10X Genomics FLEX (Fixed RNA) User guidelines

10X 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 has a cell-capture rate of ~65% and multiplet rates of 0.8% per 1,000 cells.

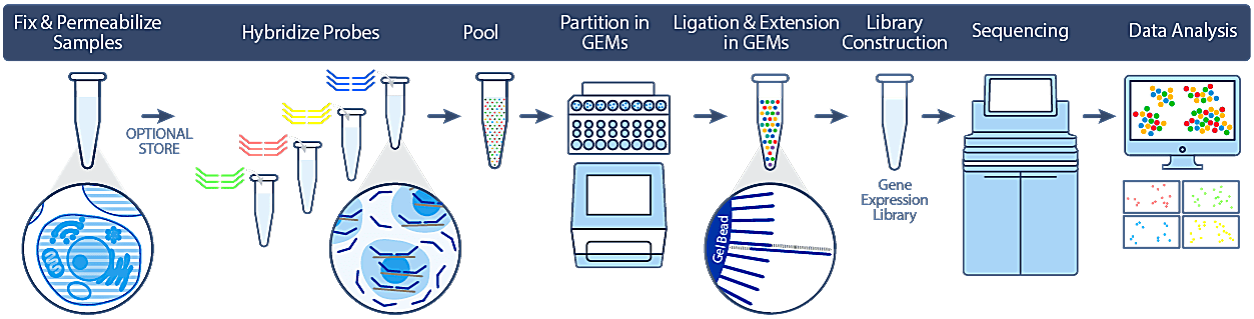


Figure 1. Workflow overview

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 sample. 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 um. Larger cells may clog the microfluidic channels of 10x Genomics chips. If cells are > 30 µm, 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 cell counts after fixation 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 x 10^6 cells whenever possible. For sample fixation, the recommended minimum of fresh cells or nuclei suspensions are

300,000 (and up to $10x10^6$) cells or 500,000 (and up to $10x10^6$) nuclei, respectively using the Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling 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:
    - Singleplex: The recommended minimum input is 200,000 cells or 400,000 nuclei per hybridization. In addition, the recommended minimum input for FFPE dissociated suspension is 400,000 cells per hybridization.
    - Multiplex: The recommended minimum input is 50,000 cells or 100,000 nuclei per hybridization (i.e., per probe barcode). In addition, the recommended minimum input for FFPE dissociated suspension is 100,000 cells per hybridization (i.e., per probe barcode).
    - The maximum input for probe hybridization is 2 x 10^6 cells/nuclei per barcode
- Fixed single cell/nuclei suspensions resuspended in Quenching Buffer, Enhancer and 50% Glycerol can be stored at -80°C for up to 6 months post-fixation and should be shipped on dry ice . Shipping at 4°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.

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)

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. Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling
2. Tissue Fixation & Dissociation for Chromium Fixed RNA Profiling This protocol is preferred if you do not have an optimized tissue dissociation protocol for single cell suspensions.
3. Isolation of Cells from FFPE Tissue Sections for Chromium Fixed RNA Profiling

It is important to note that 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 H2O (T))
2. Sigma PN 252549: Formaldehyde solution (ACS reagent, 37 wt. % in H2O, contains 10-15% Methanol as stabilizer (to prevent polymerization))
3. Sigma PN F8775: Formaldehyde solution (for molecular biology, 36.5-38% in H2O)

If the formaldehyde validated in the 10x Demonstrated Protocols Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling or Tissue Fixation & Dissociation for Chromium Fixed RNA Profiling 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 implement 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

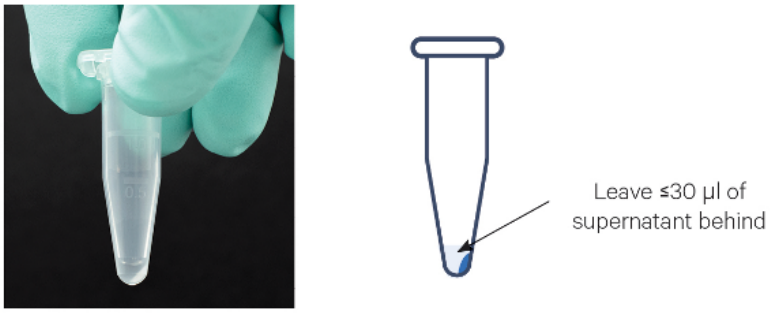


Figure 2. Wash step of cell/nuclei suspension

The expected cell/nuclei loss from all workflow steps (i.e. sample fixation through post-hybridization washes/filtration to chip loading) is ~10-50%, depending on technique/skill, and cell size. Overall, cell loss will be dependent on many factors including user, cell type, and cell number of 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 tissue is difficult to dissociate with Liberase TL, Liberase TH (1 mg/mL) may also be used as an alternative enzyme.
- 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 70 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

When using four barcodes, up to 10,000 cells can be targeted and demultiplexed per probe barcode, yielding a total of 40,000 cells recovered per GEM reaction.

When using more than four probe barcodes, up to 8,000 cells can be targeted and demultiplexed per probe barcode; thus with 16 probe barcodes, 128,000 cells (16 samples * 8,000 cells) can be recovered and demultiplexed.

128,000 cells recovered per GEM reaction represents the upper cell limit supported by the chip. Higher cell numbers may result in clogs or microfluidic failures.

We do not recommend exceeding 8,000 cells per barcode when the number of samples is 4 < n < 16. The undetected multiplet rate will increase if the recommended number of cells per Probe Barcode is exceeded.

Please note:

Even though it is possible to multiplex 10000 or 8000 cells per sample respectively for the 4 and 16 barcode kits, **the recommended starting point for both is 4000 cells** per sample (see below in "Cell counts for chip loading").

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.

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 Total-seq

See below for a table outlining the TotalSeq™ antibody type compatible with each Fixed RNA Profiling (i.e., Single Cell Gene Expression Flex) application.

Fixed RNA Profiling Application	TotalSeq™-B	TotalSeq™-C
Fixed RNA Profiling for Singleplexed samples	✓	✓
Fixed RNA Profiling for Multiplexed samples	✗	✓

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):

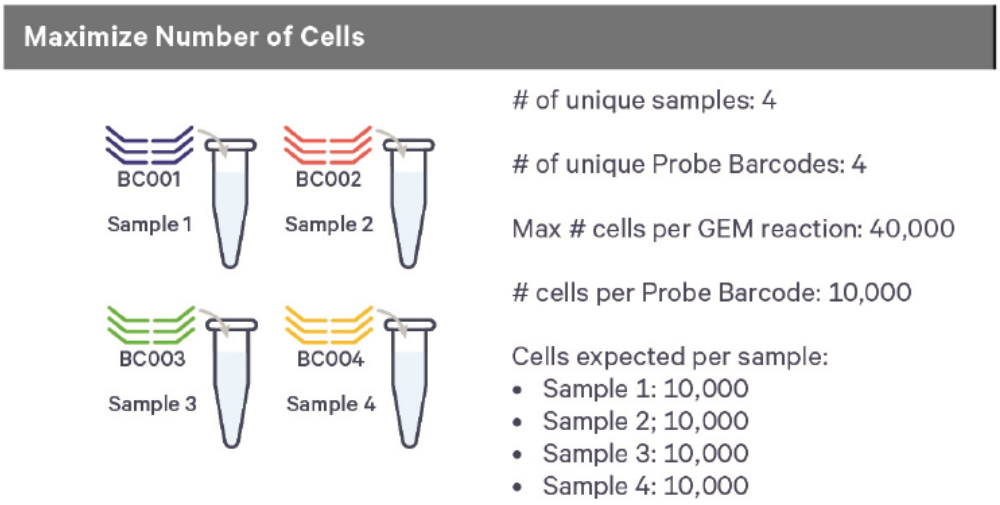
- FITC
- Brilliant Violet 605™
- PE-Cy7 (tandem dye)
- PE-Dazzel 594 (tandem dye)

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.

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.



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 16 barcodes; Max # of cells per Probe Barcode is 8,000 cells.

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.

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.

Multiplexing < 4 or < 16 samples

It is possible to multiplex fewer than 4 or 16 samples if fewer than the maximum number of samples is desired.

When pooling less than 4 or 16 samples, the number of cells targeted per GEM well should be adjusted as explained below:

- When using the Chromium Fixed RNA Kit, 4 rxns x 4 BC to pool less than 4 samples per well, the total number of cells targeted should not exceed 10,000 cells per Probe Barcode (or 10,000 * number of Probe Barcodes used/per well). For example, if pooling 3 samples hybridized with 3 unique Probe Barcodes, the total number of cells targeted should not exceed 30,000 cells/well (10,000 * 3).
- When using the Chromium Fixed RNA Kit, 4 rxns x 16 BC PN-1000476 to pool less than 16 samples per well, the total number of cells targeted should not exceed 8,000 cells per

Probe Barcode (or 8,000 * number of Probe Barcodes used/per well). For example, if pooling 14 samples hybridized with 14 unique Probe Barcodes, the total number of cells targeted should not exceed 112,000 cells/well (8,000 * 14).

The undetected multiplet rate will increase if the recommended number of cells per Probe Barcode is exceeded.

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

Cell counts for chip loading

For both singleplex and multiplexing strategies, the recommended starting point for targeted cells is 4000 cells per Probe Barcode.

When using the **Singleplex** (1 sample and 1 barcode) the recommended starting point is to target ~4000 total cells per GEM reaction (4000 cells per Probe reaction), yielding a multiplet rate of ~3,2%.

When **multiplexing 4 samples**, the recommended starting point is to target ~16,000 total cells per GEM reaction (4,000 cells per Probe Barcode), yielding a multiplet rate of ~3.2%.

When **multiplexing 16 samples**, the recommended starting point is to target ~64,000 total cells per GEM reaction (4,000 cells per Probe Barcode), yielding a multiplet rate of ~3.2%.

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 Chip Q is approximately 0.8% multiplets per 1,000 cells recovered. Up to 128,000 cells can be recovered on Chip Q 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		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*

* These cell numbers are not supported.