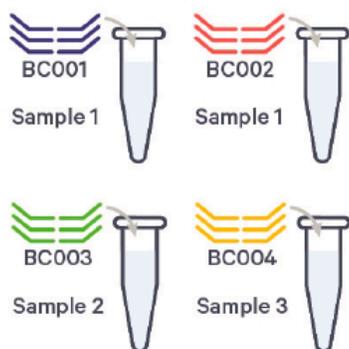
If fewer samples than the number barcodes (BC) available are used (e.g., less than 4 or 16), then it is possible to subpool a sample across multiple barcodes to use all barcodes in a single kit. Sub-pooling one sample over multiple Probe Barcodes will allow the capture of more cells from that sample, with a lower undetected multiplet rate.

### Maximize Number of Cells of a Single Sample (Sample Sub-pooling)



- # of unique samples: 1
- # of unique Probe Barcodes: 4
- Max # cells per GEM reaction: 40,000
- # cells per Probe Barcode: 10,000
- Cells expected per sample:
  - Sample 1: 40,000

### Combination



- # of unique samples: 3
- # of unique Probe Barcodes: 4
- Max # cells per GEM reaction: 40,000
- # cells per Probe Barcode: 10,000
- Cells expected per sample:
  - Sample 1: 20,000
  - Sample 2; 10,000
  - Sample 3: 10,000

This illustration provides an overview of multiplexing configurations using the 4 barcode kit. The same principle can be applied when using the 16 barcode kit; Max # of cells per Probe Barcode is 8,000 cells.

## Pool balance

It is important to be aware that since cell counting is never completely accurate, the balance of samples within a pool can not be guaranteed. For multiplexed samples, pooling is performed after probe hybridisation based on pre-hybridisation counts which could contribute to balance inaccuracy, but will minimize sample loss in subsequent washing steps.

If some samples have limiting numbers of cells, the pool will by default **not be adjusted** to match that number, unless specifically requested beforehand by the researcher. In such cases the limiting samples will be underrepresented in the final pool

## Pooling Samples with Different RNA Content

Due to the nature of a multiplexing pool, the sequencing reads for the pool will be distributed to different samples in proportion to their inherent RNA content and all the samples will have the same sequencing saturation. As a result, a sample with cells high in RNA will receive more reads per cell, whereas a sample with cells low in RNA will have proportionally fewer reads per cell, and sequencing saturation will be approximately the same for each sample. Because the distribution of reads across samples is determined by the composition of the pool, it is not possible to add reads to specific samples in the pool.

The recommendation therefore is to:

1. Pool samples when comfortable with sequencing to the same percent saturation for each sample.
2. Keep samples separate if wishing to sequence one closer to saturation than the others is preferred