protocol v1.08

CellPlex on scRNA-seq 3'GE v3.1

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

Interactions with GECF on experiment day

- IN SHORT: Bring your sample already pooled at the right concentration, on ice. Be mindful that pooled sample should not wait too long on ice or Cell Multiplexing data can be severely impacted. Tell us total number of pooled cells you want data for (="targeted cell number" of the pool), and bsl2 level if relevant.

Practical guidelines

Intro/generalities

- The CellPlex labelling procedure involves several washes/centrifugations and a 5 minutes RT° incubation, which may impact on transcriptional profile of the cells. In addition, fragile cells may specially suffer and CellPlex may not suitable in such case. 10XG therefore recommend performing a pilot experiment or at the very least a labelling test to determine if the sample type is suitable for the CellPlex assay. 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 that stressful experiment day).

- The labelling is done by users. We can provide tags if needed (at list price), but contact us/collect these reagents well in advance to avoid bad surprises.

- Collect necessary reagents in advance (in addition to the oligos tags).

- The samples must be brought to GECF already pooled, and at the right concentration:

- For less than 10'000 targeted cells in total, the concentration is the same as regular 3'GE 10XG: 700-1'200 cells/ul, but with an absolute minimum volume of 50ul (25ul not possible).
- For >10'000 targeted cells in total, the recommended cell concentration is higher: 1'300-1'600 cells/ul. Again, with an absolute minimal volume of 50ul.

- 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 the latest version of 10XG CG000391 user guide 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 CMO tag assignment. If your cells significantly diverge from the ones mentioned in these guidelines, contact us or 10XG tech support for guidance.

- Cell Multiplexing data 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 (in document CG000391).

- Input cells should be free of debris. Samples with excessive debris are not recommended as debris can contribute to high 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 (rather >150'000 if you target 30'000 cells). If the sample quantity is not limited, 0.5-2 mio cells/sample should be used. It is expected that ca 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 96-w 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 the labelling is critical to allow for efficient demultiplexing of the samples. Follow best practice guidelines for correct washing procedure. In samples with lower viability or excess debris, the effects of insufficient washing may be exacerbated and may result in a complete lack of separation between signal and noise.

- If BSA is used, do not use less than 1% BSA as this may lead to increased background in Cell Multiplexing data.

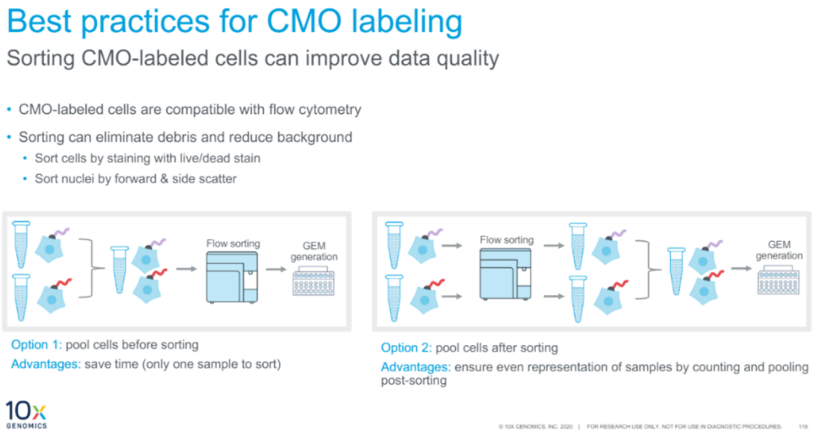
- Keep cells/nuclei on ice or 4°C at all times after CMO labelling. Higher temperature may increase background noise.

- 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 CMO 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, the Cell Multiplexing data can be severely impacted even if the single-cell behaviour of the Gene Expression data is only mildly impacted.

- CellPlex labelling is compatible with downstream FACS if needed:



- If working with nuclei, during pilot experiment, nuclei quality after CMO labelling and washing should be assessed. If nuclei clumping or low nuclei recovery after CMO labelling are observed, optimize upstream nuclei isolation protocols (e.g. lysis time, detergent concentration).

- Cellplex 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

- Based on lipids tagged with oligos, that can be used on cells and nuclei.

- How to know whether you nuclei isolation protocol is compatible: https://kb.10xgenomics.com/hc/en-us/articles/360061929592-Which-nuclei-isolation-protocols-are-supported-for-use-with-the-3-CellPlex-Kit-for-Cell-Multiplexing-?source=search

- Species-agnostic (plants not tested)

- Up to 12 multiplexed samples and 30k cells/channel (for regular Chromium chips).

- Compatible with 3'GE scRNA-seq only (and surface proteins, CRISPR barcoding or targeted panels).

- Not compatible with 5'GE+VDJ (likely soon), or other methods (ATAC, multiome).

- 10XG haven't tried with methanol fixed cells, but say "doubtful that it will work well".

- For more details regarding CellPlex technique and performances, a comprehensive tech note: https://support.10xgenomics.com/permalink/technical-note-chromium-next-gem-single-cell-3-v31-cell-multiplexing#header
- 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 it in certain cases.

Relationship between cells number, tags number and multiplets rate

- Multiplets rate increases with number of cells, and gets very high above 10k cells. For standard chips:

Targeted Cell Recovery	# of Cells Loaded	Cell Barcodes Detected	Singlets	Multiplets	Multiplet Rate
500	825	~500	~500	~3	~0.4%
1,000	1,650	~1,000	~1,000	~10	~0.8%
2,000	3,300	~2,000	~2,000	~40	~1.6%
3,000	4,950	~3,000	~2,900	~80	~2.4%
4,000	6,600	~3,900	~3,800	~140	~3.2%
5,000	8,250	~4,800	~4,600	~210	~4.0%
6,000	9,900	~5,700	~5,400	~300	~4.8%
7,000	11,550	~6,600	~6,200	~400	~5.6%
8,000	13,200	~7,500	~7,000	~510	~6.4%
9,000	14,850	~8,400	~7,700	~640	~7.2%
10,000	16,500	~9,200	~8,400	~780	~8.0%
12,000	19,800	~10,900	~9,800	~1,100	~9.6%
14,000	23,100	~12,500	~11,000	~1,500	~11.2%
16,000	26,400	~14,000	~12,100	~1,900	~12.8%
18,000	29,700	~15,500	~13,100	~2,300	~14.4%
20,000	33,000	~16,900	~14,100	~2,800	~16.0%
22,000	36,300	~18,300	~15,000	~3,300	~17.6%
24,000	39,600	~19,600	~15,800	~3,900	~19.2%
26,000	42,900	~20,900	~16,500	~4,400	~20.8%
28,000	46,200	~22,200	~17,100	~5,000	~22.4%
30,000	49,500	~23,400	~17,700	~5,600	~24.0%

At 30k targeted cells for instance, only 17’700 cells are in droplets containing a single cell.

- CellRanger can partially detect and eliminate multiplets owing to their multiple tags (not multiplets due to poor dissociation though). The ability of multiplets 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 (24%), of which only 50% will be identified and discarded by CellRanger, giving you at the end still 12% of unidentified multiplets in your data (way too high for proper analysis).

- 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).

- A practical example: if you target 20k cells, these 20k cells will be composed of 14'100 cells in droplets containing 1 cell, and the remaining 5'900 cells will be distributed in 2'800 droplets each containing several cells. All these cells will be sequenced, and therefore all the 20k cells must be included in your total number of reads calculation. This amount of multiplets is very high, but:

- with 2 tags, 50% of these multiplets are identified and bioinformatically discarded, and therefore 8% of multiplets remain in your data (still quite high).
- with 8 tags, 87.5% of multiplets are identified and bioinformatically discarded, and therefore only 1.6% of multiplets remain in your data.

- In conclusion, these relationships are not trivial, and some thought has to be given to find the sweet point between number of tags and number of cells for your experiment.

Disclaimer

Regarding putative failure issues, CellPlex 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-v1.04: Added disclaimer regarding failures. Clarified calculations. Clarified that the labelling is done on user side. Clarified concentration - targeted number of cells relationship.

- v1.05: Mentioned to mix conditions between pools (if several) to minimize batch effects. Clarified further the relationship between multiplets rates, number of cells and number of tags.

- v1.06: Added info regarding nuclei isolation compatibility with CellPlex. Suggested to look for doublets before pooling. Better description of sample preparation according to new rev of labelling user guide.

- v1.07: mentioned the new alternative protocol for labelling/washes in 96w plates, for starting from less than 100k cells. Mentioned the strategy of pooling all samples and splitting over different wells in the chip.

- v1.08: Mentioned to perform at least a labelling test if not a real pilot. Mention to prepare pooling calculations/formulas in advance.