



TotalSeq hashing and CITE-seq on scRNA-seq

These guidelines are an addendum to our 10XG guidelines (available on our web site) and to official 10XG cell preps guidelines, which all should be read carefully.

These methods are only compatible with the latest version of assays: 3'GE v4 and 5'GE v3.

Interactions with GECF on experiment day

- IN SHORT:

- Samples need to be **already pooled**
- Pools need to be at the **right concentration**,
- on **ice**
- typically in PBS 10% FBS.

IMPORTANT NOTES

- Samples need to be of very high quality (higher than for standard scRNAseq). **If cells/nuclei break after the hashing**, the antibodies will be released in the resuspension buffer, contaminating the droplets of other intact cells/nuclei and **compromising the results** for them as well
- **Nuclei** are specially challenging for totalseq hashing. **Special care** must be taken when working with them, as while the quality of cells can be easily assessed using Trypan blue, evaluating the quality of nuclei is more challenging
- **Nuclei isolation protocol must be thoroughly optimized**, ideally including a lysis time course and selecting the shortest lysis time that results in high-quality nuclei with less than 5% intact cells.

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Intro/Notes

Hashing antibodies enable **sample multiplexing** in single cell RNA-sequencing, by using a series of oligo-tagged antibodies against ubiquitously expressed surface proteins with different barcodes to uniquely label cells from distinct samples.

CITE-seq antibodies enable **quantification of cell surface protein** expression, by using oligo-tagged antibodies against the protein of interest.

Overview of Totalseq antibodies for hashing:

	10XG compatibility	Target	Antigens	N. tags available
Total-seq B	<ul style="list-style-type: none"> . Chromium 3' (v3 or later) . Flex for Singleplexed samples 	Mouse cells	CD45 and H-2 MHC Class I	24
		Human cells	CD298 and β 2- Microglobulin	24
		Nuclei (same abs for human, mouse and rat - so probably all mammals)	Nuclear pore complex mixture	24
Total-seq C	<ul style="list-style-type: none"> . Chromium 5' (v2 or later) . Flex for <ul style="list-style-type: none"> - Singleplexed samples - Multiplexed samples 	Mouse cells	CD45 and H-2 MHC Class I	24
		Human cells	CD298 and β 2- Microglobulin	24
Total-seq A	NOT to be used: it requires reagent and protocol modifications AS it is an older version not designed for 10XG			

Practical guidelines

Intro/generalities

- Totalseq hashing involves several washes/spins and incubation, which may impact on transcriptional profile. Nuclei and fragile cells may specially suffer, so Totalseq may not be suitable in such case.
 - 10XG recommend performing a titration of the hashing antibodies, which can be use also as a pilot to assess cells/nuclei quality at the end of the protocol.
 - We strongly recommend that during this test you already prepare the pooling calculations sheet/formula that you will use with real samples (to avoid having to deal with maths on the experiment day). We provide a “sandbox” pooling excel sheet on our website for you to train before the experimental day.
 - The labelling is done by users. We can provide tags if needed (at list price). Contact us/collect these reagents well in advance.
 - Collect reagents necessary for the tagging in advance (in addition to the oligos tags).
- The samples must be brought to GECF already pooled, and at the right concentration, in a minimal volume of 50ul. Refer to our calculation sandbox for details of the concentration to bring.
- Pooling ratio work best with similar amounts of cells for each sample, but down to 5% is supported by 10XG (below CellRanger may fail).

Key points for labelling

- Refer to our Totalseq hashing protocol for labelling. It is critical to follow these recommendations for optimal performance, to avoid poor signal to noise ratios in Cell Multiplexing data, which may prevent accurate tag assignment. If your cells significantly diverge from the ones mentioned in these guidelines, contact us or 10XG tech support for guidance.

- Cell Multiplexing can be severely impacted by low cell viability even if the single-cell behaviour of the gene expression data is only mildly impacted. Determine viability of each sample before pooling. Cell washing instructions & wash resuspension buffers for the labelled cells depend upon the starting viability. Choose appropriate labelling protocol according to viability (see our guidelines).
- Input cells should be free of debris (debris increase background in cell multiplexing data).
- The standard tube-based protocol is only supported for cell/nuclei inputs of at least 100'000 cells per sample. If the sample quantity is not limited, 0.5-2 mio cells/sample should be used. It is expected that about 50% of the cells will be lost in the labelling procedure. Poor visibility of the pellet may lead to incomplete washing and consequent high background in the cell multiplexing data. If you have less than 100'000 cells to start with, you must use the alternative protocol CG000426 for washes in 96w plates. This protocol can also be useful when many samples have to be labelled. 10XG note regarding these 2 protocols:

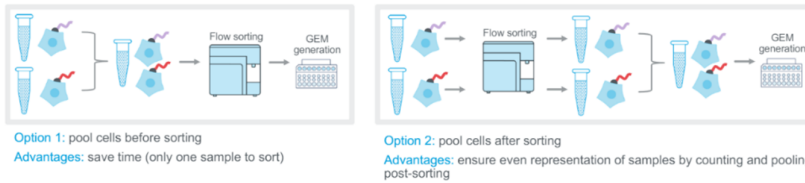
Please note that each protocol should be followed as it is written. We do not recommend interchanging aspects of the protocols:

- Using lower cell inputs with the tube-based protocol may lead to insufficient cell recovery & difficulty removing the supernatant during each wash step (pellet may not be visible)
 - Using higher cell inputs with the plate-based protocol may lead to insufficient washing (the lower wash volumes in this protocol may be insufficient for higher cell loads)
 - A vacuum pump is required for plate-based protocol, for efficient removal of supernatant
- Careful washing after labelling is critical for efficient demultiplexing of the samples. Follow best practice guidelines for correct washing procedure. In samples with lower viability or excess debris, insufficient washing may lead complete lack of separation between signal and noise.
 - Pay attention that the wash and resuspension buffer varies depending on your cell type and cell health (PBS 10% FBS for most cells, NbActiv-1 + 1% BSA for brain tissues).
 - If BSA is used, do not use less than 1% BSA as this will increase background in multiplexing data.
 - Keep cells/nuclei on ice or 4°C at all times after labelling. Higher temperatures increase noise.
 - Look for doublets before pooling and try to break them if possible. The doublets present at that stage due to poor dissociation won't be identified downstream bioinformatically (see below).
 - Spread conditions across pools (if several are planned) to minimize batch effects.
 - Pool cells/nuclei within 30 minutes of TotalSeq labelling and washing and immediately bring the cells to GECF. If cells are left for longer periods of time, either post labelling or post pooling, Cell Multiplexing can be severely impacted even if Gene Expression data is only mildly impacted.
 - TotalSeq labelling is compatible with downstream FACS if needed:

Best practices for CMO labeling

Sorting CMO-labeled cells can improve data quality

- CMO-labeled cells are compatible with flow cytometry
- Sorting can eliminate debris and reduce background
 - Sort cells by staining with live/dead stain
 - Sort nuclei by forward & side scatter



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- If working with nuclei, during pilot experiment, nuclei quality after labelling and washing should be assessed. If nuclei clumping or low nuclei recovery after labelling are observed, optimize upstream nuclei isolation protocols (e.g. lysis time, detergent concentration).
- TotalSeq labelling is not compatible with nuclei isolated from snap-frozen tissues. Isolating nuclei from snap-frozen tissues can be technically challenging, and nuclei can easily become damaged.

General description

- Antibodies available for mouse and humans.
- Up to 16 multiplexed samples and 50k cells/well.
- Compatible with 3'GE scRNA-seq v4 only (+ surface proteins) or 5'GE + VDJ v3 .
- Not compatible with ATAC, multiome.
- For crucial samples, one strategy may be considered to minimize the risk of losing a sample due to a technical issue in a specific well in the instrument (clog for instance): pooling all samples together and splitting the pool over several wells in the chip. This comes with slightly more complicated post-sequencing bioinformatic processing but may be worth for critical samples.

Relationship between cells number, tags number and multiplerts rate

- All this is taken care of in our calculation sandbox.
- Multiplerts rate increases with number of cells, and gets very high above 10k cells. For standard chips. But CellRanger can partially detect and eliminate multiplerts owing to their multiple tags (not multiplerts due to poor dissociation though). The ability of multiplerts removal by CellRanger for a given total cell number increases with the number of tags included.

Number of Tags	Multiplets Detectable
2	50.0%
4	75.0%
8	87.5%
12	91.5%

→ therefore 10XG recommend this relationship between number of tags and total cells number:

Targeted Cell Recovery	Number of Tags
500-2,500	2
2,500-10,000	2-4
10,000-20,000	4-8
20,000-30,000	8-12

Table 2. Suggested number of tags for a given Targeted Cell Recovery

Practical example: For 30k cells targeted, do not use only 2 tags with 15k cells each, as you will get a very high number of multiplets, of which only 50% will be identified and discarded by CellRanger.

- The reads from multiplets are only eliminated by CellRanger but not rescued, therefore these reads must be considered accordingly when calculating the total number of reads needed and designing the sequencing runs (especially relevant if you plan to sequence with high depth). This is taken into account in our sandbox file.

Data analysis

We sometimes observe high percentage of cells not assigned to any tag. This is due to high tag background, which is usually related to suboptimal labelling procedure. If the signal:noise ratio in the experiment is good, tag assignment metrics can be improved by lowering the "confidence threshold" in CellRanger. This can be achieved by changing the min-assignment-confidence parameter when running Cell Ranger, as described below. However, please note that this can also increase the risk of sample misassignment.

`min-assignment-confidence` *Optional.* The minimum estimated likelihood to call a sample as tagged with a Cell Multiplexing Oligo (CMO) instead of "Unassigned". Users may wish to tolerate a higher rate of mis-assignment in order to obtain more singlets to include in their analysis, or a lower rate of mis-assignment at the cost of obtaining fewer singlets. By default, this value is 0.9. Contact support@10xgenomics.com for further advice.

Disclaimer

Regarding putative failure issues, hashing requires some clarification since the labelling procedure is done on user side and cannot be QCed by the GECF before processing cells. If the experiment fails, we can in principle (but not always) assess on which side was the problem:

- if GECF gets nice libraries for both “multiplexing oligos” and GEX, and normal/good quality sequencing, but if despite that there are issues assigning reads to samples, the issue is extremely likely to come from the labelling side. The most typical issue is suboptimal washes which lead to cross-contamination of tags between samples.

- if we get good GEX libraries but no/bad oligos libraries, the issue is very likely to be at labelling step. These oligos libraries are routine at GECF, therefore it is unlikely that we specifically failed these libraries and not the GEX ones. We wouldn't be able to formally exclude an issue on our side without running a positive control library in parallel, but since it is not economically feasible to do so, we have to assume the issue is on the user side, unless of course we spotted an issue on our side.

- if we fail to get both good oligos and good GEX libraries, the issue is rather on our/10XG side, unless cells were of very poor quality and we got green light from user to proceed anyway. With nuclei it's more of a grey zone since their “viability” cannot be assessed.

Changelog:

- v1.01 (24.06.2025): initial release (based on cellplex guidelines).