TECHNICAL NOTE

Chromium Next GEM Single Cell 3' v3.1: Cell Multiplexing

Introduction

The 10x Genomics 3' CellPlex Kit provides a species-agnostic sample multiplexing solution using a set of 12 Feature Barcode oligonucleotides, each conjugated to a lipid. 3' CellPlex enables the pooling of up to 12 samples and capture of as many as 30,000 cells per channel to enable novel biomarker validation, identification and characterization of rare cell subtypes and states, and profile cell heterogeneity. This Technical Note describes concepts central to understanding Cell Multiplexing. It also highlights the utility of Cell Multiplexing across multiple species and compares data generated from Cell Multiplexing in combination with Single Cell 3' Gene Expression, Cell Surface Protein, and CRISPR Screening assays.

10x Genomics Assay-Specific Terms	
Tag	Cell Multiplexing Oligo (CMO)
Cells Loaded	Number of cells loaded into each well of the microfluidic chip. This number is higher than the desired Targeted Cell Recovery to account for cell recovery efficiency.
Targeted Cell Recovery	Number of intended captured cells for a given microfluidic chip channel.
Cell Barcodes Detected	Number of barcodes Cell Ranger analysis software has assigned to cells (signal) vs. background.
Singlets	Barcodes assigned by Cell Ranger to a single CMO.
Multiplets	Barcodes assigned by Cell Ranger to multiple CMOs.

Contents

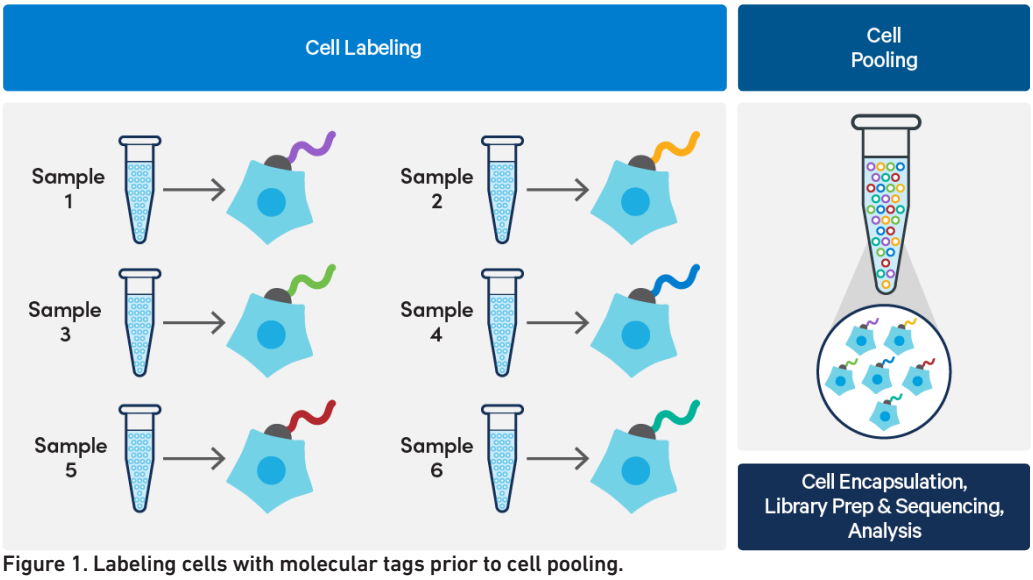


Figure 1. Labeling cells with molecular tags prior to cell pooling.

Next GEM reagents are specific to Next GEM products and should not be used interchangeably with non-Next GEM reagents.

Cell Multiplexing

Cell Multiplexing refers to a method for labeling cells (or nuclei) from individual samples with a molecular tag prior to pooling and performing single cell RNA-seq (scRNA-seq) (Figure 1). After cell encapsulation, library preparation, and sequencing, molecular tags are not only used to associate transcripts from the same cell of origin with each other, but also allow cells assigned with the same molecular tags to be associated back to the individual parent sample(s). Several multiplexing approaches have been described to label cells or nuclei, including antibodies that recognize cell surface epitopes and oligos that are incorporated into the cell membrane/interior (1-5). Despite differences in reagent/tagging strategies, each of these approaches shares a common goal: to attach a molecular barcode to a given sample. Multiplexed scRNA-seq experiments have several advantages, including:

- Increased sample throughput in a single experiment
- Increased number of cells assayed in a single experiment
- Increased number of possible replicates in a single experiment
- Detection of multiplets and their removal prior to analysis

These advantages provide greater flexibility when designing and executing scRNA-seq experiments. 10x Genomics has developed 3' CellPlex as a comprehensive Cell Multiplexing solution.

Single Cell Gene Expression with CellPlex

Single Cell Gene Expression with CellPlex provides a species agnostic, high-throughput and scalable approach to associate transcripts from the same cell/nuclei of origin with each other, while also allowing cells/nuclei assigned with the same molecular tags to be associated back to the individual parent sample(s).

3' CellPlex is enabled by the 3' CellPlex Kit which provides a set of 12 Cell Multiplexing Oligos (CMOs) each conjugated to a lipid. All 12 CMOs, or a subset, can be used together during any given experiment. When performing a Cell Multiplexing experiment, the supported Targeted Cell Recovery per channel increases to 500-30,000 cells/nuclei (the recommended range remains 500-10,000 cells/nuclei when not utilizing the 3' CellPlex Kit).

Chromium Single Cell 3' v3.1 Gel Beads

In addition to a poly(dT) primer sequence that enables the production of barcoded, full-length cDNA from polyadenylated mRNA, Single Cell 3' v3.1 Gel Beads include two additional primer sequences (Capture Sequence 1 and Capture Sequence 2) for direct capture and priming of Feature Barcode technology enabled molecules present in a cell inside a Gel-bead-in-emulsion (GEM).

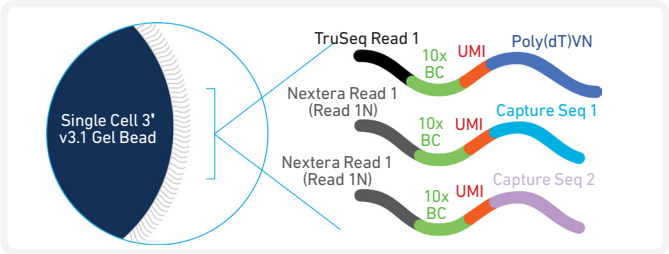


Figure 2. Chromium Single Cell 3' v3.1 Gel Bead Schematic.

3' CellPlex is built upon the existing Chromium Next GEM Single Cell 3' Gene Expression platform. The majority of the reagents required to perform 10x Genomics multiplexing are shared with the core Gene Expression assay. Refer to the Product List and Documents for the list of reagents and documentation required to generate Chromium Single Cell 3' Gene Expression Dual Index Libraries with Cell Multiplexing.

3' Feature Barcode Kit

The Single Cell Gene Expression Solution with Feature Barcode technology enables profiling of gene expression profiles in conjunction with additional cellular features from the same single cells. 3' CellPlex reagents are compatible with samples whose Cell Surface Proteins have been labeled with TotalSeq-B antibody-oligonucleotide conjugates or samples transduced with Feature Barcode technology compatible sgRNA constructs. To enable successful amplification, the 3' Feature Barcode Kit contains primers and Amp Mix required to generate:

- Gene Expression and CRISPR Screening Libraries
- Gene Expression and Cell Surface Protein Libraries
- Gene Expression and Cell Multiplexing Libraries
- Gene Expression, CRISPR Screening, and Cell Multiplexing Libraries
- Gene Expression, Cell Surface Protein, and Cell Multiplexing Libraries

3' CellPlex Kit

CMOs are provided as part of the 3' CellPlex Kit. Each CMO has sufficient volume for four staining reactions and contains a unique 15 nt Feature Barcode sequence allowing cells assigned with the same molecular tags to be associated back to the individual parent sample(s).

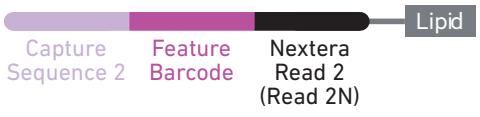


Figure 3. Cell Multiplexing Oligo

CMOs contain three distinct molecular regions:

- The reverse complement of Capture Sequence 2 enabling direct capture and priming within GEMs.
- A 15 nt Feature Barcode that provides a unique identifier for a given sample.
- Illumina Nextera Read 2 (Read 2N; read 2 sequencing primer) sequence that provides a PCR-handle that enables specific amplification of the CMOs.

For a detailed labeling protocol, consult the Demonstrated Protocol, "Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology" (Document CG000391).

Dual Index Kit NN

Cell Multiplexing libraries utilize Nextera Read 1 (from the gel bead capture oligo) and Nextera Read 2 (from the PCR handle on the CMO) sequences. The Dual Index Kit NN is required to specifically amplify the correct molecules and add on the required P5 and P7 sequences.

Workflow Overview

GEMs are generated by combining barcoded Single Cell 3' Gel Beads (v3.1), a Master Mix containing CellPlex labeled cells, and Partitioning Oil onto Next GEM Chromium Chip G. The poly(dT) and the Capture Sequence 2 primers on the gel bead are engaged simultaneously in two different reactions inside individual GEMs, generating barcoded, full-length cDNA from poly-adenylated mRNA and barcoded DNA from the CMO Feature Barcode. The 10x Barcoded cDNA molecules are then amplified via PCR, using compatible primers to generate sufficient mass for library construction. Size selection is used to separate the amplified cDNA molecules for 3' Gene Expression and Cell Multiplexing library construction. Libraries are sequenced and analyzed with Cell Ranger.

The next section discusses sample preparation tips for optimal data quality.

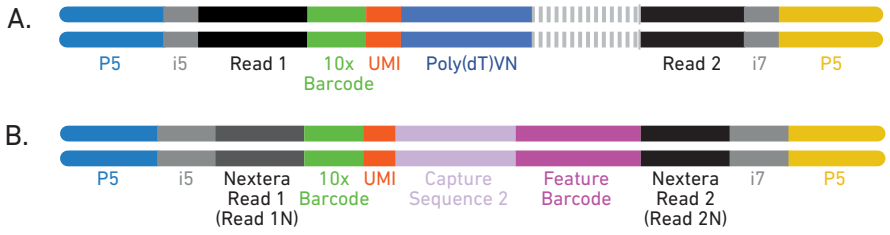


Figure 4. Products generated from library construction. Library construction in the Single Cell Gene Expression with Feature Barcode technology for Cell Multiplexing workflow generates Single Cell Gene Expression (A) and Cell Multiplexing libraries (B).

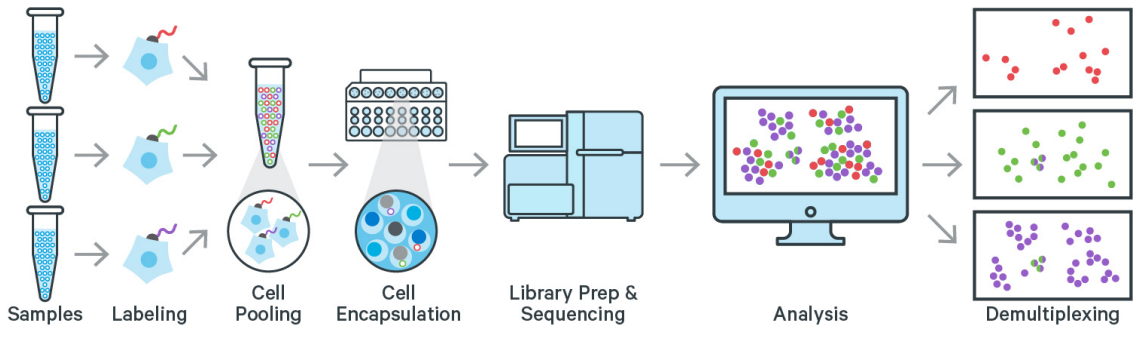


Figure 5. 3' CellPlex workflow.

Sample Preparation

- Ideally, input cell suspensions should have >90% viability.
- Robust cell washing is critical to obtaining high quality data, as it removes ambient RNA and unbound cell labeling reagents.
- Centrifugation conditions may need optimization to minimize cell loss during labeling.
- Count cells accurately before loading onto a Next GEM Chip to maximize the likelihood of achieving the desired cell recovery target.
- FACS may be used to improve cell/nuclei purity.
- Ideally, samples should be mixed in a 1:1 ratio.
- Cryopreservation of labeled cells or nuclei is not recommended.

Cell Ranger Metrics	
No Tag Assigned	Number of cells not assigned to any particular CMO
Fraction Reads in Cell-associated Barcodes	The fraction of valid-barcode, valid-UMI, recognized multiplexing-barcode reads with cell-associated barcodes
Fraction CMO Reads Usable	Fraction of read pairs that contain a recognized CMO sequence, a valid UMI, and a cell-associated barcode.

Cell Quality

Input cell suspensions for Cell Multiplexing Oligo labeling should contain more than 90% viable cells at all points during the experiment. A high fraction of non-viable, stressed, or dying cells may negatively impact Feature Barcode metrics and may increase the frequency of "No Tag Assigned" reported by Cell Ranger.

Cell Washing

High levels of ambient RNA or labeling reagents (Cell Surface Protein or 3' CellPlex) partitioned into all (cell containing and non cell containing) GEMs contribute to background noise in single cell assays and can compromise Feature Barcode data quality. Complete removal of supernatant during washing steps is critical and results in fewer GEMs containing unbound labeling reagents and an increase in the "Fraction Reads in Cell-associated Barcodes" reported by Cell Ranger.

The recommended cell washing and resuspension buffer for use in the cell labeling protocol is 1X PBS (calcium and magnesium free) containing 1% weight/volume BSA (10 mg/ml). BSA is added to minimize cell losses and aggregation. Primary cells, stem cells, and other sensitive cell types may require washing and suspension in alternative buffers to maximize viability. If necessary, PBS can be replaced with most common cell culture buffers. Consult the 10x Demonstrated Protocol for Cell Multiplexing Oligo Labeling (CG000391) for complete guidance.

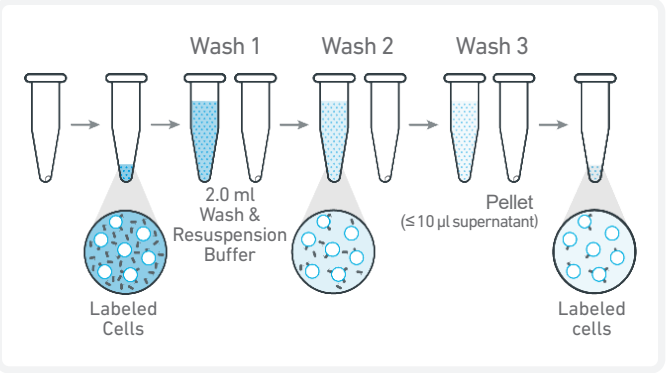


Figure 6. Overview of the 3' CellPlex washing protocol. Wash steps have been optimized to remove background and improve tag assignment efficacy by Cell Ranger.

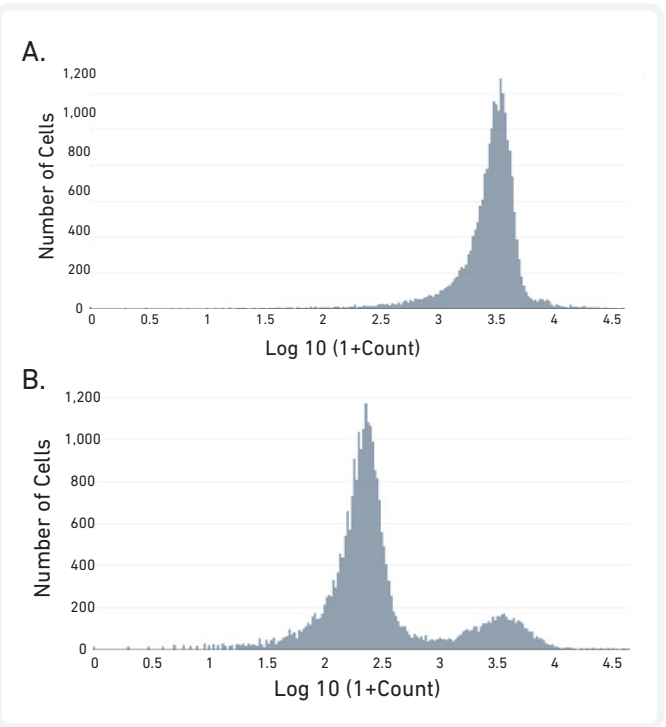


Figure 7. Histogram of tag counts from a controlled experiment where all reagents and cells were shared. Poor sample washing results in only a single peak (A). This causes Cell Ranger to assign all cells to the same tag. Two distinct populations are readily seen after stringent washing (left peak = background, right peak = signal (B). This separation allows for accurate cell/sample assignments.

Cell Recovery

Cell recovery/pelleting efficiency at a given speed, time, and temperature is heavily influenced by the number of cells, their concentration, size, and density. Therefore, centrifugation conditions may need to be optimized for specific sample types. Under optimized conditions, cell/nuclei recovery post labeling is ~50%, with the majority of samples falling in the 30-70% range.

Ideal centrifugation conditions will generate a solid, moderately packed cell pellet with minimal cells in the supernatant. Minimizing cells in the supernatant is critical for maintaining an accurate representation of heterogeneous samples that contain cells of variable sizes. However, excessive centrifugation may decrease viability and increase the risk of cell shearing due to extra pipetting. If necessary, the use of a swinging-bucket rotor as well as optimized centrifugation time and speed can help minimize cell loss while preserving sample integrity. Recommended centrifugation conditions for several sample types are provided in the Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode Technology demonstrated protocol (Document CG000391).

Cell Counting

After cell washing, a cell counting device such as a hemocytometer or a Countess II FL Automated Cell Counter can be used for cell quantification. These devices provide accurate cell counts that are critical for calculating the volume of cell suspension required to pool labeled samples in desired ratios. Over or under estimation of cell concentrations at this step can lead to inaccurate pooling of labeled samples, which can compromise application performance and quality of Feature Barcode data. To obtain accurate cell counts, the cell concentration and viability of the final, pooled sample should be determined prior to GEM generation. To maximize the likelihood of achieving the desired recovery target, the optimal cell concentration is 1,300-1,600 cells (or nuclei)/µl when targeting 10,000-30,000 cells. If targeting 500 to 10,000 cells, the recommended input cell concentration is 700-1,200 cells/µl.

Cell Sorting

Fluorescence-Activated Cell Sorting (FACS) may be used to remove subcellular debris, aggregates/clumps, ambient

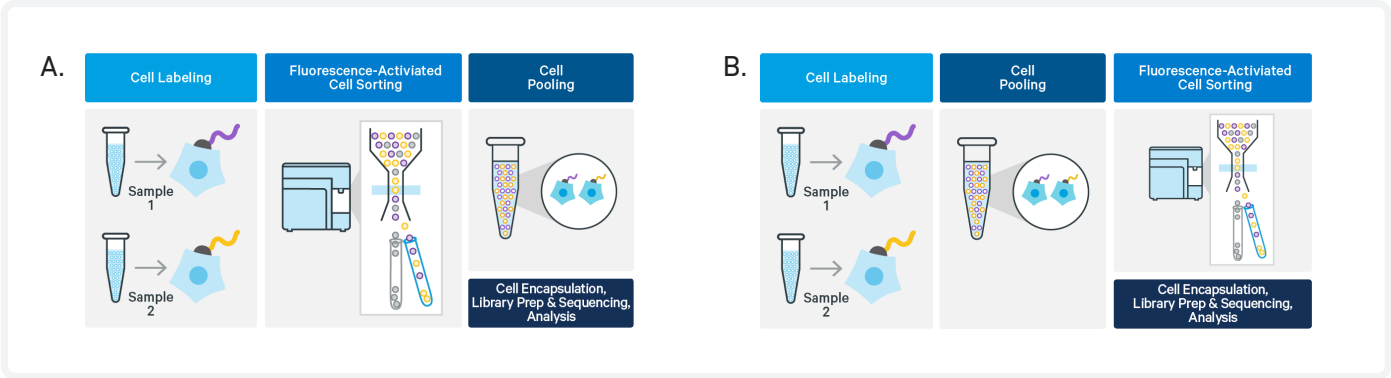


Figure 8. Fluorescence-Activated Cell Sorting is compatible with Cell Multiplexing. FACS may be performed pre (A) or post (B) sample pooling.

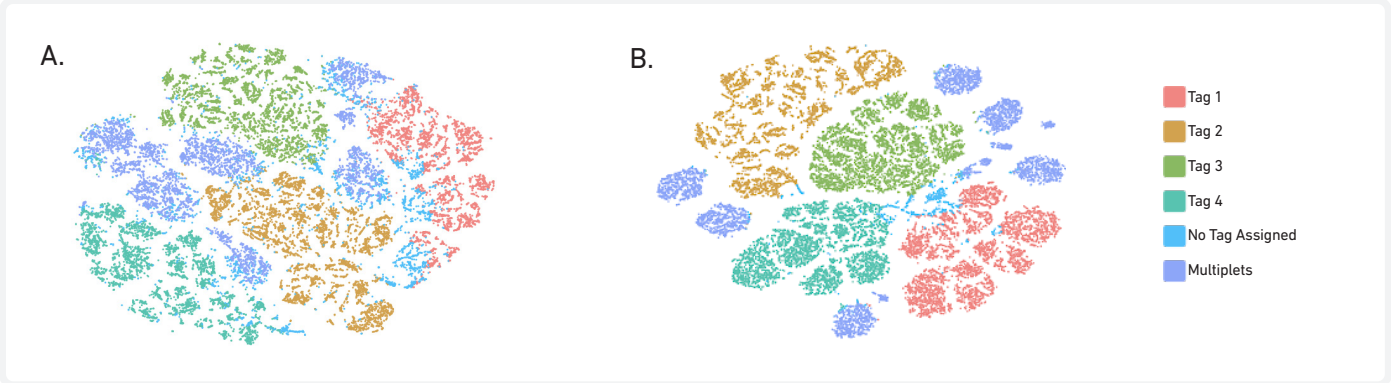


Figure 9. Fluorescence-Activated Cell Sorting results in improved multiplexing performance. Four dissociated tumor cell (DTC) samples were labeled with 1 tag each before pooling and running in a chip. Samples were labeled by their tag assignments. Tag-based clustering of pooled DTCs without sorting (A) and the same samples shown in A sorted to remove dead/dying cells prior to pooling (B). Cells that were sorted show better separation of tag clusters and fewer cells with 'No Tag Assigned' (Unsorted = 12.1%, Sorted = 3.4%).

RNA/DNA, and unbound labeling reagents from samples. Forward and side scatter should be used to exclude multiplets and other clumps. Inclusion of a dead cell marker will ensure identification and removal of non-viable cells. As shown in Figure 8, samples can be sorted either prior to or post pooling of labeled samples.

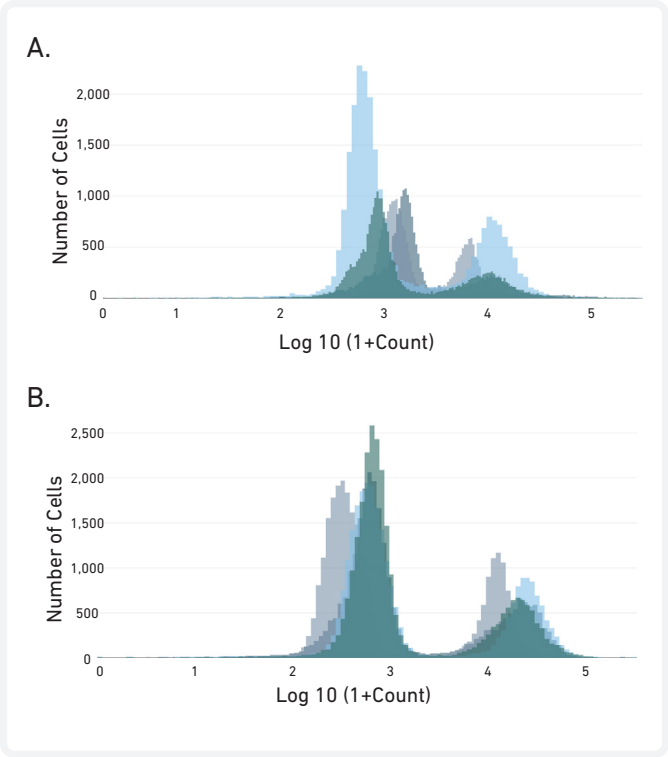


Figure 10. Histogram of tag counts showing that Fluorescence-Activated Cell Sorting results in improved multiplexing performance. The separation between background/noise (left peak) and foreground/signal (right peak) is improves in samples that have gone through flow cytometry (B) vs. those that have not (A). This results in more robust tag assignments.

The fraction of non-viable cells in a single cell suspension can vary significantly depending on sample type and sample preparation method. Ambient RNA due to lysed dead cells contributes to background noise and compromises data quality.

By reducing the fraction of non-viable cells from samples with FACS, a significant improvement in the following metrics may be obtained:

Single Cell Gene Expression

- Improved cell recovery
- Improved Fraction Reads in Cells
- Higher complexity libraries
- Reduction in mitochondrial/stress based transcripts

Cell Multiplexing

- Improved cell recovery
- Improved Fraction Reads in Cell-associated Barcodes
- Improved Fraction CMO Reads Usable
- Higher signal to noise ratio
- Reduction in "No Tag Assigned" assignments reported by Cell Ranger

Sample Mixing

The 3' CellPlex assay supports sample mixing from 50:50 to 5:95 ratios. Consideration of sample mixing ratios is critical if maximum multiplet detection is desired, as optimal multiplet detection occurs when samples are mixed in equal ratios. Further discussion of sample mixing, including example data, can be found in the Data Analysis section.

Sample Storage

Freezing or cryopreservation of labeled cell or nuclei samples is not recommended, as freezing can damage cellular/nuclear membranes. If cryopreservation is necessary, perform prior to labeling.

GEM Generation

After sample preparation and labeling, samples are loaded in a Chromium Chip G where GEM generation occurs. GEMs are generated by combining barcoded Single Cell 3' v3.1 Gel Beads, a Master Mix, and Cell Multiplexing Oligo labeled cells. To achieve single cell resolution, cells are delivered at a limiting dilution, such that the majority of generated GEMs contain no cell, while the remainder largely contain a single cell (singlet). This ensures that the transcriptome of only one cell is indexed by a common 10x barcode. Cells are loaded into GEMs following a Poisson distribution; thus, loading more cells into a channel increases the probability of a GEM forming with multiple cells. Figure 11 also shows that higher targeted cell recoveries result in higher multiplet rates. The detection of multiplets is discussed in the Data Analysis section.

Targeted Cell Recovery	10,000 cells	30,000 cells
Cells Loaded	16,500	49,500
Cell Barcodes Detected	~9,200	~23,400
Singlets	~8,400	~17,700
Multiplets	~780	~5,600

Table 1. Cell recovery metrics at a given Targeted Cell Recovery. The number of cells loaded into the GEM-RT Master Mix is higher than the desired Targeted Cell Recovery to account for recovery efficiency.

After GEM generation, amplified DNA from Cell Multiplexing Oligos and cDNA from poly-adenylated mRNA are used for library construction. If combining Cell Multiplexing with Cell Surface Protein or CRISPR Screening Feature Barcode technology, amplified cDNA from cell surface protein Feature Barcodes or sgRNA molecules are used to generated Cell Surface Protein libraries or CRISPR Screening libraries respectively.

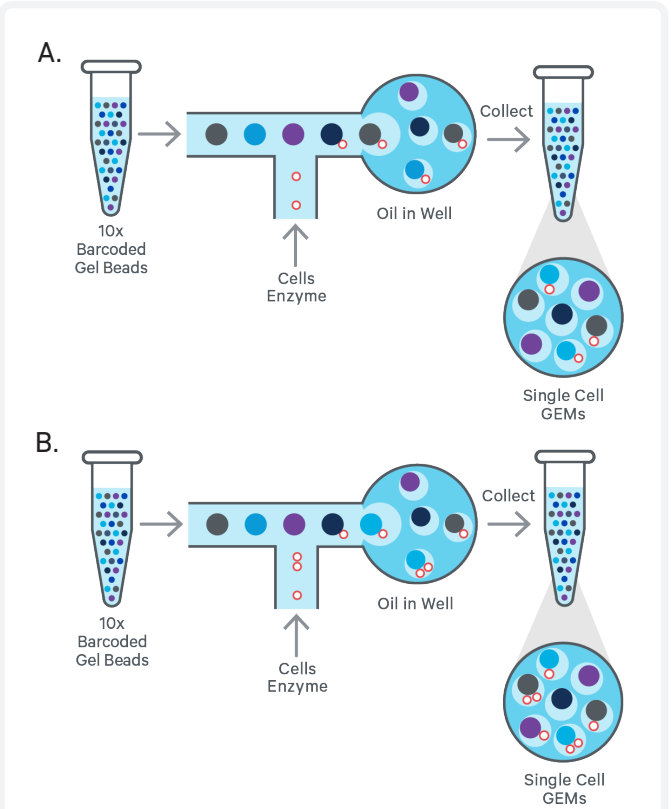


Figure 11. Higher cell loads increases the chance for multiplet formation. Under ideal GEM generation conditions, a single cell is captured in a GEM (A). At higher cell loads, multiplet rates increase (B).

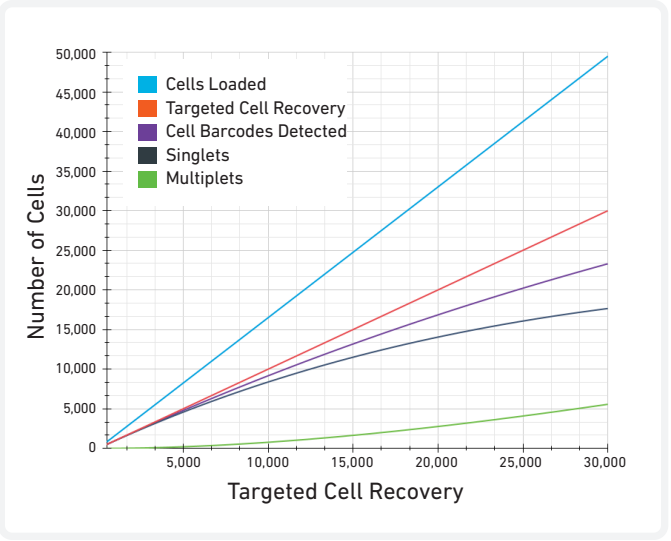


Figure 12. Multiplet rate increases with higher Targeted Cell Recovery. Cell Barcodes detected deviates from Targeted Cell Recovery at high cell recoveries due to more GEMs containing multiple cells, each of which Cell Ranger sees as a single cell barcode.

Library Pooling

Chromium Single Cell 3' v3.1 Gene Expression, CRISPR Screening, Cell Surface Protein, and Cell Multiplexing libraries can be pooled together for sequencing, taking into account the differences in cell number and per-cell read depth requirements between each library. Samples utilizing the same sample index should not be pooled together, or run on the same flow cell lane, as this would not enable correct library demultiplexing.

Sequencing

Sequencing Parameters	
Library	**Sequencing Depth**
Gene Expression	Minimum 20,000 read pairs per cell
Cell Multiplexing	Minimum 5,000 read pairs per cell (Minimum required Read 2 length is 15 bp)
CRISPR Screening	Minimum 5,000 read pairs per cell (Minimum required Read 2 length is 70 bp)
Cell Surface Protein	Minimum 5,000 read pairs per cell (Minimum required Read 2 length is 25 bp)
Sequencing Type	
Paired-end, dual indexing	
Sequencing Read	Recommended Number of Cycles
Read 1	28 cycles
i7 Index	10 cycles
i5 Index	10 cycles
Read 2	90 cycles

For more information, contact support@10xgenomics.com.

Data Analysis

Data from sequenced Cell Multiplexing libraries may be analyzed with Cell Ranger, a set of analysis pipelines used to align reads, perform clustering and gene expression analysis, and more. This section highlights Cell Ranger analysis topics relevant to Cell Multiplexing.

Multiplet Detection

For a scRNA-seq experiment containing cells from a single species, Cell Ranger cannot typically identify if an individual partition contains more than a single cell. Thus, Cell Ranger supports multi-species experiments when 3' CellPlex is not used, where cells from two different species can be mixed together prior to GEM generation*. This allows a subset of multiplets to be detected on the basis that some reads with a given cell barcode will align to one reference genome, and a different set of reads with the same cell barcode will align to another reference genome. This is typically done with a 50:50 mixture of human and mouse cells, with mixtures deviating from 50:50 being less accurate. GEMs containing a combination of mouse and human cells will be detected, while GEMs that contain multiple cells from the same species will not be detected. As shown in Figure 13, the multiplet rate reported by Cell Ranger accounts for this. This concept of using genetic information to identify and demultiplex samples within a GEM channel is analogous to how 3' CellPlex works, with genetic information replaced by molecular tags used to stain input samples.

*When using 3' CellPlex, mixing multiple species together in the same pool is not supported.

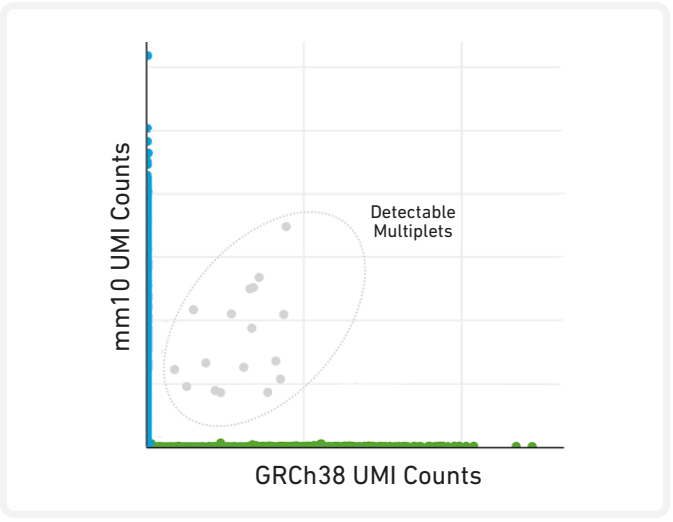


Figure 13. Multiplet detection reported by Cell Ranger from a 50:50 mixture of human and mouse cells. Detectable multiplets are barcodes with total UMI counts that exceed the 10th percentile of human and mouse distributions. For experiments not utilizing 3'CellPlex, Cell Ranger can only observe (human, mouse) multiplets; thus, it estimates the total number of multiplets to include hidden multiplets (human, human or mouse, mouse). This plot is to illustrate detectable multiplets - 10x Genomics does not support multiplexing with multiple species.

To demonstrate the detection of multiplets within a single species experiment, Jurkat (human T lymphoblast) and Raji (human B lymphoblast) cell lines were individually tagged (tag 1 = Jurkat, tag 2 = Raji) and combined in various mixing ratios. In the samples where an equal number of Jurkat and Raji cells were mixed, two distinct clusters of equal size were identified (Figure 14). Based on the expression of cell type-specific markers, cluster 1 was identified as Jurkat cells (preferentially expressing CD3E), while cluster 2 was identified as Raji cells (preferentially expressing CD79A).

Points located between the two clusters are likely multiplets, as they express both CD3E and CD79A. This marker based classification was verified by overlaying tag assignments by Cell Ranger. Cells in cluster 1 categorized as tag 1 and cells in cluster 2 categorized as tag 2. Cells assigned both tag 1 and tag 2 are considered multiplets, in agreement with the marker based assignment.

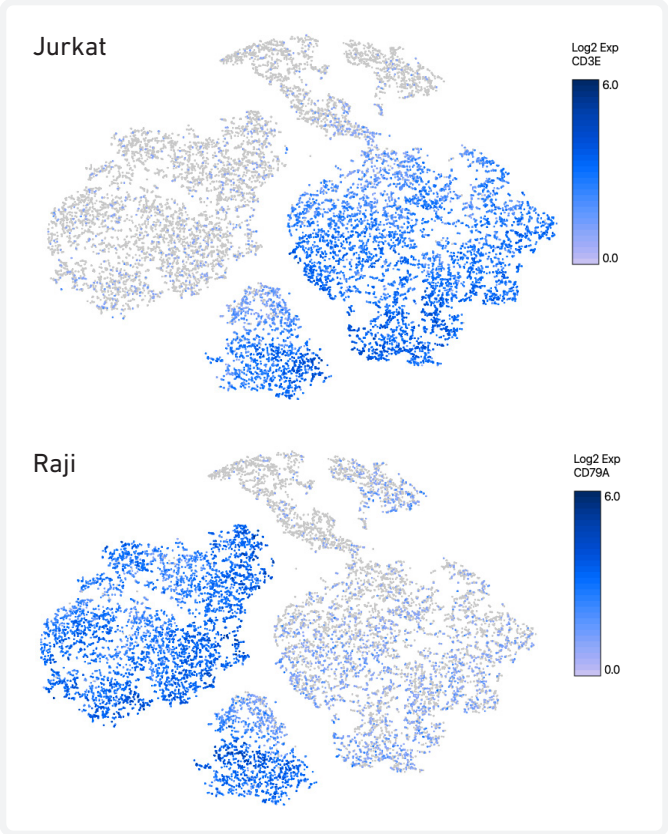


Figure 14. Jurkat and Raji cells, each labeled with one tag, are distinguishable via their gene expression based clustering.

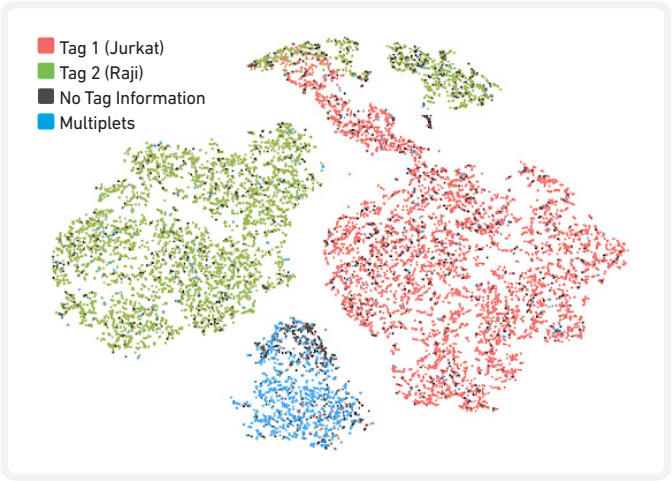


Figure 15. Jurkat and Raji cells mixed 50:50. Tag labeling is highly concordant with gene expression based clustering.

Assuming equal mixing of individually tagged samples, the probability of a multiplet containing cells from different samples can be described by the following:

$$P_{robability} = (1 - 1/N_{umber\ of\ tagged\ samples})$$

Selecting Number of Tags

When determining the number of tags needed for an experiment, consider that a smaller number of tags may be appropriate for maximizing channel throughput while a higher number of tags may be appropriate for maximizing multiplet detection. 10x Genomics recommends 1 tag for every 2,500 cells targeted for recovery. The following guidelines help ensure robust sample demultiplexing, but are not absolute requirements.

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

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

Table 3. Multiplets detectable per number of tags.

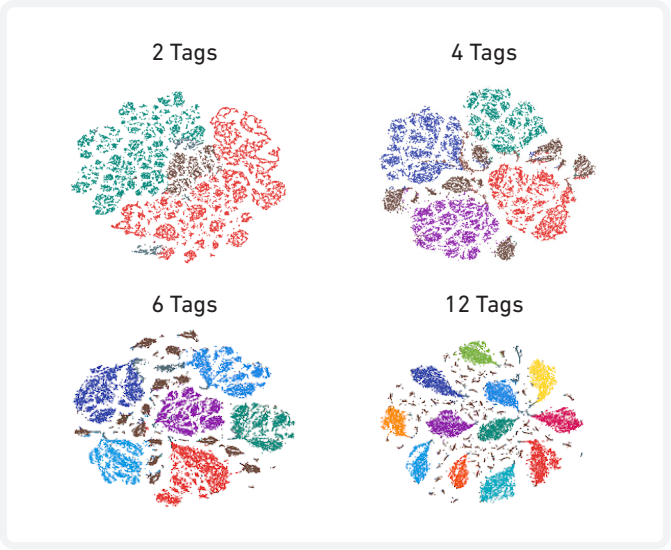


Figure 16. Tag based clustering for pools with 2, 4, 6, or 12 samples. Brown dots indicate multiplets. Increasing the number of tags, when possible, ensures robust sample demultiplexing.

As shown in Figure 17, 10x Genomics Chromium Next GEM Single Cell 3' assays have a microfluidic rate of 0.8% per 1,000 targeted cells recovered. This linear relationship is consistent with Poisson loading.

Cell Ranger can only detect multiplets when two different tags are assigned to a given cell barcode. For example, if two cells labeled with tag 1 are in the same GEM, Cell Ranger will assign that cell barcode tag 1. The number of multiplets remaining in a demultiplexed sample will depend on the number of tags used. Thus, by increasing the number of tags used in a given pool, the higher the likelihood of detecting multiplets.

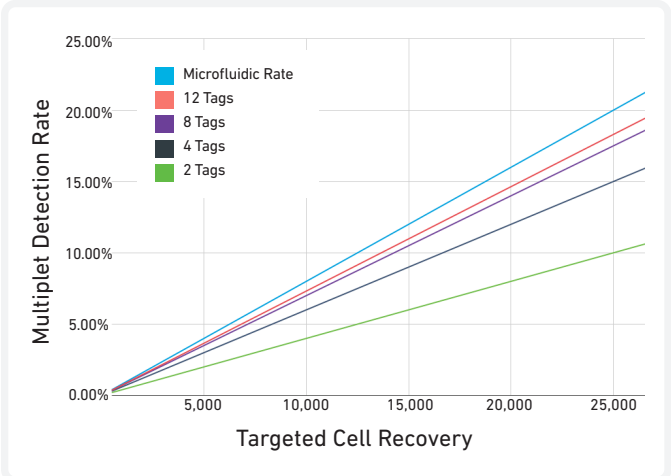


Figure 17. Multiplet detection vs. expected microfluidic rate. Data represent theoretical calculations and are supported by cell titration experiments.

Sample Mixing

The 3' CellPlex assay supports sample mixing from 50:50 to 5:95 ratios. If two samples are multiplexed in a single GEM channel, Cell Ranger will correctly assign cells to the appropriate sample as long as a sample makes up at least 5% of the total cell count and sample 2 makes up the remaining 95%.

Consideration of sample mixing ratios is critical if maximum multiplet detection is desired, as optimal multiplet detection occurs when samples are mixed in equal ratios. To demonstrate this, Jurkat and Raji cells were multiplexed in six different ratios (Figure 18). Cell Ranger was able demultiplex both samples in all instances, demonstrating its ability to perform unbiased detection of tagged samples.

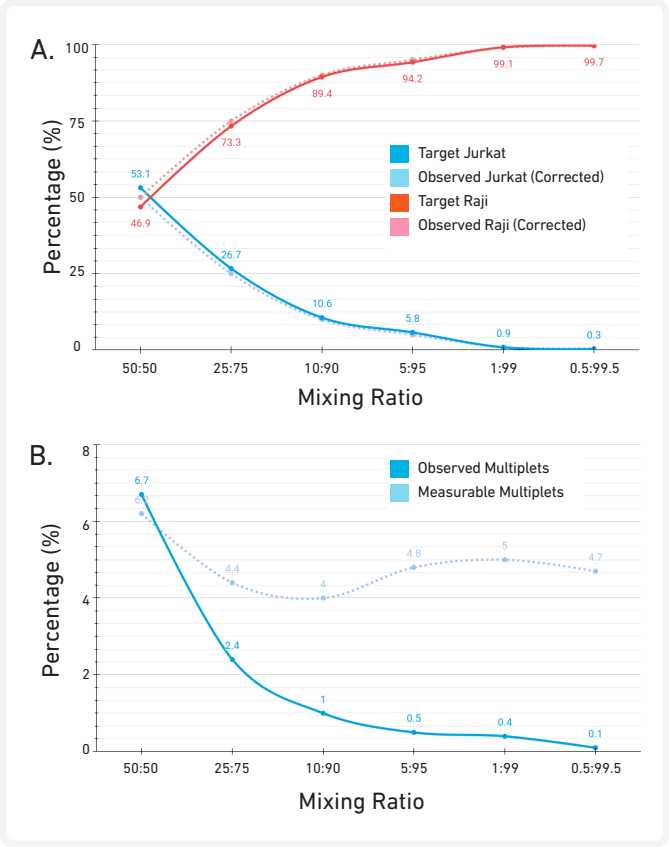


Figure 18. Multiplexed Jurkat and Raji cell lines, each individually tagged with a single tag. Measurable vs. observed singlets (A). Measurable vs. observed multiplets (B). Corrected = a small percentage of cells with no tag information have been excluded such that Tag 1/Jurkat + Tag 2/Raji = 100%. As the deviation from a 50:50 mixing ratio increases, the observed percentage of multiplets decreases. 1% and 0.5% data points are shown for demonstration purposes only. The lower supported limit for sample mixing is 5%.

Tag Assignments

The output of the 3' CellPlex workflow are two physical next-generation sequencing libraries. The first is a Single Cell Gene Expression library and the second is a Cell Multiplexing library. These two libraries are sequenced together on an Illumina sequencer and are provided as inputs to Cell Ranger so that accurate tag assignments can be made and single cell transcriptome data can be generated. Accurate tag assignments are critical to the success of experiments utilizing 3' CellPlex as it ensures high fidelity sample demultiplexing. Each physical library contributes different pieces of information that are used for analysis. The gene expression library is used to determine the 10x cell barcodes that are associated with cell containing GEMs (cell calling). The second physical library produced is the Cell Multiplexing library, which contains tag molecules originally captured during GEM-RT. Reads generated from a Cell Multiplexing library go through several levels of filtering before being used by Cell Ranger to assign tags to cells. 3' CellPlex reads that contain a valid cell barcode, match with a cell containing GEM, and contain a valid UMI are used to perform tag assignments.

When these filtered 3' CellPlex reads are plotted as a histogram, two major populations appear. Data shown below is from a 3' CellPlex experiment where the population on the left is referred to as the 'Background' (or 'Noise'), while the population on the right as 'Foreground' (or 'Signal') (Figure 19A). A simplified view of this can be seen in Figure 19B where, for a particular tag, only cells that fall into the foreground population are assigned that tag. The further the foreground population is from the background population (the higher the signal to noise ratio), the more robust tag assignments can be. The background population is generated from cells picking up a small number of tag UMIs from tags other than those used to originally stain a particular sample. Background levels are higher when washing during the 3' CellPlex demonstrated protocol is incomplete, or when the pooled sample has been left to sit too long before running in a 10x channel.

While it is possible to make tag assignments by only considering data from one tag at a time, Cell Ranger is able to make more confident tag calls by jointly considering data from all tags observed in a given experiment. Figure 19C below illustrates how Cell Ranger assigns cells in an experiment where two samples were pooled together. Cell barcodes are plotted based on their number of tag UMIs for both tag 1 and tag 2. Four possible states are observable: cells assigned one tag, multiplets, cells confidently assigned no tags and cells that are not confidently assigned to any of the previously listed states. Jointly considering data from all tags allows Cell Ranger to apply a confidence interval threshold; 90% or greater is required for assignment and cells not meeting this threshold are marked as 'No Tag Assigned'. Only cells confidently assigned a single tag will be included in demultiplexed samples.

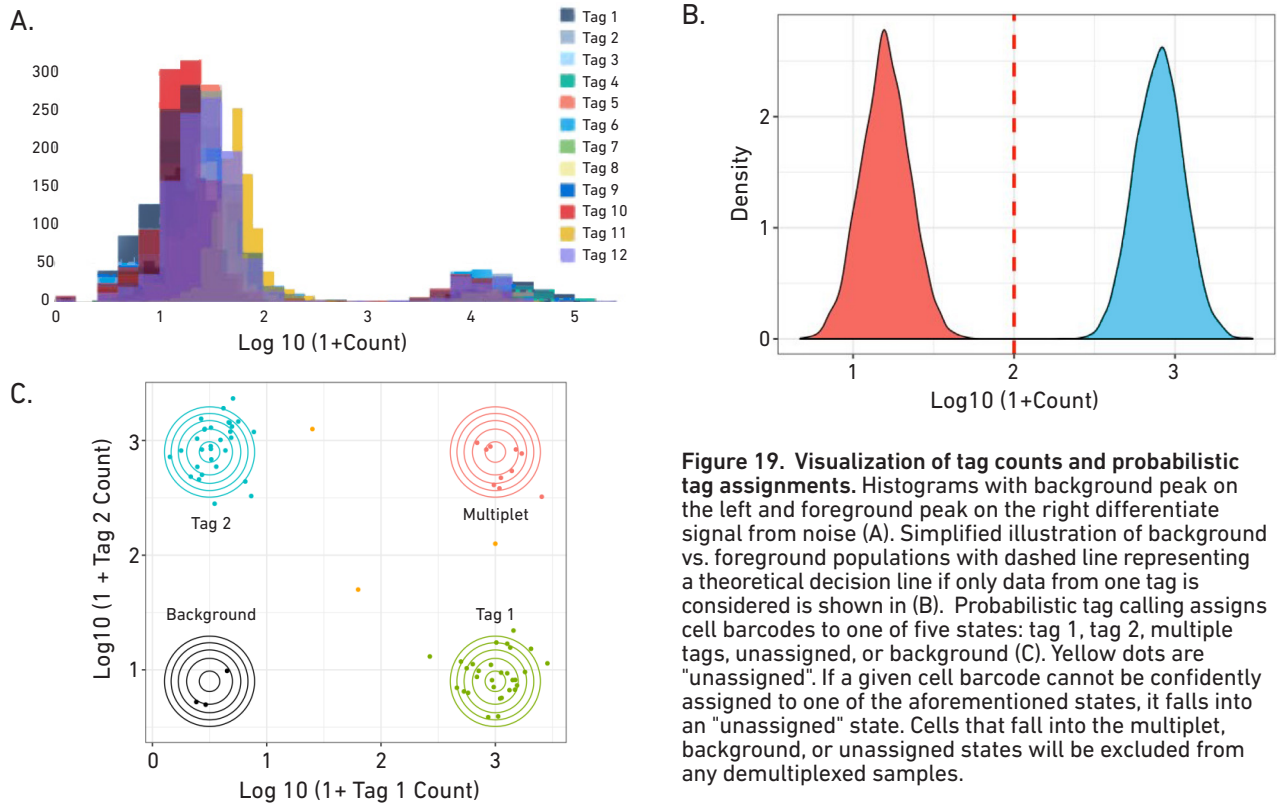


Figure 19. Visualization of tag counts and probabilistic tag assignments. Histograms with background peak on the left and foreground peak on the right differentiate signal from noise (A). Simplified illustration of background vs. foreground populations with dashed line representing a theoretical decision line if only data from one tag is considered is shown in (B). Probabilistic tag calling assigns cell barcodes to one of five states: tag 1, tag 2, multiple tags, unassigned, or background (C). Yellow dots are "unassigned". If a given cell barcode cannot be confidently assigned to one of the aforementioned states, it falls into an "unassigned" state. Cells that fall into the multiplet, background, or unassigned states will be excluded from any demultiplexed samples.

Representative Data Highlights

Representative Data Highlights 1-4 each provide an overview of experimental methods along with a comparison of key results. Data Highlight 1 demonstrates the utility of Cell Multiplexing in an across-species experiment. Data Highlight 2 demonstrates the use of 12 tags in a single experiment. Data Highlights 3-4 show how Cell Multiplexing may be combined with Cell Surface Protein or CRISPR Screening Feature Barcode technology. Together, these data highlights show the versatility and robustness of the 3' CellPlex assay.

All data highlights made use of the following protocols/ parameters for library construction, data analysis, and visualization:

Library Construction

- Chromium Single Cell 3' Reagent Kits v 3.1 (Dual Index) User Guide (CG000315)
- Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) with Feature Barcode Technology for CRISPR Screening User Guide (CG000316)
- Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) with Feature Barcode Technology for Cell Multiplexing User Guide (CG000388)
- Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) with Feature Barcode Technology for CRISPR Screening and Cell Multiplexing User Guide(CG000389)
- Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) with Feature Barcode Technology for Cell Surface Protein and Cell Multiplexing User Guide (CG000390)

Sequencing Depth*

- Gene Expression/Cell Multiplexing Library = 30,000 read pairs per cell
- CRISPR Screening/Cell Surface Protein Library = 10,000 read pairs per cell
- Cycles: Read 1–28; i5–10; i7–10; Read 2–90

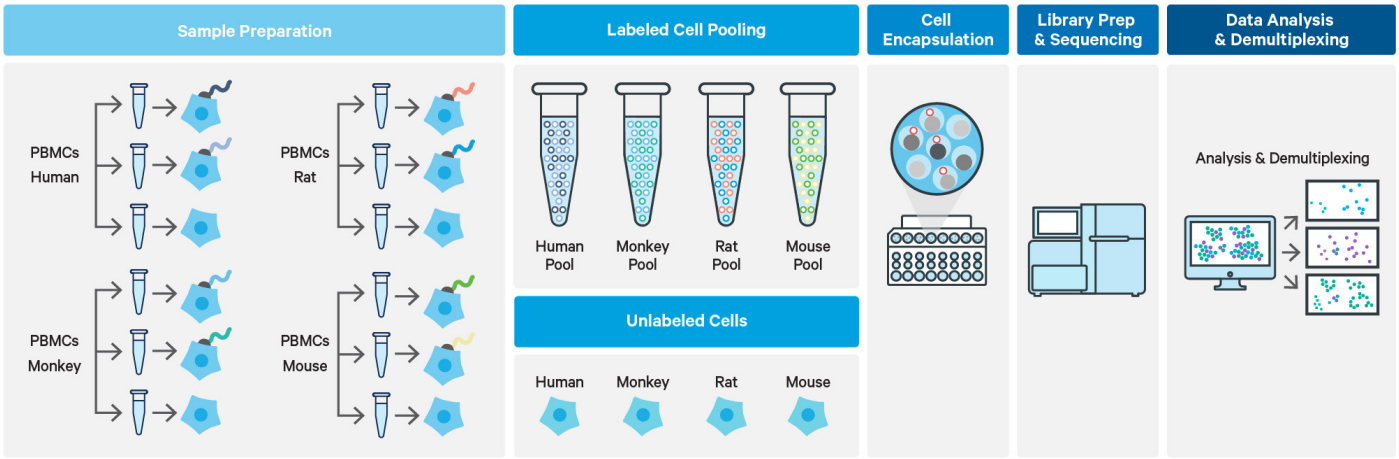
Analysis and Visualization:

- Cell Ranger 6.0
- Loupe Browser 6.0

*Data Highlight samples were sequenced at higher than recommended read depths for demonstration purposes. Refer to Sequencing section for minimum recommended sequencing depth.

Representative Data Highlight 1

Cell Multiplexing is Species Agnostic



Sample Preparation

- Peripheral Blood Mononuclear Cells (PBMC)s taken from four species (Human, Rhesus Monkey, Wistar Rat, C57BL/6 Mouse) were labeled with two tags per sample. Samples from the same species were pooled 1:1 into four distinct pools and loaded separately onto a Single Cell 3' v3.1 Next GEM chip targeting 10,000 cells (5,000 per tag).
- In parallel, unlabeled samples taken from all four species were loaded separately onto a Single Cell 3' v3.1 Next GEM chip targeting 5,000 cells.

Representative Data Highlight 1 contd.

Results

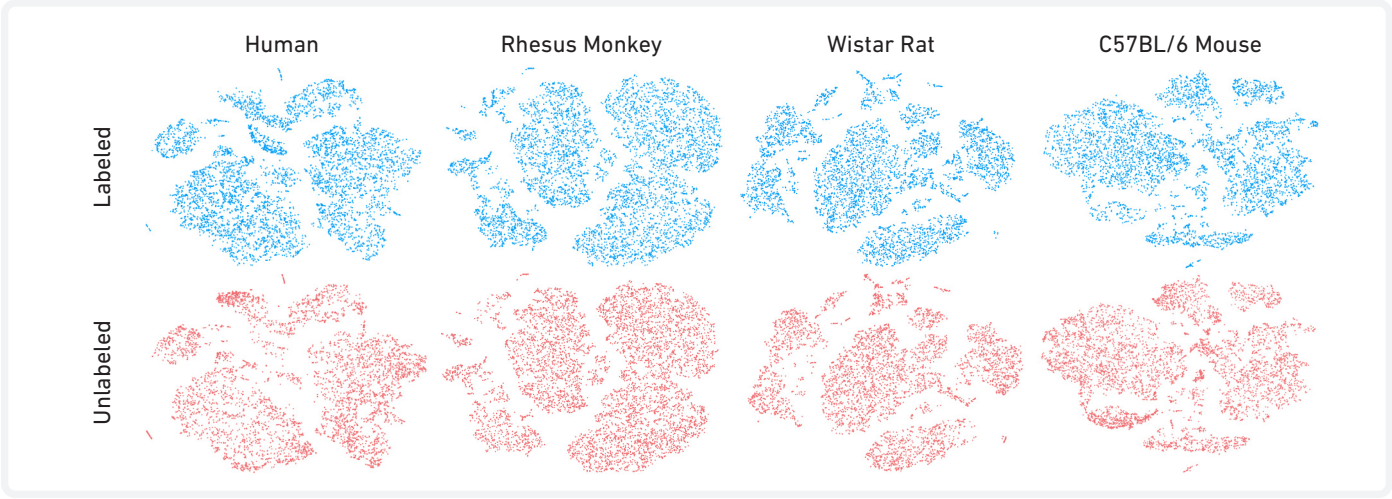


Figure 20. Loupe gene expression clustering of labeled and unlabeled PBMCs. Labeled and unlabeled samples show similar clustering across species, demonstrating that no gene expression changes are induced by cell labeling.

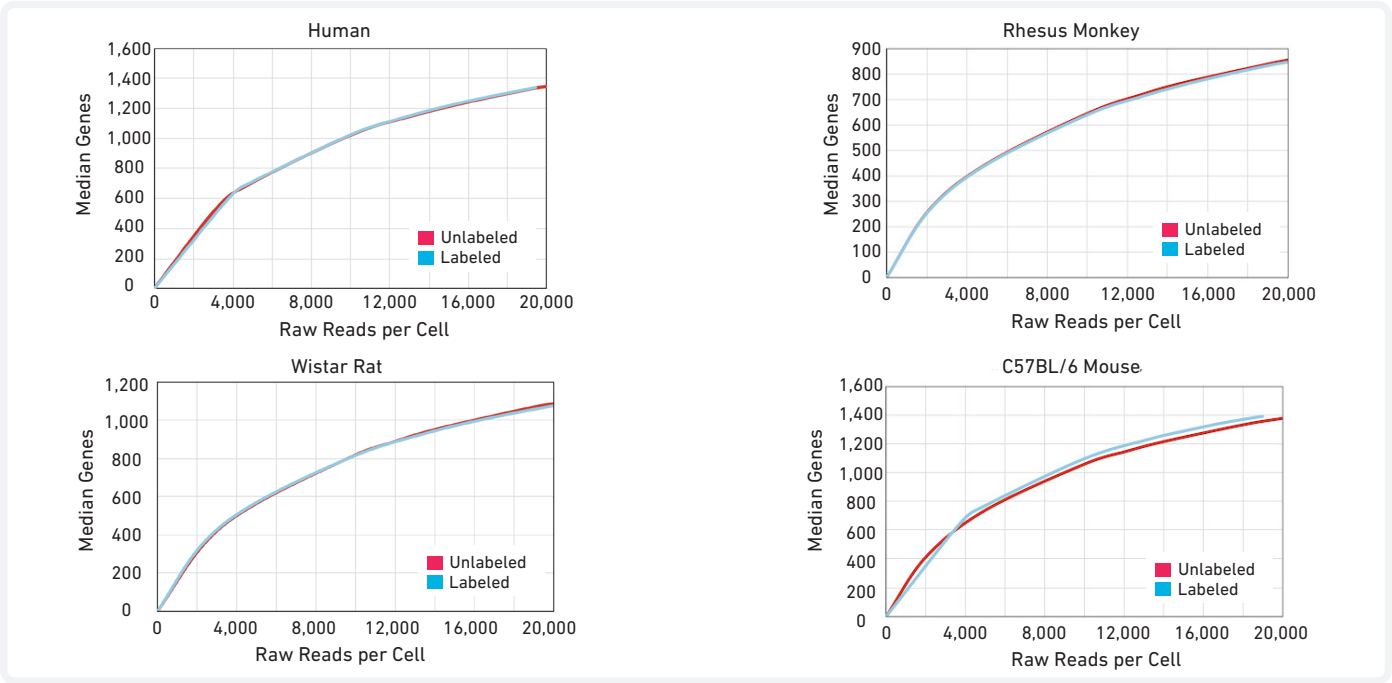
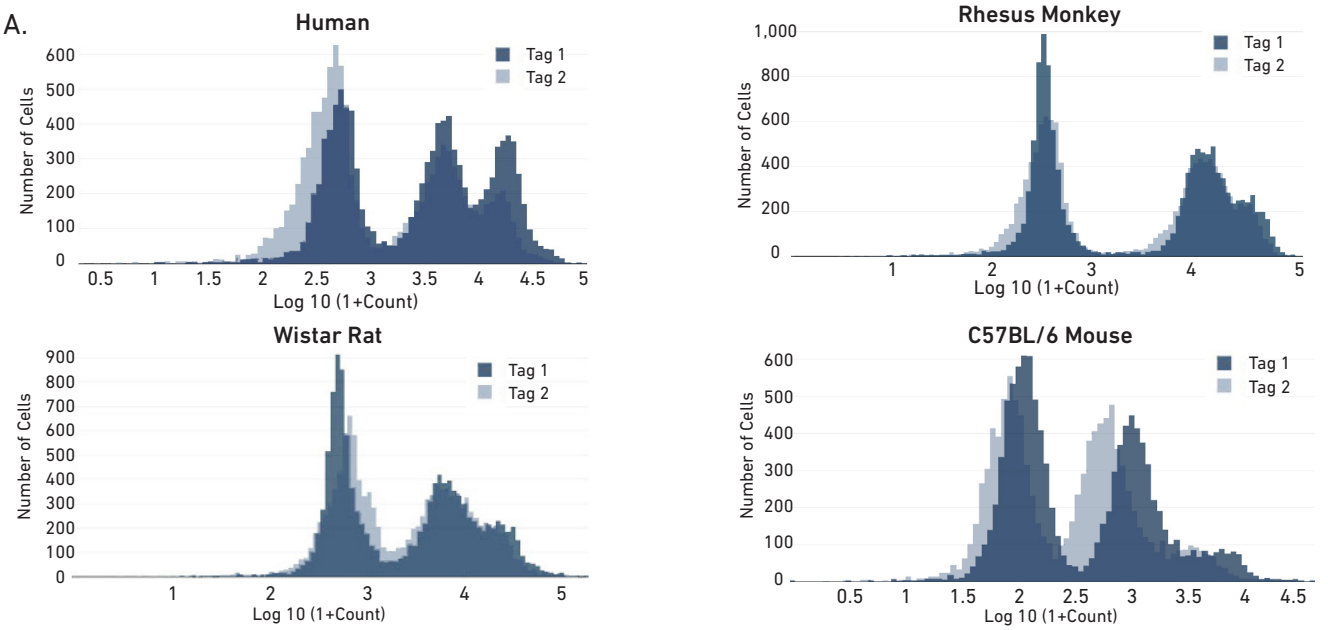


Figure 21. Sensitivity graphs for labeled and unlabeled PBMCs. Comparable library complexity was observed between labeled and unlabeled PBMCs.

Representative Data Highlight 1 contd.

Results

A.



B.

Tags Assigned	Human	Rhesus Monkey	Wistar Rat	C57BL/6 Mouse
1 Tag	93.6%	92.4%	87.9%	92.9%
>1 Tag	3.6%	5.4%	3.3%	3.0%
None	2.9%	2.2%	8.8%	4.1%

Figure 22. Tag counts histograms for labeled PBMCs. Histograms of tag counts show separation between background (left peak) and foreground (right peak) signal for each tag (A). The background population is interpreted as noise and the foreground population is interpreted as signal. Number of tags assigned to each sample (B). The foreground population can appear multimodal in heterogenous samples.

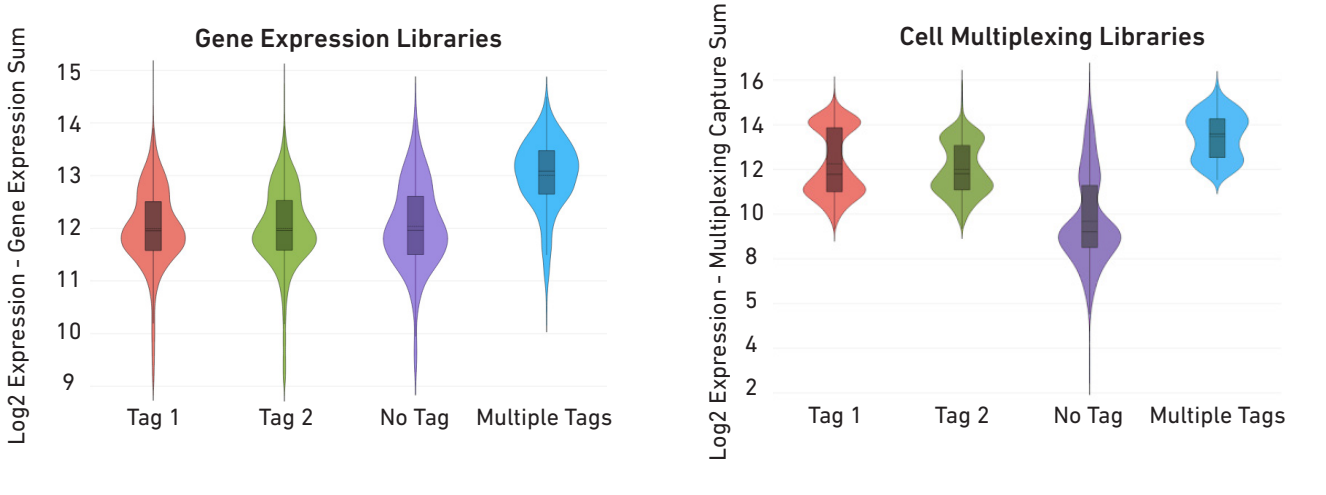
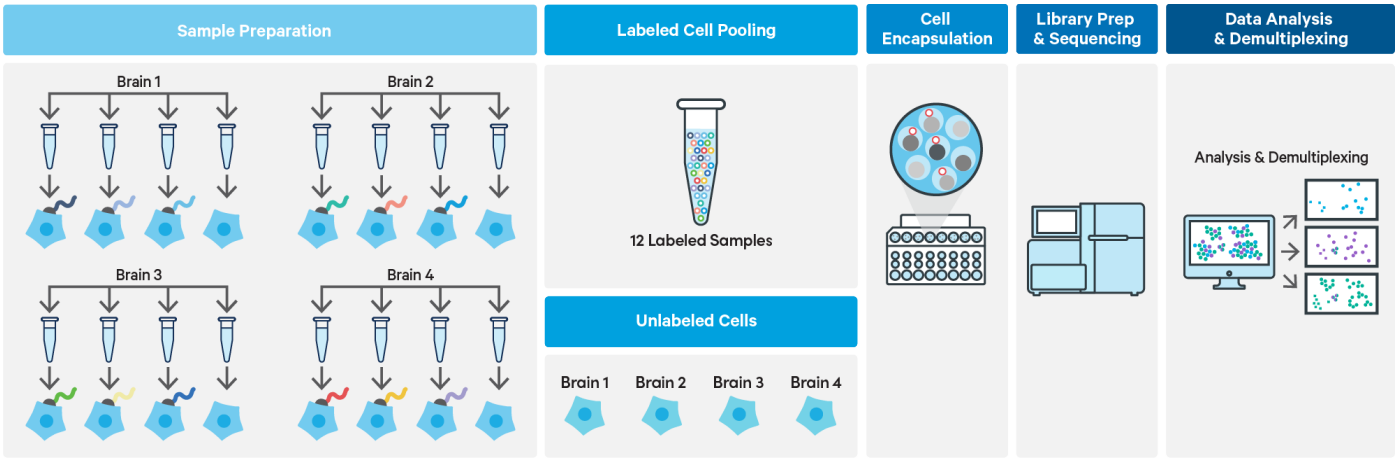


Figure 23. Log2 expression of the Gene Expression and Cell Multiplexing Capture Sums by tag assignment demonstrate comparable UMI density between cells labeled with different tags. Comparable gene expression UMI density between labeled human PBMCs assigned to tag 1, tag 2, and no tag. Higher gene expression UMI density is seen in cells with multiple tags. Comparable cell multiplexing UMI density between labeled human PBMCs with tag 1 and tag 2, demonstrating comparable performance across different tags.

Representative Data Highlight 2

Cell Multiplexing can be performed with up to 12 Cell Multiplexing Oligos



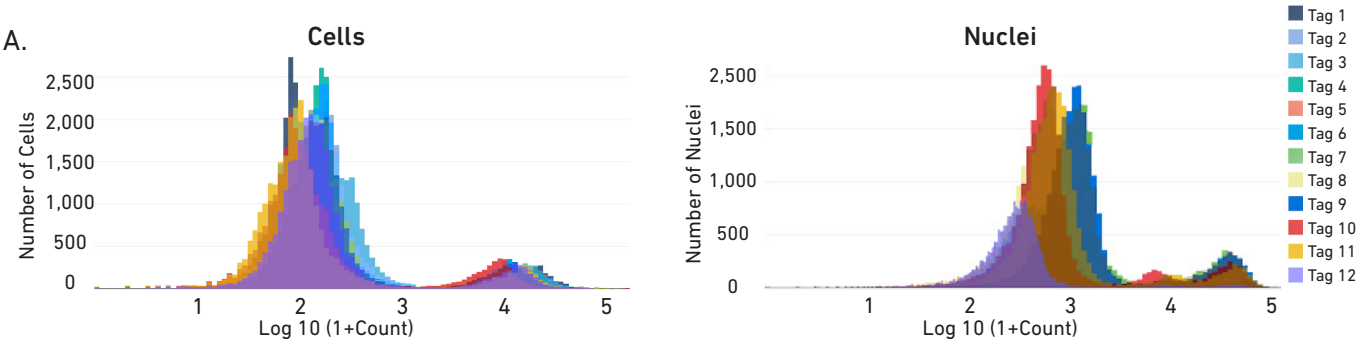
Nuclei isolation is not depicted in this workflow.

Sample Preparation

- Four E18 brains from C57BL/6 mice were dissociated according to the Dissociation of Mouse Embryonic Neural Tissue for Single Cell RNA Sequencing Demonstrated Protocol (Document CG00055). Neurons from each brain were aliquoted into three tubes and each labeled with one tag before pooling at a 1:1 ratio, for a total of 12 labeled samples. The pooled sample was run on a single channel of a Single Cell 3' v3.1 Next GEM chip targeting 20,000 cells. In parallel, unlabeled neurons from each brain were run in separate channels targeting 5,000 cells.
- Neurons derived from the four brains were lysed to generate nuclei according to the Isolation of Nuclei for Single Cell RNA Sequencing Demonstrated Protocol (Document CG000124). Isolated nuclei were labeled and run in a similar manner to the isolated neurons discussed above.

Results

A.



B.

Tags Assigned	Cells	Nuclei
1 Tag	72.8%	82.4%
>1 Tag	23.7%	9.6%
None	3.5%	7.9%

Figure 24. Chromium Single Cell 3' Cell Multiplexing libraries generated from a multiplexed sample of E18 neurons and a multiplexed sample of E18 neuronal nuclei. Clear signal (right peak) to noise (left peak) separation is observed for each tag (A). Tag assignments for E18 neurons and neuronal nuclei (B).

Representative Data Highlight 2 contd.

Results

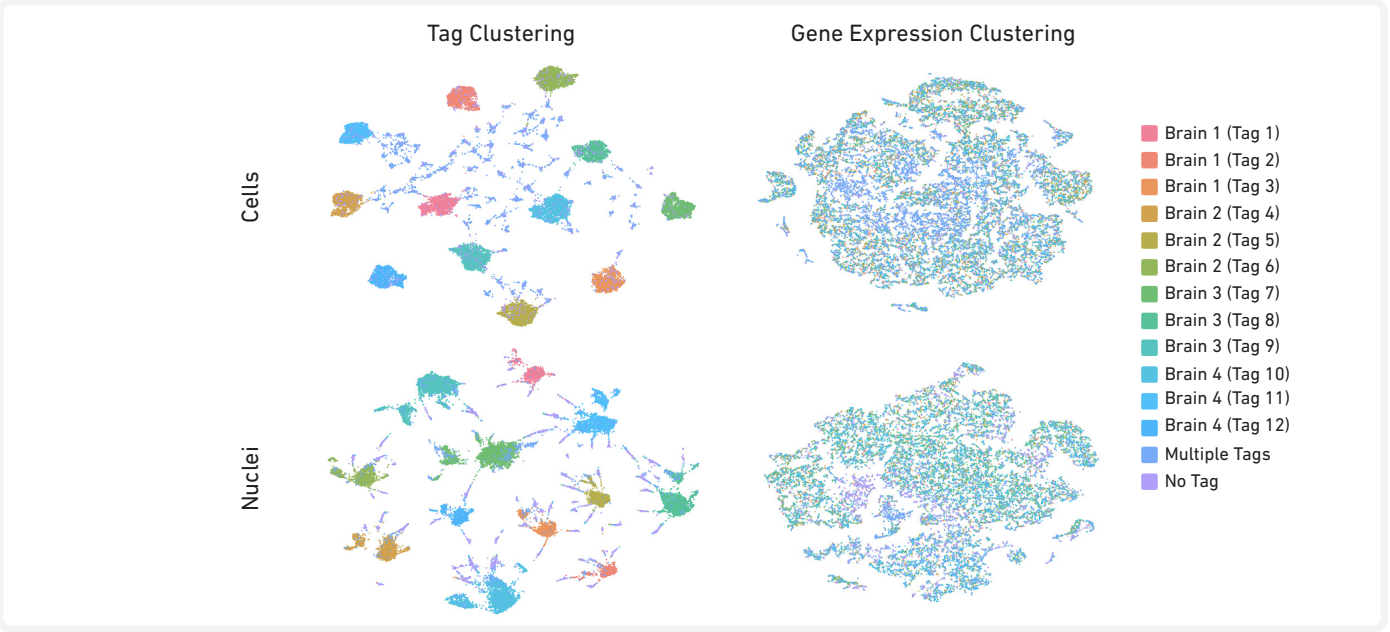


Figure 25. Chromium Single Cell 3' Cell Multiplexing libraries generated from a multiplexed sample of E18 neurons and a multiplexed sample of E18 neuronal nuclei show distinct tag clustering and comparable gene expression clustering in cells and nuclei.

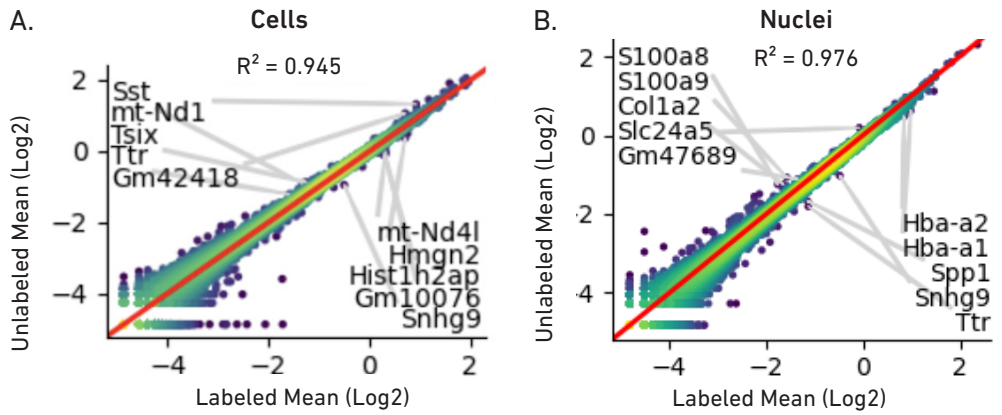
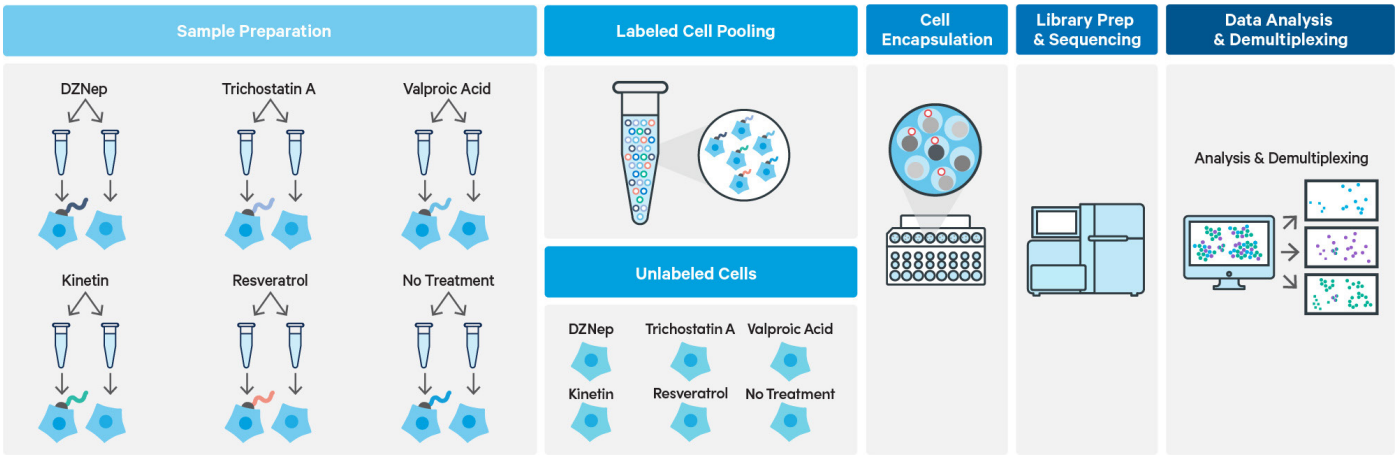


Figure 26. Correlation plots for E18 neurons and E18 neuronal nuclei. Comparable chemistry correlation was observed between unlabeled and labeled E18 neurons (A) and E18 neuronal nuclei (B).

Representative Data Highlight 3

Cell Multiplexing is compatible with CRISPR Screening Feature Barcode technology

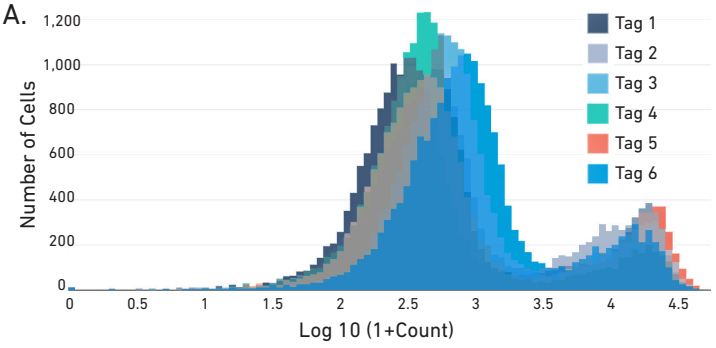


Sample Preparation

- A549 cells expressing dCas9-KRAB were transduced with a pool of 93 sgRNAs, including three non-targeting control sgRNAs.
- Cells were split into six conditions: five conditions where cells were treated with a single drug, and one control condition. Each condition was individually labeled with a single tag before pooling together and loading into a single channel of a Single Cell 3'v3.1 Next GEM chip, targeting 30,000 cells.
- In parallel, unlabeled samples corresponding to each condition were individually loaded into a channel of a Single Cell 3'v3.1 Next GEM chip, targeting 5,000 cells.

Results

A.



B.

Tags Assigned	Multiplexed Pool (6 cell lines)
1 Tag	81.2%
>1 Tag	4.5%
No Tag	14.4%

Figure 27. Chromium Single Cell 3' Cell Multiplexing library generated from a multiplexed sample composed of six A549 cell lines expressing dCas9-KRAB. Clear signal (right peak) to noise (left peak) separation for each tag is observed (A). Tag assignments for the multiplexed pool of six A549 cell lines (B).

Representative Data Highlight 3 contd.

Results

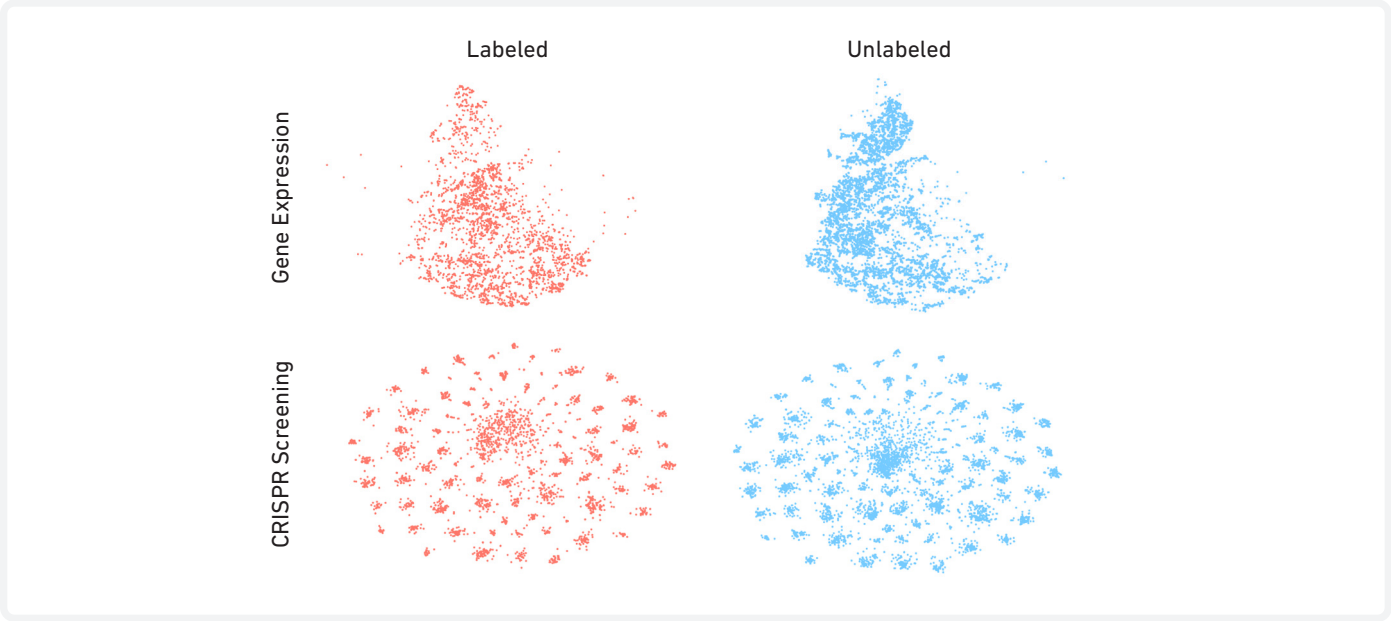


Figure 28. Loupe Gene Expression and CRISPR Screening tSNE clustering. Comparison tSNE plots for unlabeled and labeled samples. Cell Ranger was used to demultiplex the pooled treatment conditions.

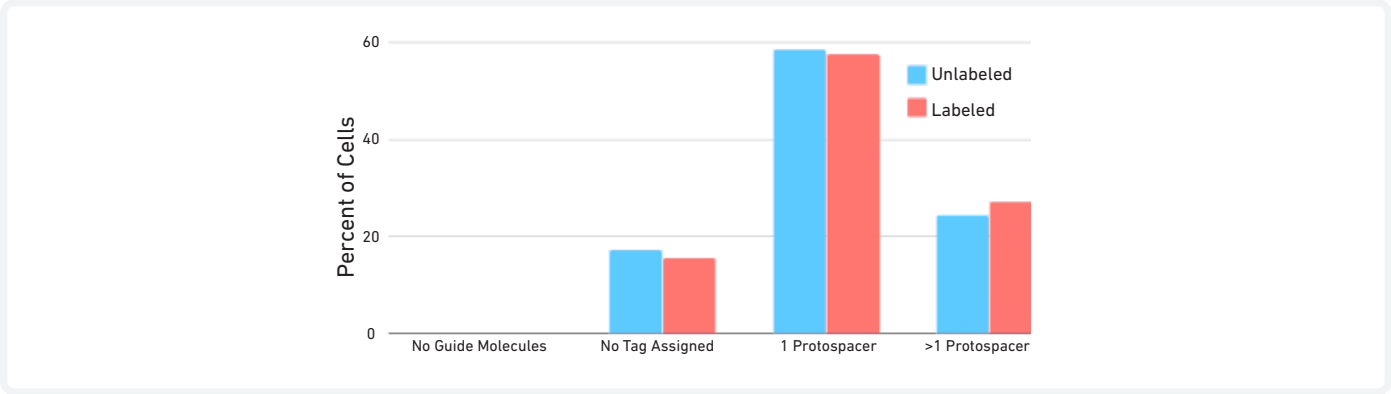
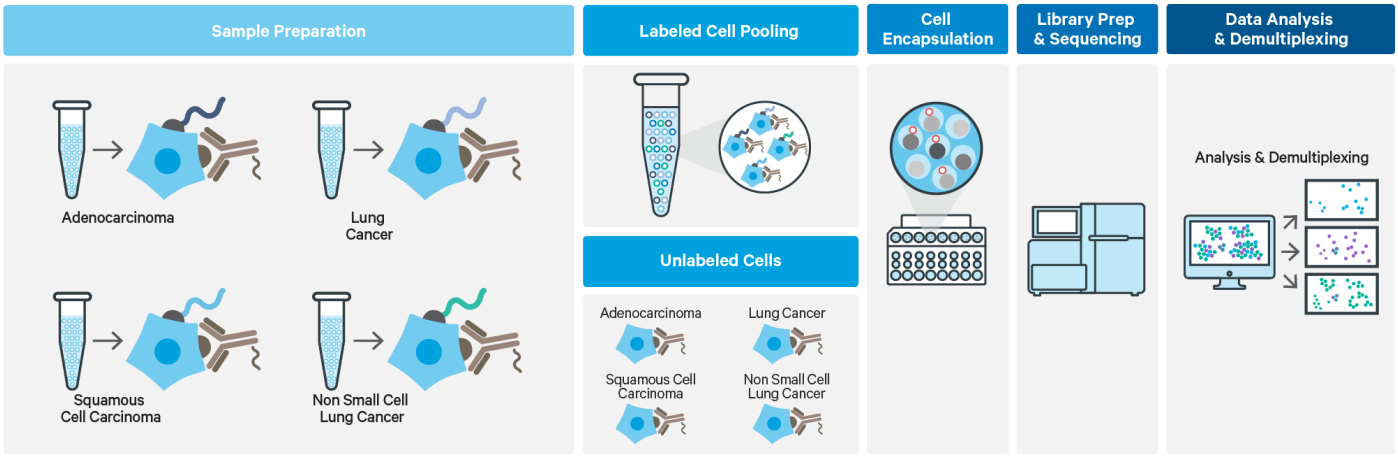


Figure 29. Guide assignment summary for no treatment cell line. Unlabeled sample was run in a single channel targeting 5,000 cells. Labeled sample was pooled with five other cell lines and run on a single channel targeting 30,000 cells.

Figure 30. Gene expression heatmap. Transcriptional profiles of cells across six different treatment conditions are affected when a given gene is knocked down. Comparison of cells receiving a non-targeting guide vs cells receiving a guide targeting ELOF1, across treatment conditions.

Representative Data Highlight 4

Cell Multiplexing is compatible with Cell Surface Protein Feature Barcode technology

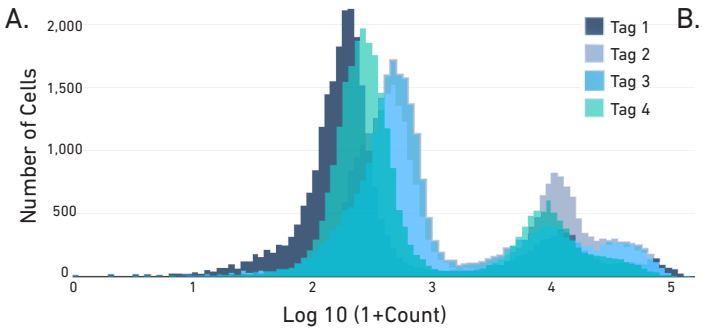


Data from unlabeled samples are not shown.

Sample Preparation

- Cells from four dissociated lung tumor samples (Adenocarcinoma, Non Small Cell Lung Cancer, Squamous Cell Carcinoma, and Lung Cancer) were dissociated and labeled with 17 TotalSeqB antibody-oligoconjugates and one tag, then pooled back together 1:1. The pool was sorted to remove dead/dying cells and loaded into a Single Cell 3'v3.1 Next GEM chip targeting 30,000 cells.
- In parallel, unlabeled samples corresponding to each dissociated lung tumor sample were labeled with 17 TotalSeqB antibody-oligoconjugates and were individually loaded into a channel of a Single Cell 3'v3.1 Next GEM chip targeting 5,000 cells.

Results



B.

Tags Assigned	Multiplexed Pool
1 Tag	79.24%
>1 Tag	13.05%
No Tag	7.71%

Figure 31. Chromium Single Cell 3' Cell Multiplexing library generated from a multiplexed sample composed of cells from four dissociated lung tumor samples. Clear signal (right peak) to noise (left peak) separation for each tag is observed (A). Tag assignments for the multiplexed pool of TotalSeqB-labeled dissociated lung tumor cells (B).

Representative Data Highlight 4 contd.

Results

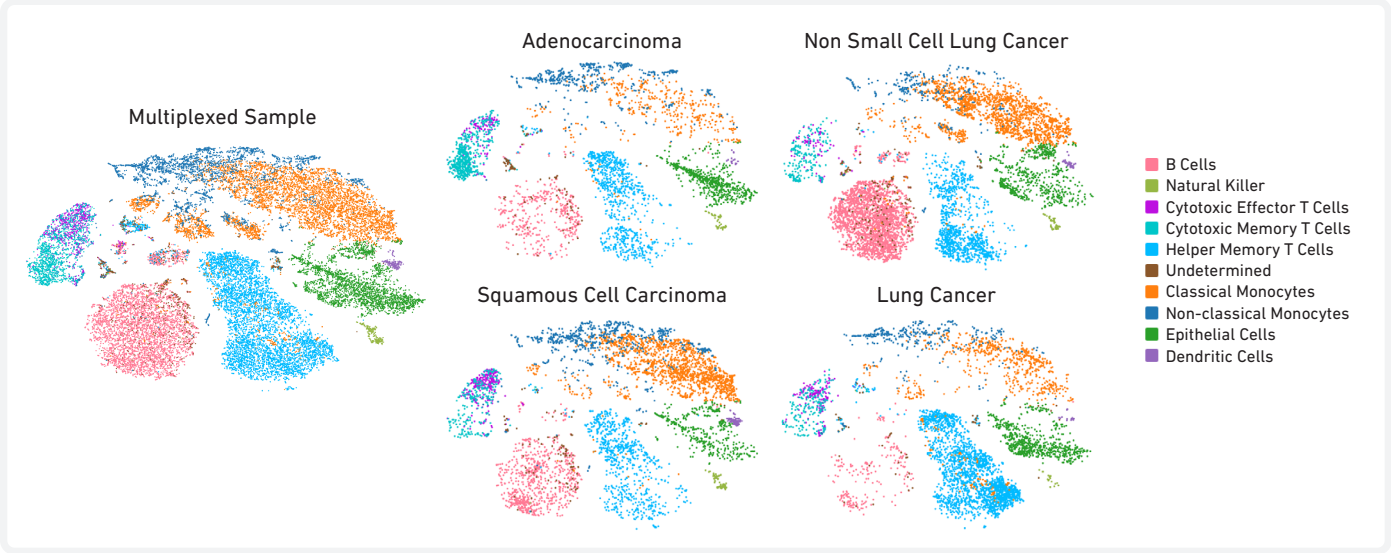


Figure 32. Detection of protein markers in a multiplexed sample composed of cells from four lung dissociated tumors labeled with a panel of 17 TotalSeqB antibody-oligoconjugates. Visualization with Loupe Browser of protein expression from the antibody tSNE of the multiplexed sample and individual samples.

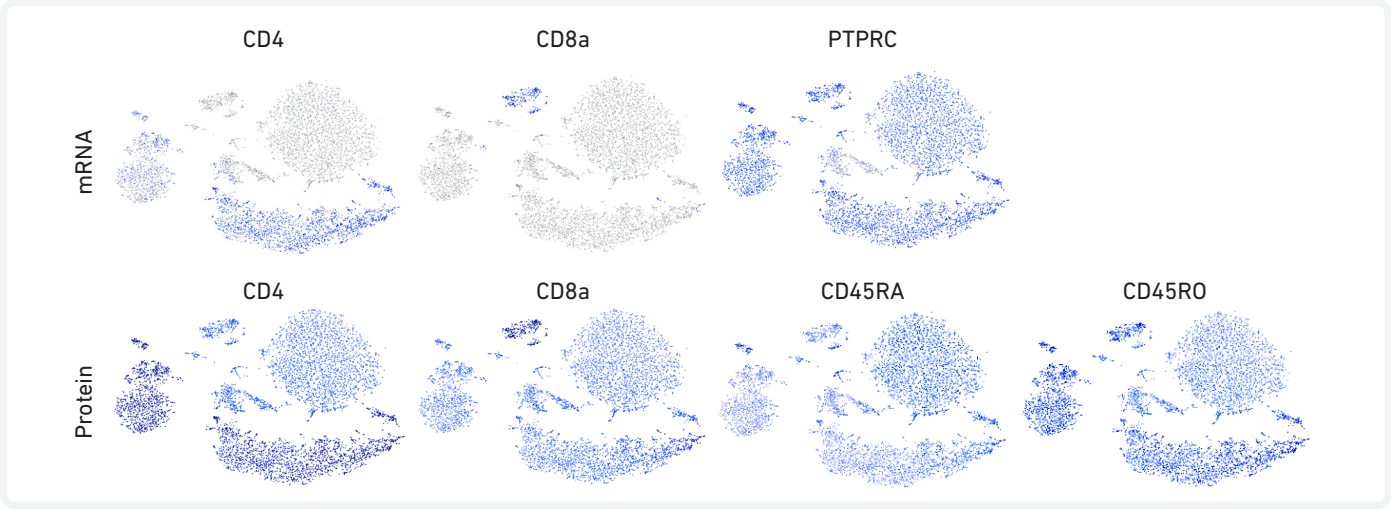


Figure 33. Detection of gene expression and protein markers in Non Small Cell Lung Cancer dissociated cells labeled with TotalSeqB antibody-oligo conjugates from a sample multiplexed with three additional lung cancer samples. Visualization with Loupe Browser of CD4, CD8A, and PTPRC transcripts and protein expression. CD45RA and CD45 RO are isoforms of PTPRC.

Chromium Next GEM Single Cell 3' v3.1 – Product List & Documents (Dual Index)

Product List (for generating Chromium Single Cell 3' Gene Expression Dual Index Libraries with Cell Multiplexing)		
Reagent Kits	**Reactions**	**Part Number (PN)**
Chromium Next GEM Single Cell 3' Kit v3.1	16 rxns 4 rxns	1000268 1000269
Chromium Next GEM Chip G Single Cell Kit	48 rxns 16 rxns	1000120 1000127
3' Feature Barcode Library Kit	16 rxns	1000262
3' CellPlex Kit Set A	48 rxns	1000261
Dual Index Kit TT Set A	96 rxns	1000215
Dual Index Kit NN Set A	96 rxns	1000243
Instrument		
Chromium Controller & Next GEM Accessory Kit	-	120223 (12 month warranty) 120246 (24 month warranty)
Software		
Cell Ranger Analysis Pipeline (DOWNLOAD)		
Loupe Browser (DOWNLOAD)		
Documents (for Single Cell 3' Gene Expression + Single Cell 3' Cell Multiplexing Dual Index Libraries ONLY)		
User Guide: Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) with Feature Barcode technology for Cell Multiplexing (CG000388)		
Demonstrated Protocol: Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocols (CG000391)		

If using Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) protocols with Feature Barcode technology for Cell Surface Protein or CRISPR screening, the reagent below is required in addition to all the products listed above. Refer to the indicated documents for specific guidance.

Additional Product List (with Feature Barcode technology for Cell Surface Protein or CRISPR screening)		
Reagent Kits	**Reactions**	**Part Number (PN)**
Dual Index Kit NT Set A	96 rxns	1000242
Documents (for Single Cell 3' Gene Expression + Single Cell 3' Cell Multiplexing + Single Cell 3' CRISPR Screening Dual Index Libraries ONLY)		
User Guide: Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) with Feature Barcode technology for CRISPR Screening and Cell Multiplexing (CG000389)		
Documents (for Single Cell 3' Gene Expression + Single Cell 3' Cell Multiplexing + Single Cell 3' Cell Surface Protein Dual Index Libraries ONLY)		
User Guide: Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) with Feature Barcode technology for Cell Surface Protein and Cell Multiplexing (CG000390)		

References

1. M Stoeckus et al, Cell Hashing with barcoded antibodies enables multiplexing and doublet detection for single cell genomics. Genome Biol. 19, 224 (2018).
2. C McGinnis et al, MULTI-seq: sample multiplexing for single-cell RNA sequencing using lipid tagged indices. Nat. Methods. 16, 619-626 (2019).
3. J Gehring et al, Highly multiplexed single-cell RNA-seq by DNA oligonucleotide tagging of cellular proteins. Nat. Biotechnol. 38, 35-38 (2020).
4. D Shin et al, Multiplexed single-cell RNA-seq via transient barcoding for simultaneous expression profiling of various drug perturbations. Science Advances. 5, EAAV2249 (2019).
5. C Guo et al, CellTag Indexing: genetic barcode-based sample multiplexing for single-cell genomics. Genome Biol. 20, 90 (2019).

Contact:
support@10xgenomics.com
10x Genomics
6230 Stoneridge Mall Road
Pleasanton, CA 94588 USA