

Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocols *with Feature Barcode technology*

Introduction

The 10x Genomics 3' CellPlex Kit provides a species agnostic sample multiplexing solution through the use of a set of 12 Feature Barcode oligonucleotides each conjugated to a lipid. These Cell Multiplexing Oligos (CMOs) can be used to label individual cells or nuclei samples, and the labeled cells can be pooled together prior to loading onto a 10x Genomics chip. The Feature Barcode molecules can be directly captured by oligonucleotides present on the Gel Beads inside a GEM during GEM-RT, subsequently amplified, and used to generate Cell Multiplexing libraries.

This protocol provides guidance for:

- Labeling cells/nuclei with CellPlex reagents (see Cell Multiplexing Oligo Labeling) for use with Single Cell 3' v3.1 (Dual Index) protocols with Feature Barcode technology for Cell Multiplexing and Single Cell 3' v3.1 (Dual Index) protocols with Feature Barcode technology for CRISPR Screening & Cell Multiplexing.
- Labeling cells with antibody-oligonucleotide conjugates and CellPlex reagents (see Cell Surface Protein & Cell Multiplexing Oligo Labeling) for use with Single Cell 3' v3.1 (Dual Index) protocols with Feature Barcode technology for Cell Surface Protein & Cell Multiplexing.

See Reference section for the compatible user guides.

Consult *Technical Note Chromium Single Cell 3' v3.1 Cell Multiplexing (Document CG000383)* for additional details on experimental design considerations for cell multiplexing.

Additional Guidance

This protocol was demonstrated using primary cells including peripheral blood mononuclear cells (PBMCs), dissociated tumor cells (DTCs), and dissociated brain tissue), cell lines (including Jurkat, Raji, A20, and EL4) as well as cell lines that have been transduced with CRISPR machinery (including A549, A375, SKOV3, and U2OS), and nuclei isolated from fresh cells or tissues. Optimize this protocol based on sample type (e.g., centrifugation conditions and wash buffers).

Consult Demonstrated Protocol Cell Preparation Guide (Document CG00053) for Tips & Best Practices on handling cells and Technical Note Guidelines on Accurate Target Cell Counts (Document CG000091) for determining accurate cell counts.

Cells carry potentially hazardous pathogens. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage and disposal of biological materials.

Specific Reagents & Consumables

Vendor	Item	Part Number
10x Genomics	3' CellPlex Kit Set A	1000261
Thermo Fisher Scientific	UltraPure Bovine Serum Albumin (BSA, 50 mg/ml)	AM2616
	Trypan Blue Stain (0.4%)	T10282
	Fetal Bovine Serum (FBS), qualified, heat inactivated	16140071

Vendor	Item	Part Number
Corning	Phosphate-Buffered Saline, 1X without Calcium and Magnesium	21-040-CV
Millipore Sigma	Bovine Serum Albumin In DPBS (10%) (alternative to Thermo Fisher product)	A1595
	Protector RNase Inhibitor	3335399001
BioLegend	Human TruStain FcX (Fc Receptor Blocking Solution)	422301
	TotalSeq™ Antibody-Oligonucleotide Conjugates (TotalSeq™-B)	-
BrainBits	NbActiv-1 Neuronal culturing medium	NbActive1 100
VWR	Premium Grade Fetal Bovine Serum (FBS) (alternative to Thermo Fisher product)	97068-085
	Disposable Transfer Pipets, 5 ml, extended fine tip, large bulb	414004-042
Equipment		
Eppendorf	DNA LoBind Tubes 2.0 ml	022431048
Thermo Fisher Scientific	Countess II FL Automated Cell Counter	AMAQAF1000
	Countess II FL Automated Cell Counting Chamber Slides	C10228

This list may not include some standard laboratory equipment.

Preparation - Buffers

Buffers
Prepare fresh, maintain at 4°C
Wash & Resuspension Buffers for Cells*
PBS + 1% BSA <i>(for PBMCs, cell lines, and dissociated tumor cells)</i>
PBS + 10% FBS <i>(for samples with <80% viable cells)</i>
NbActiv-1 + 1% BSA <i>(for dissociated brain tissues)</i>
Wash & Resuspension Buffers for Nuclei
PBS + 1% BSA + RNase Inhibitor (0.2 U/μl)
Additional Buffers
PBS + 0.04% BSA <i>(maintain at room temperature or 4°C; see labeling protocols for details)</i>

**Wash & resuspension buffers depend upon the sample type. Use appropriate buffer.*

Tips & Best Practices

The following recommendations are critical for optimal performance of the Single Cell 3' Cell Multiplexing assay. Failure to adhere to these guidelines may lead to poor signal to noise ratios in Cell Multiplexing data, which may prevent accurate CMO tag assignment.

Sample Preparation Guidelines

- Input samples in this protocol were prepared by using following Demonstrated Protocols:

Sample Type	Demonstrated Protocol
PBMCs	Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing (Document CG00039)
Cell Lines	Single Cell Suspensions from Cultured Cell Lines for Single Cell RNA Sequencing (Document CG000054)
Dissociated Tumor Cells	Thawing Dissociated Tumor Cells for Single Cell RNA Sequencing (Document CG000233)
Dissociated Brain Tissue	Dissociation of Mouse Embryonic Neural Tissue for Single Cell RNA Sequencing (Document CG00055)
Nuclei	Isolation of Nuclei for Single Cell RNA Sequencing (Document CG000124)

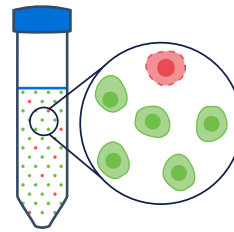


Nuclei isolated using alternative nuclei isolation protocols and nuclei isolated from flash frozen tissues may not be compatible with CMO labeling.

Sample Viability & Appropriate Labeling Protocol

- For labeling nuclei isolated from fresh cells or tissues, follow Protocol 1.
- For labeling cells, determine cell viability before labeling. Cell washing instructions & wash resuspension buffers for the labeled cells depend upon the starting viability. Choose appropriate labeling protocol for the sample.

Samples with >80% viable cells



E.g., Cell lines, PBMCs, and other less fragile cells

Follow appropriate protocol

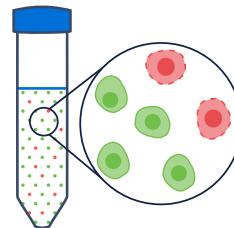
***Protocol 1: Cell Multiplexing Oligo Labeling for Samples with >80% Viable Cells**

OR

***Protocol 2: Cell Surface Protein & Cell Multiplexing for Samples with >80% Viable Cells**

***Three washes after CMO labeling**

Samples with <80% viable cells



E.g., Tumor cells and other more fragile cells

Follow appropriate protocol

****Protocol 3: Cell Multiplexing Oligo Labeling for Samples with <80% Viable Cells**

OR

****Protocol 4: Cell Surface Protein & Cell Multiplexing Oligo Labeling for Samples with <80% Viable Cells**

****One wash after CMO labeling + FACS enrichment of viable cells**

Sample Quality

- Use high-quality single cell or nuclei suspensions that can withstand the CMO labeling and washing steps.
- Perform pilot experiments to determine if the sample type is suitable for the 3' CellPlex assay.
- Use single cell suspensions with >80% (ideally >90%) viability. If one or more samples in the

pool has lower viability, follow protocol 3 or 4 to perform fluorescence activated cell sorting (FACS) after CMO labeling to increase viability (Figure 1).

- Cell Multiplexing data can be severely impacted by low cell viability even if the single-cell behavior of the gene expression data is only mildly impacted (Figure 1).



Figure 1. Histogram of CMO counts and gene expression barcode rank plot for samples with and without FACS enrichment of viable cells.

CMO tags

- Up to 12 CMO labeled samples can be pooled per well on a 10x Genomics chip. Ensure that a different CMO tag is used for each sample in the pool.
- For optimal multiplet detection and optimal signal-to-noise ratios, pool samples at 1:1 ratios after CMO labeling and use no more than ~2,500 cells/nuclei per CMO tag.
- To increase the number of CMO tags per sample, split a sample into aliquots and label each aliquot separately with a different CMO tag. DO NOT tag one aliquot simultaneously with multiple CMO tags.
- DO NOT pool CMO-labeled samples with unlabeled samples.

Nuclei Isolation Protocols

- The 3' CellPlex assay has ONLY been validated on nuclei isolated from fresh cells or tissues using Demonstrated Protocol CG000124 - Isolation of Nuclei for Single Cell RNA Sequencing.
- Nuclei isolated from frozen tissues, or nuclei isolated using alternative isolation protocols, have not been tested for compatibility with Cell Multiplexing assay.
- Prolonged nuclei lysis can damage nuclei, impacting Cell Multiplexing data.
- Perform pilot experiments to assess nuclei quality after CMO labeling and washing.
- If nuclei clumping or low nuclei recovery after CMO labeling are observed, optimize upstream nuclei isolation protocols (eg. lysis time, detergent concentration).

Centrifugation & Pellet Resuspension

- Use of a swinging-bucket rotor is recommended for higher cell/nuclei recovery.
- After each centrifugation step, gently mix cells/nuclei 10-15x, or until the pellet is completely resuspended, without introducing bubbles.
- To facilitate pellet resuspension in 2 ml volumes of Wash & Resuspension Buffer, add 1 ml buffer first, pipette mix to resuspend the pellet, add the remaining 1 ml buffer, then pipette mix again.

Number of Input cells/nuclei

- This protocol is only supported for cell/nuclei inputs of at least 0.1×10^6 per sample. If the sample quantity is not limited, $0.5-2 \times 10^6$ per sample should be used.
- Using too few cells/nuclei as input may result in:
 - High background in the Cell Multiplexing data caused by incomplete removal of supernatant after each wash step due to poor visibility of the cell/nuclei pellet
 - Insufficient cells/nuclei remaining for accurate counting and pooling (loss of 30-70% of cells/nuclei is expected after CMO labeling and washing).

Wash & Resuspension Buffer

- Refer to Table 2: Sample Type Specific Wash & Resuspension Buffers for recommended buffers.
- When working with a cell type that does not have optimal viability in PBS + 1% BSA, replace PBS with most types of cell culture media and/or replace 1% BSA with 10% serum, to maximize viability.
- If BSA is used, do not use less than 1% BSA as this may lead to increased background in Cell Multiplexing data.

Debris

- Input single cell/nuclei suspensions should be free from debris.
- Samples with minor debris may be used if FACS is performed to remove debris after CMO labeling.
- Samples with excessive debris are not recommended as debris can contribute to high background in cell multiplexing data.

Wash Steps & Supernatant Removal

- Thorough washing of cells post labeling is critical to obtain high-quality data and to remove background from unbound CMOs.
- After each wash step, remove the supernatant (no more than 10 µl remaining) without touching the bottom of the tube to avoid dislodging the cell/nuclei pellet.
- Leaving behind excess supernatant may decrease the separation between signal and noise and may

lead to a large decrease in the fraction of CMO reads usable (Figure 2).

- 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.
- Transfer pipettes may be used to fully remove the supernatant to minimize disturbance to the pellet.

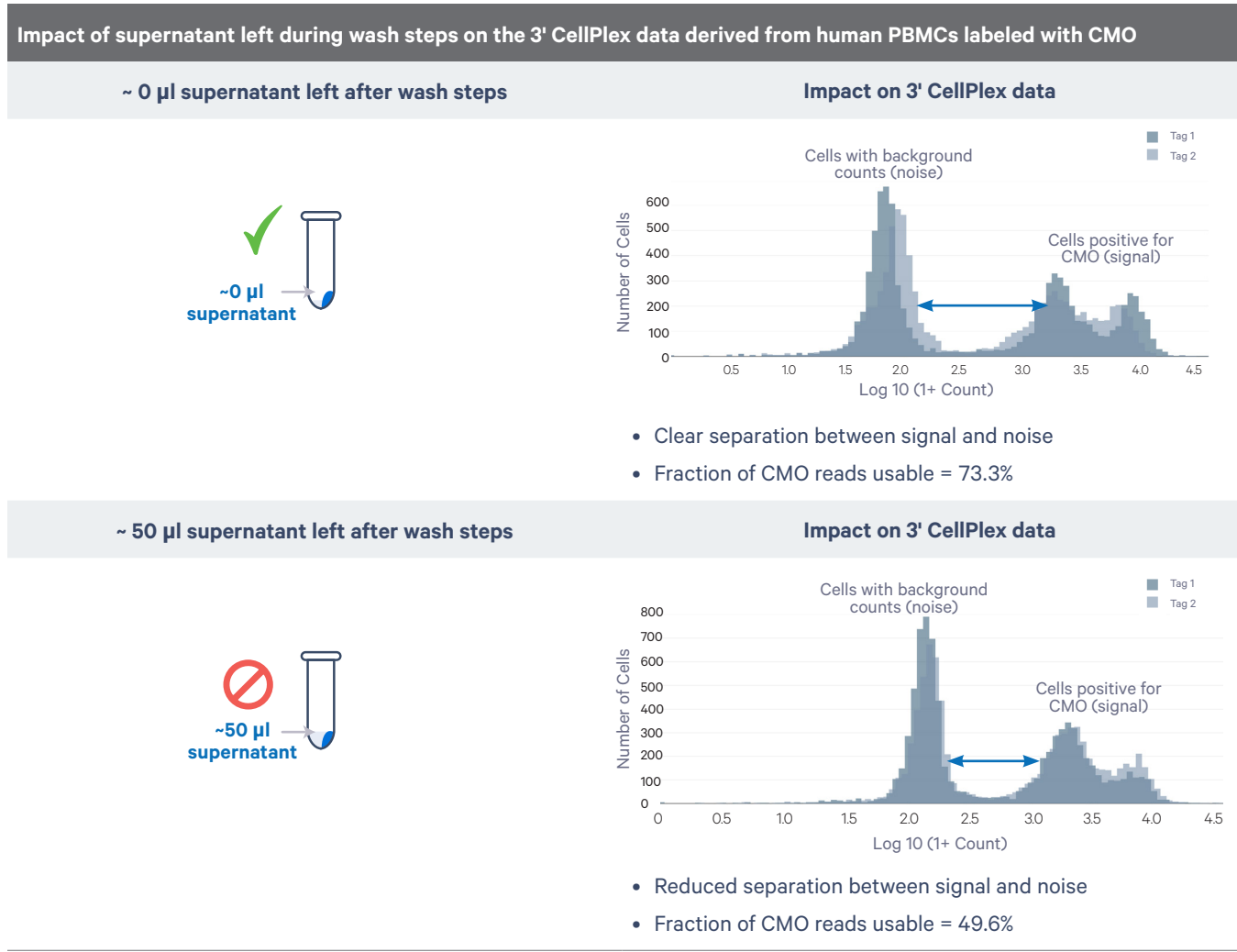


Figure 2. Histogram of CMO counts for cells with 0 µl and 50 µl supernatant left behind.

Time After Labeling

- Work efficiently and avoid letting cells/nuclei sit for extended periods, as this may decrease sample quality and increase background noise in CellPlex data.
- Pool cells/nuclei within 30 min of CMO labeling and washing. After pooling, immediately load cells/nuclei onto the 10x Genomics chip.
- Keep cells/nuclei on ice or 4°C at all times after CMO labeling.
- Use chilled Wash & Resuspension Buffers and perform centrifugation steps at 4°C.
- DO NOT let CMO labeled cells/nuclei sit at room temperature, as this may increase background noise in CellPlex data (Figure 3).
- If cells are left for longer periods of time, either post labeling or post pooling, the Cell Multiplexing data can be severely impacted even if the single-cell behavior of the Gene Expression data is only mildly impacted.

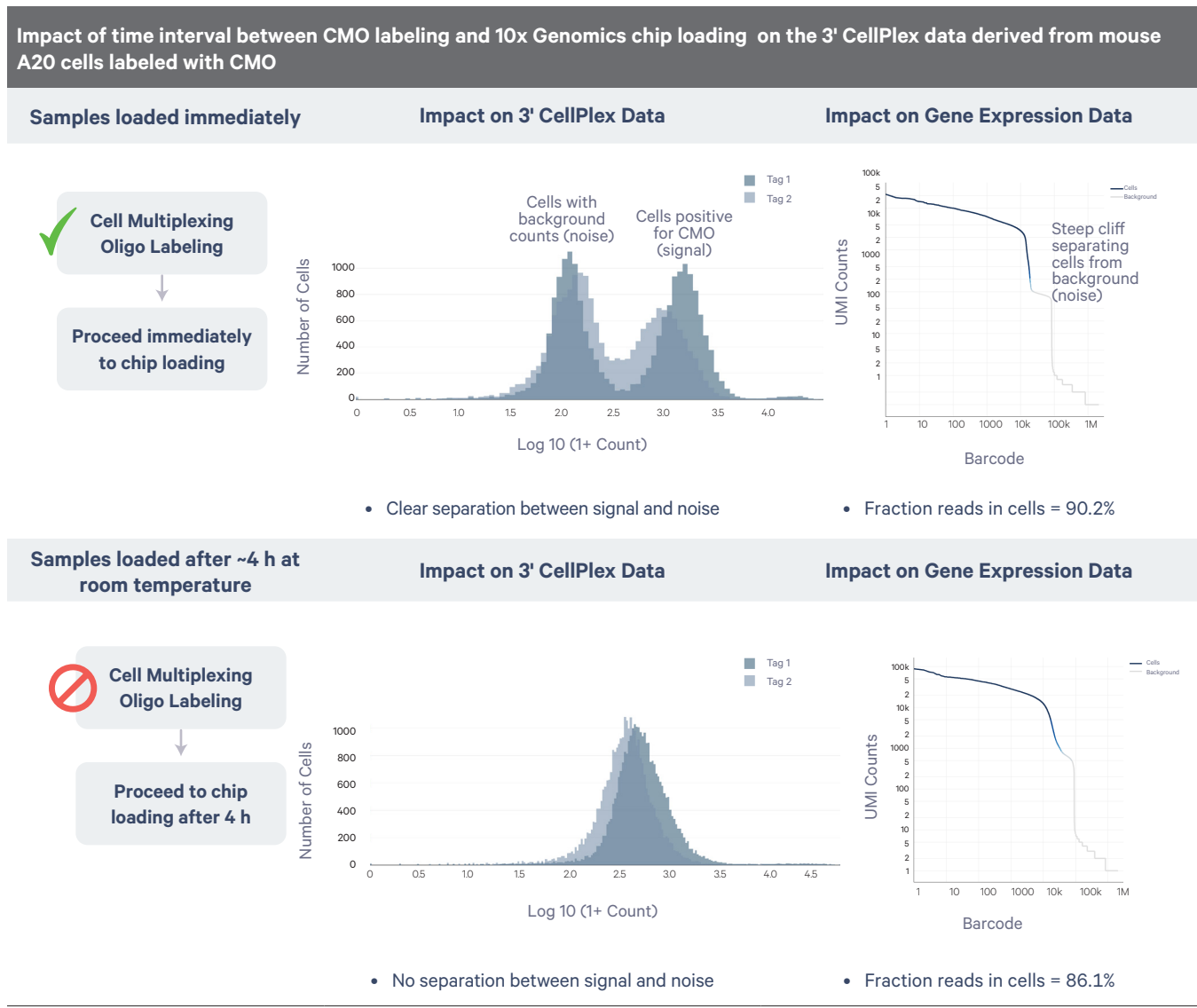
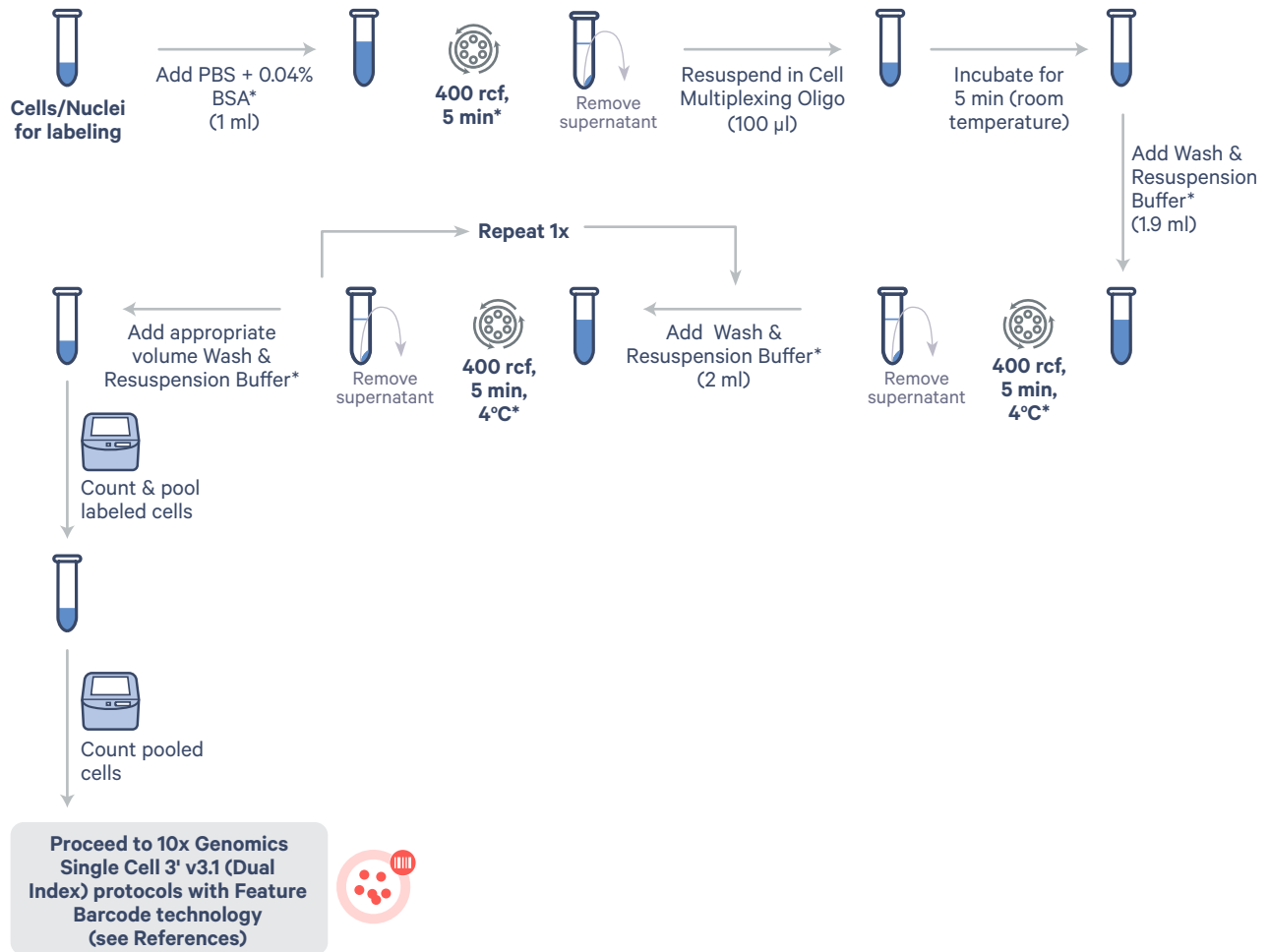


Figure 3. Effect of time before loading on 3' CellPlex data shown in the histogram of CMO Counts and gene expression barcode rank plot.

Protocol 1 Overview : Cell Multiplexing Oligo Labeling

for Samples with >80% Viable Cells & for Nuclei Isolated from Fresh Cells/Tissues

Label Cells/Nuclei



*Centrifugation conditions and buffer composition depend upon the sample type. See Labeling Protocol for details.

Protocol 1: Cell Multiplexing Oligo Labeling

for Samples with >80% Viable Cells & for Nuclei Isolated from Fresh Cells/Tissues

3' CellPlex Kit Set A (PN-1000261) was used for cell/nuclei multiplexing. Cell Multiplexing Oligos are supplied at -20°C.

Prepare Cell Multiplexing Oligo

Before use, thaw Cell Multiplexing Oligo at room temperature. Vortex 5 sec at maximum speed and centrifuge briefly for 5 sec.

Cells

This protocol was demonstrated using 0.1-2 x 10⁶ cells. Use 0.5-2 x 10⁶ cells, if the number of cells is not limited. Wash cells according to the appropriate 10x Genomics Demonstrated Protocol for the cell type being prepared. See Tips & Best Practices.

Nuclei

This protocol was demonstrated using 0.1-2 x 10⁶ nuclei. Use 0.5-2 x 10⁶ nuclei, if the number of nuclei is not limited. Wash nuclei according to the appropriate 10x Genomics Demonstrated Protocol. See Tips & Best Practices.

- a. Transfer cells/nuclei to a 2-ml microcentrifuge tube and add appropriate buffer for a total 1 ml volume and gently pipette mix.
 - For cells, add room temperature PBS + 0.04% BSA.
 - For nuclei, add chilled PBS + 1% BSA + RNase Inhibitor (0.2 U/μl).
- b. Centrifuge cells/nuclei. Centrifuge cells at **room temperature** and nuclei at **4°C**. Use of swinging-bucket rotor is recommended for higher cell recovery. Centrifugation speed and time depends upon the sample type. Use following table for guidance.

Table 1. Sample Type Specific Centrifugation Conditions

Sample Types	Speed (rcf)	Time (min)
Cell Lines	300	5
PBMCs/Dissociated Brain Tissue	400	5
Dissociated Tumor Cells	150	10
Nuclei	500	10

- c. Remove the supernatant without disturbing the pellet. Transfer pipettes may be used to remove supernatant after centrifugation to minimize disturbance to the pellet.
- d. Add **100 μl** Cell Multiplexing Oligo (room temperature) to the cells/nuclei. Gently pipette mix 10-15x to resuspend. Store unused Cell Multiplexing Oligo at -20°C and avoid more than 12 freeze-thaw cycles.
- e. Incubate for **5 min** at **room temperature**. When working with multiple samples, start the 5 min incubation after the last sample has been resuspended in Cell Multiplexing Oligo.



After labeling samples with the CMO, ensure that samples are kept on ice/chilled/4°C at all times.

- f. **Wash 1**
Wash by adding **1.9 ml** appropriate chilled Wash & Resuspension Buffer (for a total 2 ml volume) to the sample. Gently pipette mix.

Table 2. Sample Type Specific Wash & Resuspension Buffers

Sample Types	Wash & Resuspension Buffer (maintain at 4°C)
PBMCs/Cell Lines/ Dissociated Tumor Cells	PBS + 1% BSA
Dissociated Brain Tissue	NbActiv-1 + 1% BSA
Nuclei	PBS + 1% BSA + RNase Inhibitor (0.2 U/μl)

- g. Centrifuge cells/nuclei at **4°C**. Centrifugation speed and time depends upon the sample type. See Table 1 for guidance.

- h.** Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet.



DO NOT leave >10 µl supernatant behind.

Leaving behind excess supernatant may cause non-specific binding, which may result in increased background reads during sequencing.



- q.** Proceed immediately to relevant Chromium Single Cell RNA Sequencing protocols with Feature Barcode technology for Cell Multiplexing (see References).

Fluorescence Activated Cell Sorting (FACS) can be used to sort out live cells by staining with a live/dead cell staining or nuclei by forward or side scatter to remove debris.

Cell Multiplexing Oligo labeling is compatible with FACS at either the pre or post pooling of labeled cells.

- i. Wash 2**

Resuspend the pellet in **2 ml** chilled Wash & Resuspension Buffer. Gently pipette mix.

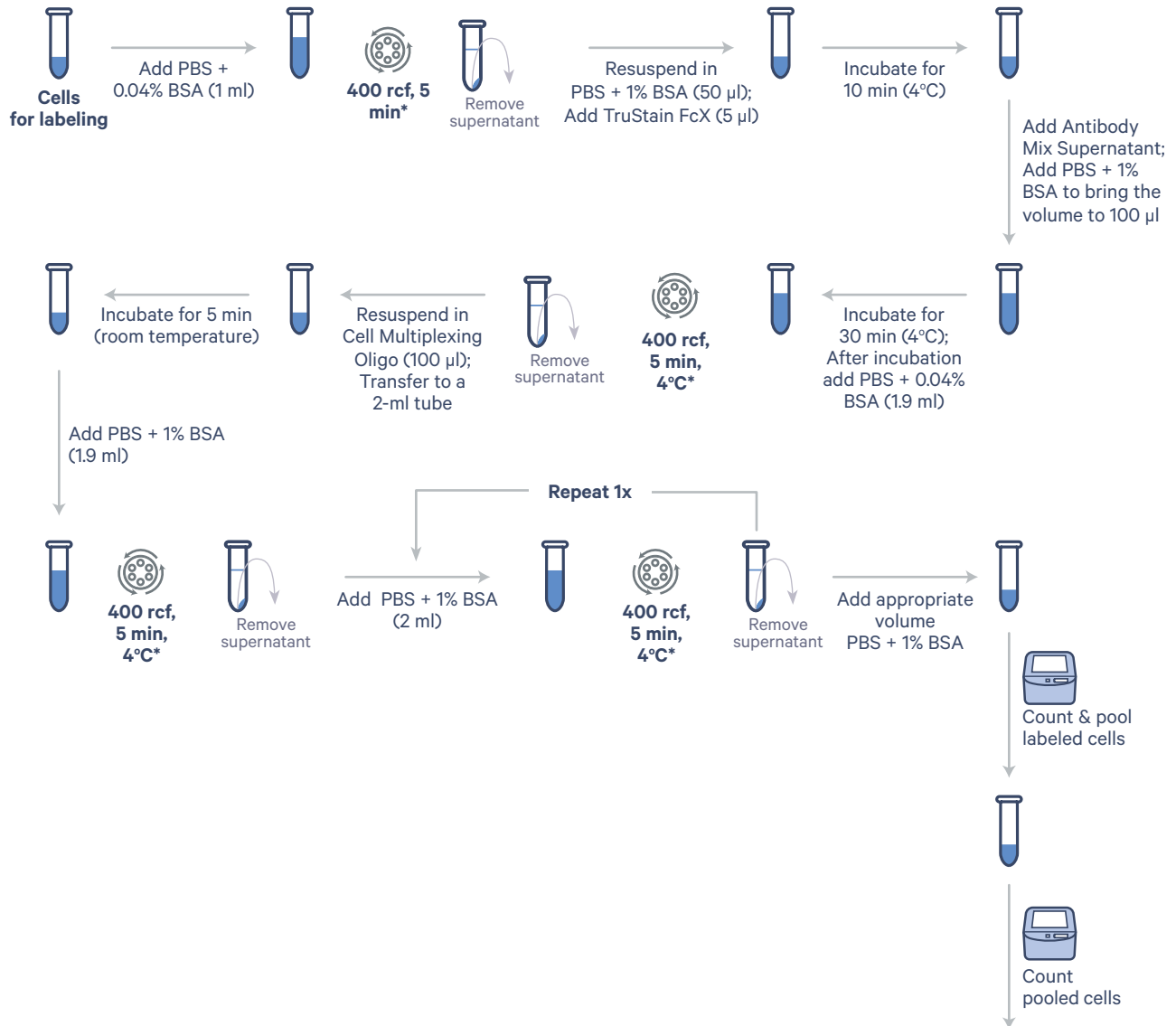
- j.** Centrifuge at **4°C**. Centrifugation speed and time depends upon the sample type. See Table 1 for guidance.
- k.** Remove the supernatant without disturbing the pellet.
- l. Wash 3**
Repeat i-k for a total of three washes.
- m.** Based on starting concentration and assuming ~50% cell loss, add an appropriate volume chilled Wash & Resuspension buffer. The final concentration depends up on the targeted cell recovery and the assay type performed. Gently pipette mix 10-15x.

Assay Type	Targeted Cell Recovery	Final Concentration
3' v3.1 Assay	500-10,000 cells	700-1,200 cells/µl
	10,000-30,000 cells	1,300-1,600 cells/µl
3' HT v3.1 assay	2,000-20,000 cells	700-1,200 cells/µl
	20,000-60,000 cells	1,300-1,600 cells/µl

- n.** Determine cell/nuclei concentration and viability using a Countess II Automated Cell Counter or a hemocytometer.
- o.** Pool labeled cells/nuclei using the calculation provided in the Appendix.
- p.** Determine cell concentration and viability of the pooled sample using a Countess II Automated Cell Counter or a hemocytometer.

Protocol 2 Overview : Cell Surface Protein & Cell Multiplexing Oligo Labeling for Samples with >80% Viable Cells

Label Cells



*Centrifugation conditions depend upon the sample type. See Labeling Protocol for details.



Proceed to 10x Genomics Single Cell 3' v3.1 (Dual Index) protocols with Feature Barcode technology (see References)

Protocol 2: Cell Surface Protein & Cell Multiplexing Oligo Labeling

for Samples with >80% Viable Cells

This protocol was optimized using TotalSeq-B™ antibody-oligonucleotide conjugates from BioLegend and 3' CellPlex Kit from 10x Genomics.

For guidance on custom antibody-oligonucleotide conjugation, refer to Demonstrated Protocol Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (Document CG000149).

All steps can be performed in 2-ml microcentrifuge tubes.

3' CellPlex Kit Set A (PN-1000261) was used for cell multiplexing. Cell Multiplexing Oligos are supplied at -20°C.

Prepare Cell Multiplexing Oligo

Before use, thaw Cell Multiplexing Oligo at room temperature. Vortex 5 sec at maximum speed and centrifuge briefly for 5 sec.

Prepare Antibody Mix Supernatant

Add appropriate/manufacturer's recommended amount of antibody-oligonucleotide conjugates to a 1.5-ml microcentrifuge tube. Centrifuge the mix at 14,000 rcf for 10 min at 4°C. Transfer the supernatant (containing Antibody Mix) to a new tube and maintain at 4°C.

Cells

This protocol was demonstrated using 0.2-2 x 10⁶ PBMCs. Use 0.5-2 x 10⁶ cells, if the number of cells is not limited. Wash cells according to the appropriate 10x Genomics Demonstrated Protocol for the cell type being prepared.

- a. Transfer cells to a 2-ml microcentrifuge tube and add chilled PBS + 0.04% BSA for a total **1 ml** volume.

- b. Centrifuge cells at **4°C**. Use of swinging-bucket rotor is recommended for higher cell recovery. Centrifugation speed and time depends upon the sample type. Use following table for guidance.

Table 3. Sample Type Specific Centrifugation Conditions

Sample Types	Speed (rcf)	Time (min)
Cell Lines	300	5
PBMCs	400	5
Dissociated Tumor Cells	150	10

- c. Remove the supernatant without disturbing the pellet.
- d. Resuspend cell pellet in **50 µl** chilled PBS + 1% BSA.
- e. Add **5 µl** Human TruStain FcX. Gently pipette mix.
- f. Incubate for **10 min** at **4°C**.
- g. Add the prepared Antibody Mix supernatant.
- h. Add chilled PBS + 1% BSA to the cells to bring the total volume to 100 µl. Gently pipette mix 10x (pipette set to 90 µl).
- i. Incubate for **30 min** at 4°C. Recommended incubation temperature for most sample types is 4°C. However, incubation temperature is sample type dependent and should be chosen accordingly.
- j. Wash by adding **1.9 ml** chilled PBS + 0.04% BSA (for a total volume of 2.0 ml).
- k. Centrifuge at **4°C**. Larger or fragile cell types may require slower centrifugation speeds. See Table 3 for guidance.
- l. Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet.




DO NOT leave >10 µl supernatant behind.

Leaving behind excess supernatant may cause non-specific binding, which may result in increased background reads during sequencing.



- m.** Add **100 µl** Cell Multiplexing Oligo (room temperature) to the cells. Gently pipette mix 10-15x to resuspend and transfer to a new 2-ml microcentrifuge tube. After use, Cell Multiplexing Oligo can be stored at -20°C.
- n.** Incubate for 5 min at room temperature. When working with multiple samples, start the 5 min incubation after the last sample has been resuspended in Cell Multiplexing Oligo.

 After labeling samples with the CMO, ensure that samples are kept on ice/chilled/4°C at all times.

- o. Wash 1** Wash by adding **1.9 ml** chilled PBS + 1% BSA (for a total 2 ml volume) to the sample.
- p.** Centrifuge at **4°C**. Centrifugation speed and time depends upon the sample type. See Table 3 for guidance.
- q.** Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet.
- r. Wash 2** Wash by adding **2.0 ml** chilled PBS + 1% BSA to the sample.
- s.** Centrifuge at **4°C**. Centrifugation speed and time depends upon the sample type. See Table 3 for guidance.
- t.** Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet.
- u. Wash 3** Repeat r-t for a total of three washes.
- v.** Based on starting concentration and assuming ~50% cell loss, add an appropriate volume chilled PBS + 1% BSA. The final concentration depends up on the targeted cell recovery and the assay type performed.

Assay Type	Targeted Cell Recovery	Final Concentration
3' v3.1 Assay	500-10,000 cells	700-1,200 cells/µl
	10,000-30,000 cells	1,300-1,600 cells/µl
3' HT v3.1 assay	2,000-20,000 cells	700-1,200 cells/µl
	20,000-60,000 cells	1,300-1,600 cells/µl

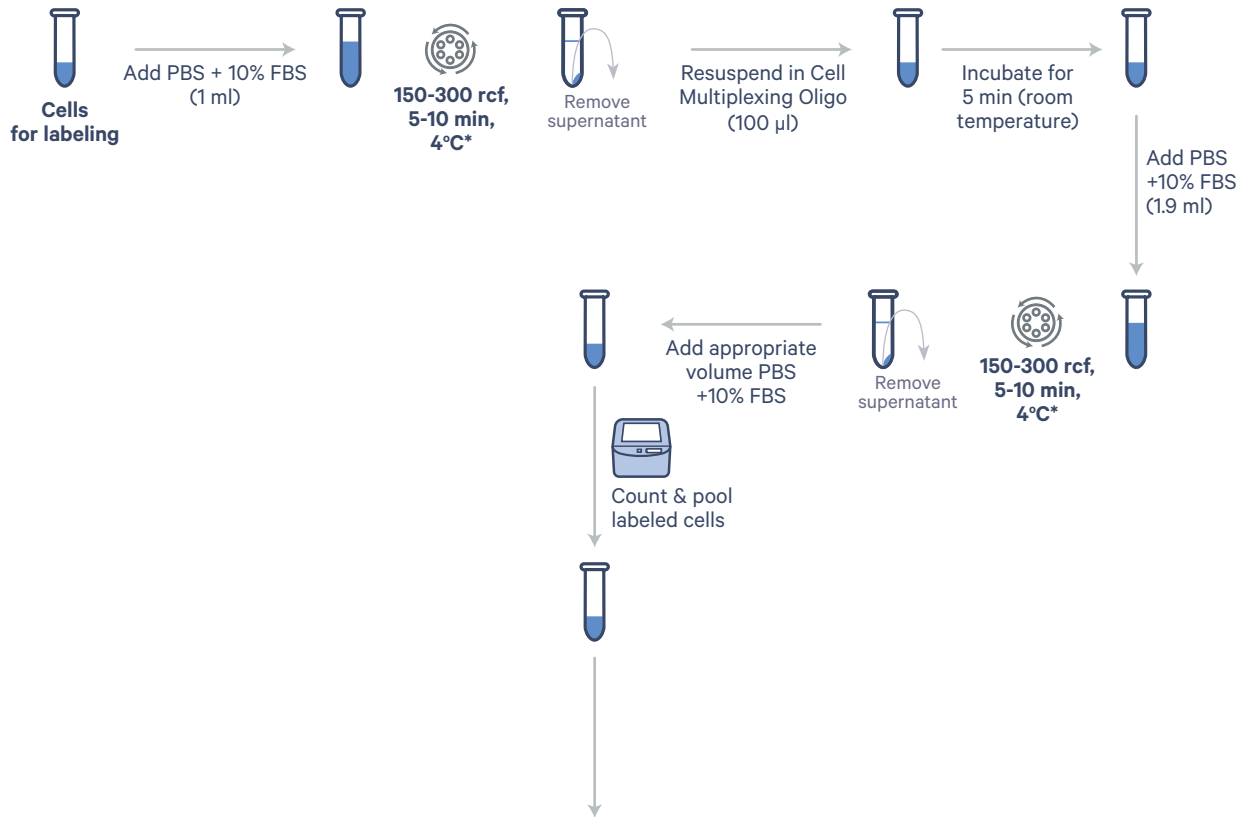
Gently pipette mix.

- x.** Determine cell concentration and viability of the sample using a Countess II Automated Cell Counter or a hemocytometer
- y.** Pool labeled cells using the calculation provided in the Appendix.
- z.** Determine cell concentration and viability of the pooled sample using a Countess II Automated Cell Counter or a hemocytometer.
- aa.** Proceed immediately to Chromium Single Cell RNA Sequencing protocols with Feature Barcode technology for Cell Surface Protein & Cell Multiplexing.
Fluorescence Activated Cell Sorting (FACS) can be used to sort out live cells by staining with a live/dead stain to remove debris. Cell Multiplexing Oligo labeling is compatible with FACS at either the pre or post pooling of labeled cells.

Protocol 3 Overview : Cell Multiplexing Oligo Labeling

for Samples with <80% Viable Cells

Label Cells



Enrichment of Viable Cells by FACS

- Enrich viable cells by FACS
- Centrifuge sorted cells at 150-300 rcf for 10 min
- Add appropriate volume PBS + 10% FBS

Count cells

Proceed to 10x Genomics Single Cell 3' v3.1 (Dual Index) protocols with Feature Barcode technology (see References)

*Centrifugation conditions depend upon the sample type. See Labeling Protocol for details.

Protocol 3: Cell Multiplexing Oligo Labeling

for Samples with <80% Viable Cells

This protocol describes Cell Multiplexing Oligo labeling samples that contain <80% viable cells.

3' CellPlex Kit Set A (PN-1000261) was used for cell-multiplexing. Cell Multiplexing Oligos are supplied at -20°C.

Prepare Cell Multiplexing Oligo

Before use, thaw Cell Multiplexing Oligo at room temperature. Vortex 5 sec at maximum speed and centrifuge briefly for 5 sec.

Cells

This protocol was demonstrated using cell suspensions containing 0.2-2 x 10⁶ viable cells. Cell viability was determined using a Countess II Automated Cell Counter or a hemocytometer. Use 0.5-2 x 10⁶ viable cells if the number of cells is not limited.

- a. Transfer cells to a 2-ml microcentrifuge tube and add chilled PBS +10% FBS for a total 1 ml volume and gently pipette mix.
- b. Centrifuge cells at **150-300 rcf** for **5-10 min** at **4°C**. Centrifugation speed and time depends on the sample type. Larger or fragile cells may require slower centrifugation speeds. Use of a swinging bucket rotor is recommended for higher cell recovery
- c. Remove the supernatant without disturbing the pellet. Transfer pipettes may be used to remove supernatant after centrifugation to minimize disturbance to the pellet.
- d. Add **100 µl** Cell Multiplexing Oligo (room temperature) to the cells. Gently pipette mix 10-15x to resuspend. Store unused Cell Multiplexing Oligo at -20°C and avoid more than 12 freeze-thaw cycles.

- e. Incubate for **5 min** at **room temperature**. When working with multiple samples, start the 5 min incubation after the last sample has been resuspended in Cell Multiplexing Oligo.



After labeling samples with the CMO, ensure that samples are kept on ice/chilled/4°C at all times.

- f. **Wash 1**
Wash by adding **1.9 ml** chilled PBS + 10% FBS (for a total 2 ml volume) to the sample. Gently pipette mix.
- g. Centrifuge cells at **150-300 rcf** for **5-10 min** at **4°C**.
- h. Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet.



DO NOT leave >10 µl supernatant behind.

Leaving behind excess supernatant may cause non-specific binding, which may result in increased background reads during sequencing.



- i. **Enrichment of viable cells by FACS**
 - Based on starting concentration and assuming ~10-20% cell loss, add an appropriate volume chilled PBS + 10% FBS to obtain a final concentration of 5-10 x 10⁶ cells/ml.
 - Determine cell concentration and viability using a Countess II Automated Cell Counter or a hemocytometer.
 - Pool labeled cells based on the viable cell concentration and add a dead cell marker.
 - Proceed to FACS (see FACS Guidance).
- j. After FACS, centrifuge cells at **150-300 rcf** for **10 min** at **4°C**. Use of a longer centrifugation time (10 min) is recommended post FACS.
- k. Remove the supernatant without disturbing the pellet.

- l.** Add an appropriate volume of chilled PBS + 10% FBS. The final concentration depends up on the targeted cell recovery and the assay type performed.

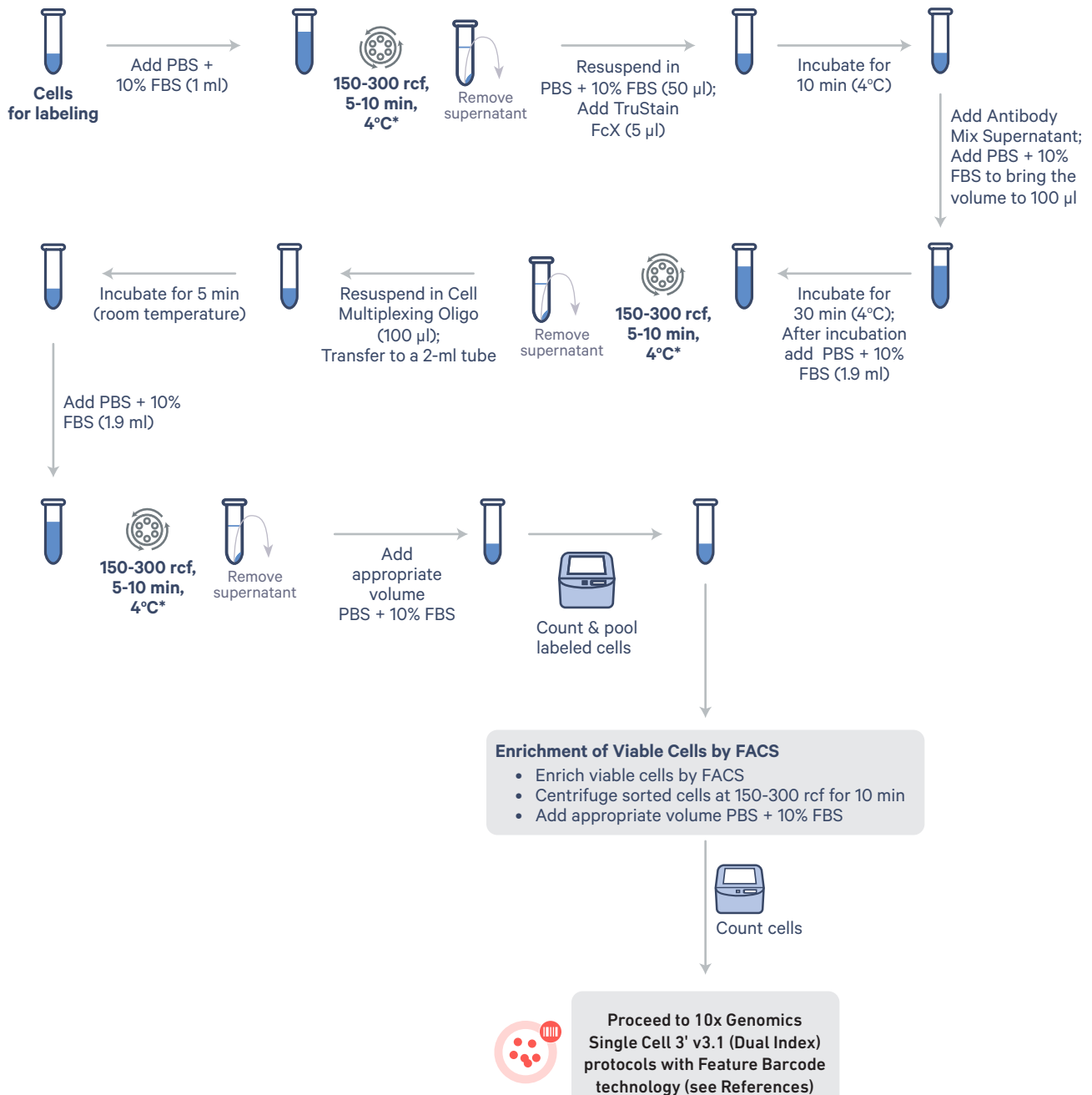
Assay Type	Targeted Cell Recovery	Final Concentration
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	10,000-30,000 cells	1,300-1,600 cells/ μ l
3' HT v3.1 assay	2,000-20,000 cells	700-1,200 cells/ μ l
	20,000-60,000 cells	1,300-1,600 cells/ μ l

Gently pipette mix 10-15x.

- n.** Determine cell concentration and viability using a Countess II Automated Cell Counter or a hemocytometer.
- o.** Proceed immediately to relevant Chromium Single Cell RNA Sequencing protocols with Feature Barcode technology for Cell Multiplexing (see References).

Protocol 4 Overview : Cell Surface Protein & Cell Multiplexing Oligo Labeling for Samples with <80% Viable Cells

Label Cells



*Centrifugation conditions depend upon the sample type. See Labeling Protocol for details.

Protocol 4: Cell Surface Protein & Cell Multiplexing Oligo Labeling

for Samples with <80% Viable Cells

This protocol was optimized using TotalSeq-B™ antibody-oligonucleotide conjugates from BioLegend and 3' CellPlex Kit from 10x Genomics.

For guidance on custom antibody-oligonucleotide conjugation, refer to Demonstrated Protocol Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (Document CG000149).

All steps can be performed in 2-ml microcentrifuge tubes.

3' CellPlex Kit Set A (PN-1000261) was used for cell multiplexing. Cell Multiplexing Oligos are supplied at -20°C.

Prepare Cell Multiplexing Oligo

Before use, thaw Cell Multiplexing Oligo at room temperature. Vortex 5 sec at maximum speed and centrifuge briefly for 5 sec.

Prepare Antibody Mix Supernatant

Add appropriate/manufacturer's recommended amount of antibody-oligonucleotide conjugates to a 1.5-ml microcentrifuge tube. Centrifuge the mix at 14,000 rcf for 10 min at 4°C. Transfer the supernatant (containing Antibody Mix) to a new tube and maintain at 4°C.

Cells

This protocol was demonstrated using cell suspensions containing 0.2-2 x 10⁶ viable cells. Cell viability was determined using a Countess II Automated Cell Counter or a hemocytometer. Use 0.5-2 x 10⁶ viable cells if the number of cells is not limited. Wash cells according to the appropriate 10x Genomics Demonstrated Protocol for the cell type being prepared.

- a. Transfer cells to a 2-ml microcentrifuge tube and add chilled PBS + 10% FBS for a total 1 ml volume.
- b. Centrifuge cells **150-300 rcf** for **5-10 min** at **4°C**. Use of swinging-bucket rotor is recommended for higher cell recovery. Centrifugation speed and time depends upon the sample type. Larger or fragile cells may require slower centrifugation speeds.
- c. Remove the supernatant without disturbing the pellet.
- d. Resuspend cell pellet in **50 µl** chilled PBS + 10% FBS.
- e. Add **5 µl** Human TruStain FcX. Gently pipette mix.
- f. Incubate for **10 min** at **4°C**.
- g. Add the prepared Antibody Mix supernatant.
- h. Add chilled PBS + 10% FBS to the cells to bring the total volume to 100 µl. Gently pipette mix 10x (pipette set to 90 µl).
- i. Incubate for **30 min** at **4°C**. Recommended incubation temperature for most sample types is 4°C. However, incubation temperature is sample type dependent and should be chosen accordingly.
- j. Wash by adding **1.9 ml** chilled PBS + 10% FBS (for a total volume of 2.0 ml).
- k. Centrifuge at **150-300 rcf** for **5-10 min** at **4°C**. Larger or fragile cell types may require slower centrifugation speeds.
- l. Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet.



DO NOT leave >10 µl supernatant behind.



Leaving behind excess supernatant may cause non-specific binding, which may result in increased background reads during sequencing.

m. Add **100 µl** Cell Multiplexing Oligo (room temperature) to the cells. Gently pipette mix 10-15x to resuspend and transfer to a new 2-ml microcentrifuge tube. After use, Cell Multiplexing Oligo can be stored at -20°C.

n. Incubate for **5 min** at **room temperature**. When working with multiple samples, start the 5 min incubation after the last sample has been resuspended in Cell Multiplexing Oligo.



After labeling samples with the CMO, ensure that samples are kept on ice/chilled/4°C at all times.

o. Wash 1 Wash by adding **1.9 ml** chilled PBS + 10% FBS (for a total 2 ml volume) to the sample.

p. Centrifuge at **150-300 rcf** for **5-10 min** at **4°C**. Centrifugation speed and time depends upon the sample type.

q. Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet.

r. Enrichment of viable cells by FACS

- Based on starting concentration and assuming ~10-20% cell loss, add an appropriate volume chilled PBS + 10% FBS to obtain a final concentration of 5-10 x 10⁶ cells/ml.
 - Determine cell concentration and viability using a Countess II Automated Cell Counter or a hemocytometer.
 - Pool labeled cells based on the viable cell concentration and add a dead cell marker.
 - Proceed to FACS (see FACS Guidance).
- s.** After FACS, centrifuge cells at **150-300 rcf** for **10 min** at **4°C**. Use of a longer centrifugation time (10 min) is recommended post FACS.
- t.** Remove the supernatant without disturbing the pellet.
- u.** Add an appropriate volume chilled PBS + 10% FBS. The final concentration depends up on the targeted cell recovery and the assay type performed.

Assay Type	Targeted Cell Recovery	Final Concentration
3' v3.1 Assay	500-10,000 cells	700-1,200 cells/µl
	10,000-30,000 cells	1,300-1,600 cells/µl
3' HT v3.1 assay	2,000-20,000 cells	700-1,200 cells/µl
	20,000-60,000 cells	1,300-1,600 cells/µl

Gently pipette mix 10-15x.

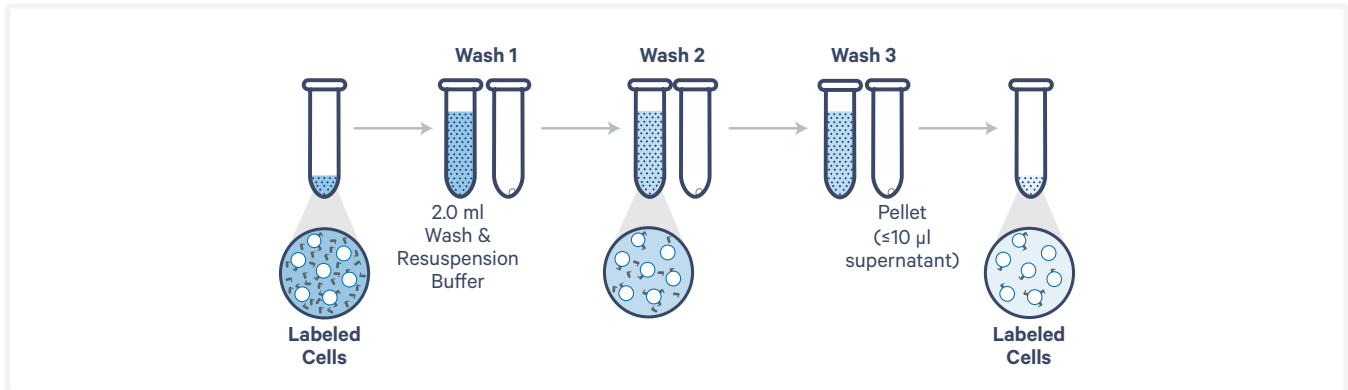
- w.** Determine cell concentration and viability of the sample using a Countess II Automated Cell Counter or a hemocytometer
- x.** Proceed immediately to Chromium Single Cell RNA Sequencing protocols with Feature Barcode technology for Cell Surface Protein & Cell Multiplexing.

Appendix

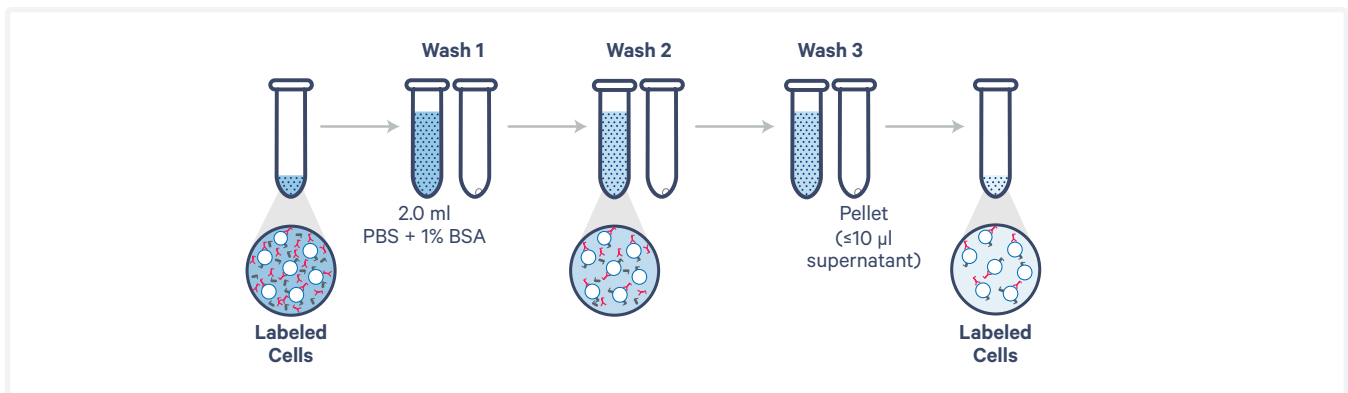
Cell Washing Overview

To eliminate non-specific binding with comparable efficiency, wash steps should be performed in 2-ml microcentrifuge tubes using indicated buffer volumes. Non-specific binding contributes to increased background reads during sequencing.

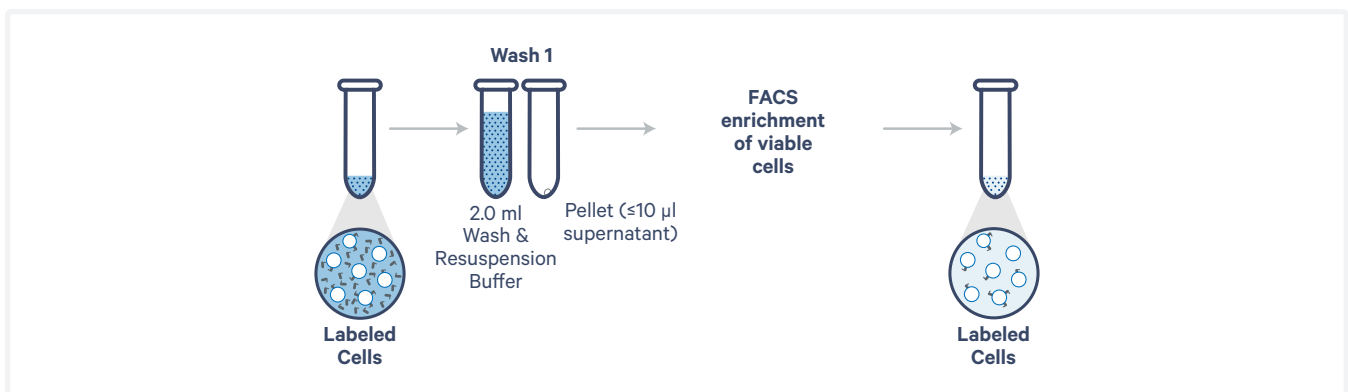
Cells labeled with Cell Multiplexing Oligo Only – Protocol 1 (For Samples with >80% Viable Cells)



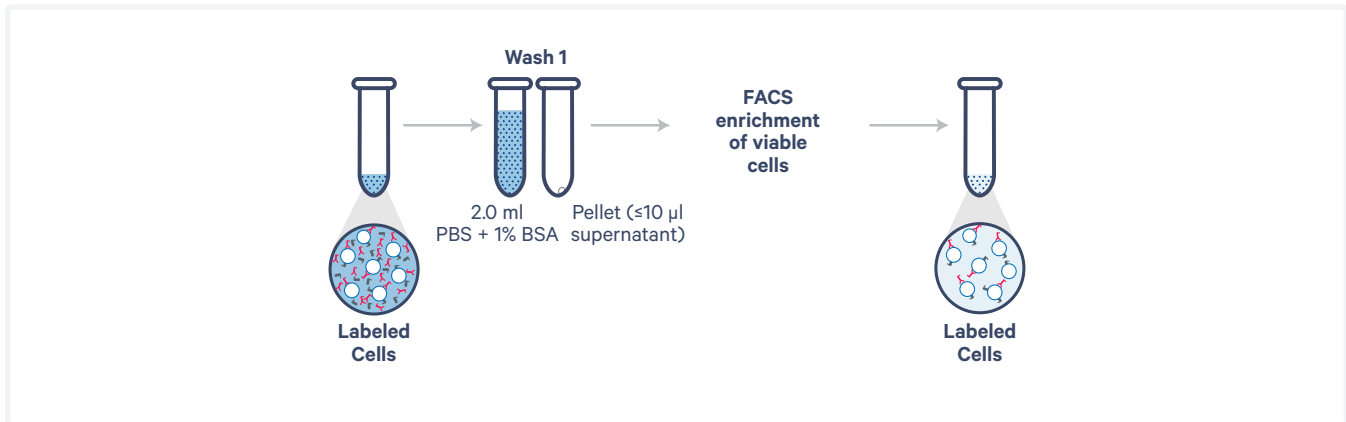
Cells labeled with Cell Surface Protein & Cell Multiplexing Oligo – Protocol 2 (For Samples with >80% Viable Cells)



Cells labeled with Cell Multiplexing Oligo Only – Protocol 3 (For Samples with <80% Viable Cells)



Cells labeled with Cell Surface Protein & Cell Multiplexing Oligo – Protocol 4 (For Samples with <80% Viable Cells)



FACS Guidance

This protocol was demonstrated using the viability dye 7-AAD.

FACS Cell Collection

It is recommended to collect FACS enriched cells in up to 20% FBS to maintain cell viability. Cells should be collected either in 20 μl volume in the collection tube/plate (96-well plate) or in 300 μl volume in a 5-ml tube. Collection buffer should be serum rich or use an optimal buffer for fragile cells to maintain a high cell viability.

The sort stream should be adjusted so that the cell-droplet falls into the collection buffer. Sorted cells must be counted and viability measured before proceeding to the 10x Genomics Single Cell protocols. If necessary, the collected cells may be concentrated by centrifugation at 150-300 rcf for 10 min at 4°C and by removing the supernatant.

Cell loss during FACS is common. Optimize the protocol steps accordingly.



Once sorting is complete, proceed immediately to relevant Chromium Single Cell RNA Sequencing protocols with Feature Barcode technology (see References).

Pooling Calculations

- Labeled samples can be pooled in desired ratios. Ensure pooling of sufficient cells/volumes to fulfill the experimental needs. Refer to the cell suspension volume calculator table in the relevant User Guide to determine target cell recovery and corresponding minimum cell load. For example, to recover 10,000 cells, a minimum of 16,500 cells should be loaded.
- The protocol has been demonstrated with labeled cells/nuclei populations from individual samples making up between 5%-95% of the total. The following calculation can be used to calculate the volume of each labeled sample to be pooled.
- Example calculation for a cell multiplexing experiment involving four Cell Multiplexing Oligo labeled samples and a Targeted Cell Recovery of 30,000 cells for one well of a 10x Genomics chip.

Targeted Cell Recovery = 30,000

Number of Cells to be Loaded = 49,500

Total number of cells to be pooled, with 3x coverage = $49,500 \times 3 = 148,500$

3x coverage is suggested starting point and can be adjusted accordingly. Ensure that sufficient volume is available for counting and viability assessment post pooling and for a re-run in case of a rare run failure (e.g., wetting failure or clog).

	1/Number of samples to be pooled
Pooling ratio =	1/4
	0.25

Cell concentration sample 1 = 1,500 cells/ μ l

Cell concentration sample 2 = 1,400 cells/ μ l

Cell concentration sample 3 = 1,300 cells/ μ l

Cell concentration sample 4 = 1,200 cells/ μ l

Sample volume (μ l) =	$\frac{\text{Total number of cells to be pooled} \times \text{Pooling ratio}}{\text{Cell concentration of sample}}$
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Sample 1 volume = $(148,500 \times 0.25) / 1500 = 24.8 \mu$ l

Sample 2 volume = $(148,500 \times 0.25) / 1400 = 26.5 \mu$ l

Sample 3 volume = $(148,500 \times 0.25) / 1300 = 28.6 \mu$ l

Sample 4 volume = $(148,500 \times 0.25) / 1200 = 30.9 \mu$ l

Pool the cell volumes from the calculations provided for sample 1, sample 2, sample 3, and sample 4.

Poolled cell volumes from samples 1-4 = 24.8μ l + 26.5μ l + 28.6μ l + 30.9μ l = 110.8μ l

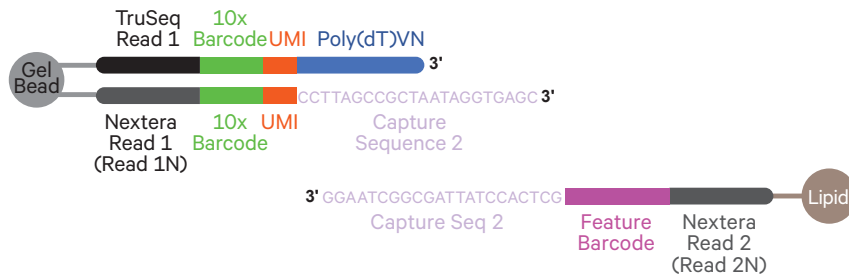
- Determine cell concentration and viability of the pooled sample using a Countess II Automated Cell Counter or a hemocytometer. Proceed immediately to relevant Chromium Single Cell RNA Sequencing protocols with Feature Barcode technology for Cell Multiplexing (see References).

Illustrative Overview of Cell Multiplexing Oligo Capture

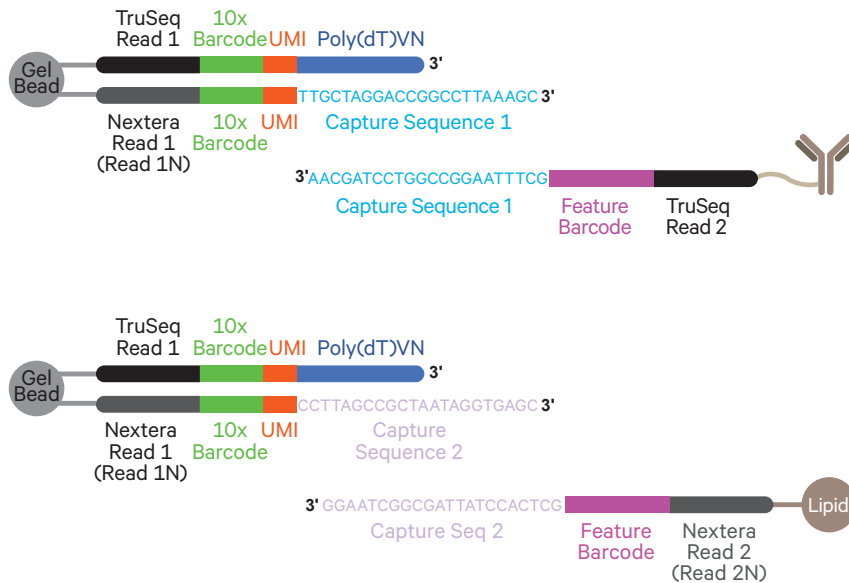
Illustrative overview of Cell Multiplexing Oligo capture by protocol specific Gel Bead primers is illustrated below.

Illustrative Overview of Cell Multiplexing Oligo Capture

Single Cell 3' v3.1 (Dual Index) - Cell Multiplexing (CG000388, CG000389, CG000419 & CG000421)



Single Cell 3' v3.1 (Dual Index) - Cell Surface Protein & Cell Multiplexing (CG000390 & CG000420)



References

The Cell Multiplexing Oligo Labeling protocol outlines labeling cells/nuclei with Cell Multiplexing Oligo for use with:

1. Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) with Feature Barcode technology for Cell Multiplexing User Guide (CG000388)
2. 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)
3. Chromium Next GEM Single Cell 3' HT Reagent Kits v3.1 User Guide with Feature Barcode technology for Cell Multiplexing (CG000419)
4. Chromium Next GEM Single Cell 3' HT Reagent Kits v3.1 User Guide with Feature Barcode technology for CRISPR Screening and Cell Multiplexing (CG000421)

The Cell Surface Protein and Cell Multiplexing Oligo Labeling protocol outlines labeling cells with antibody-oligonucleotide conjugates and Cell Multiplexing Oligo for use with:

1. 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)
2. Chromium Next GEM Single Cell 3' HT Reagent Kits v3.1 (Dual Index) with Feature Barcode technology for Cell Surface Protein and Cell Multiplexing User Guide (CG000420)

Document Revision Summary

Document Number	CG000391
Title	Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology
Revision	Rev A to Rev B
Revision Date	November 2021
Specific Changes	Updated Tips & Best Practices section Added Protocol 3 & 4 for samples with <80% viable cells Updated Pooling Calculations Section in the Appendix Updated Reference section to include high-throughput User Guides
General Changes	Updated for general minor consistency of language and terms throughout.

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