

## DEMONSTRATED PROTOCOL

# Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocols

with Feature Barcode technology

## Overview

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 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 page 2-3, Cell Multiplexing Oligo Labeling) for use with Single Cell 3' v3.1 (Dual Index) protocols with Feature Barcode technology for Cell Multiplexing (CG000388) and Single Cell 3' v3.1 (Dual Index) protocols with Feature Barcode technology for CRISPR Screening & Cell Multiplexing (CG000389).
- Labeling cells with antibody-oligonucleotide conjugates and CellPlex reagents (see page 4-5, 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 (CG000390).

This protocol was demonstrated using primary cells (including peripheral blood mononuclear cells (PBMCs), dissociated tumor cells, 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). Modifications to this protocol may be required when working with other cell types (e.g., centrifugation speed and time). For additional information on the preparation of specific sample types, consult 10x Genomics Demonstrated Protocols available on the 10x Genomics support website.

## Additional Guidance

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.

## Preparation – Buffers

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

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

## 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
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
BrainBits	NbActiv-1 Neuronal culturing medium	NbActive1 100
BioLegend	Human TruStain FcX (Fc Receptor Blocking Solution)	422301
	TotalSeq™ Antibody-Oligonucleotide Conjugates (TotalSeq™-B)	-

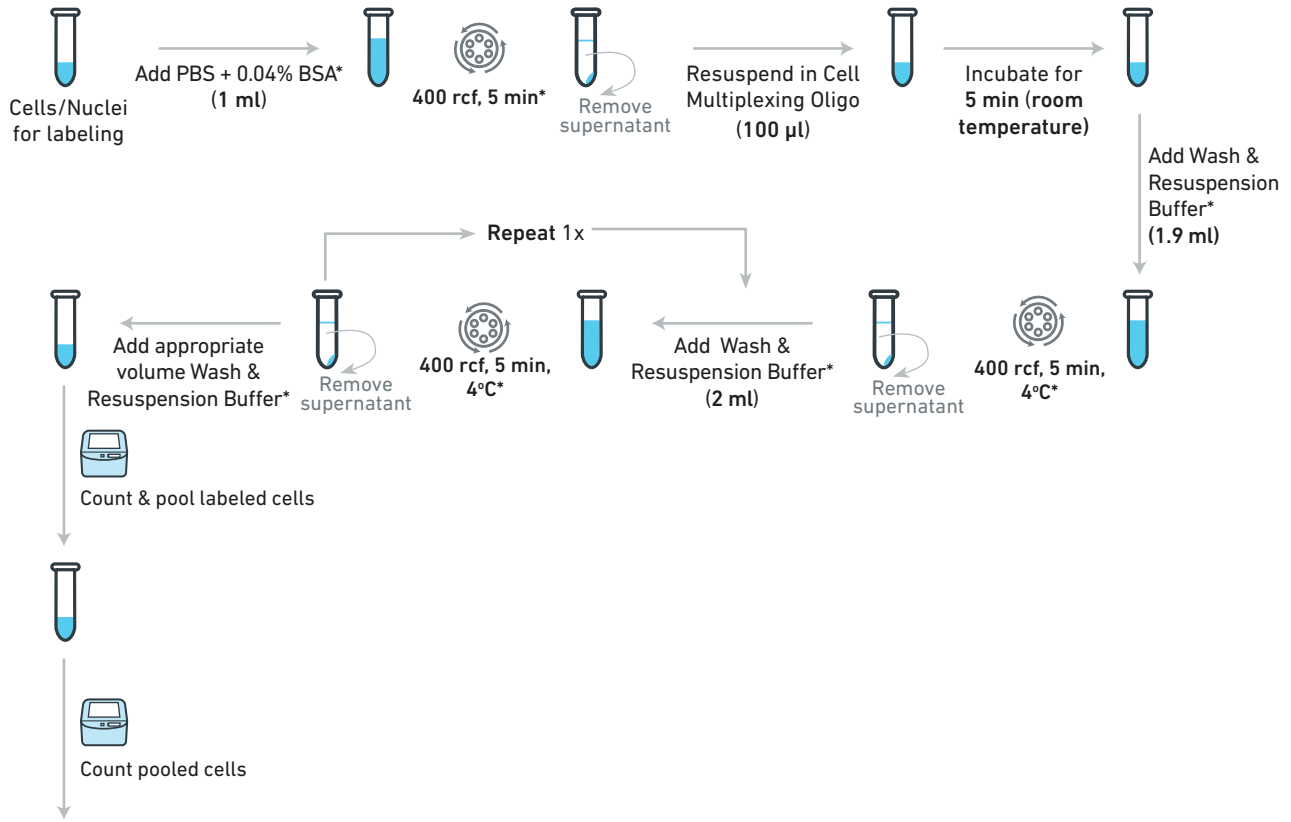
Equipment		
Thermo Fisher Scientific	Countess II FL Automated Cell Counter	AMAQAF1000
	Countess II FL Automated Cell Counting Chamber Slides	C10228

This list may not include some standard laboratory equipment.



## Protocol Overview: Cell Multiplexing Oligo Labeling

### Label Cells/Nuclei



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



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

## Tips & Best Practices

- **Pellet Resuspension:** Gently mix cells/nuclei 10-15x, or until the pellet is completely resuspended without introducing bubbles.
- **Supernatant Removal:** Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet. Leaving behind excess supernatant may result in increased background reads during sequencing. Transfer pipettes may be used to remove supernatant after centrifugation to minimize disturbance to the cell/nuclei pellet.

## 1. Cell Multiplexing Oligo Labeling Protocol

3' CellPlex Kit Set A (PN-1000261) was used for cell/nuclei multiplexing. Cell Multiplexing Oligos are supplied at  $-20^{\circ}\text{C}$ .

**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  $0.1\text{-}2 \times 10^6$  cells. Use  $0.5\text{-}2 \times 10^6$  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.

**Nuclei:** This protocol was demonstrated using  $0.1\text{-}2 \times 10^6$  nuclei. Use  $0.5\text{-}2 \times 10^6$  nuclei, if the number of nuclei is not limited. Wash nuclei according to the appropriate 10x Genomics Demonstrated Protocol.

- Transfer cells/nuclei to a 2-ml microcentrifuge tube and add appropriate buffer for a total **1 ml** volume.  
For cells, add room temperature PBS + 0.04% BSA.  
For nuclei, add chilled PBS + 1% BSA + RNase Inhibitor ( $0.2 \text{ U}/\mu\text{l}$ ). Gently pipette mix.
- Centrifuge cells/nuclei. Centrifuge cells at **room temperature** and nuclei at  $4^{\circ}\text{C}$ . 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

- Remove the supernatant without disturbing the pellet. Transfer pipettes may be used to remove supernatant after centrifugation to minimize disturbance to the pellet.
- Add **100  $\mu\text{l}$**  Cell Multiplexing Oligo (**room temperature**) to the cells/nuclei. Gently pipette mix 10-15x to resuspend. Store unused Cell Multiplexing Oligo at  $-20^{\circ}\text{C}$  and avoid more than 12 freeze-thaw cycles.

- 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.
- 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:

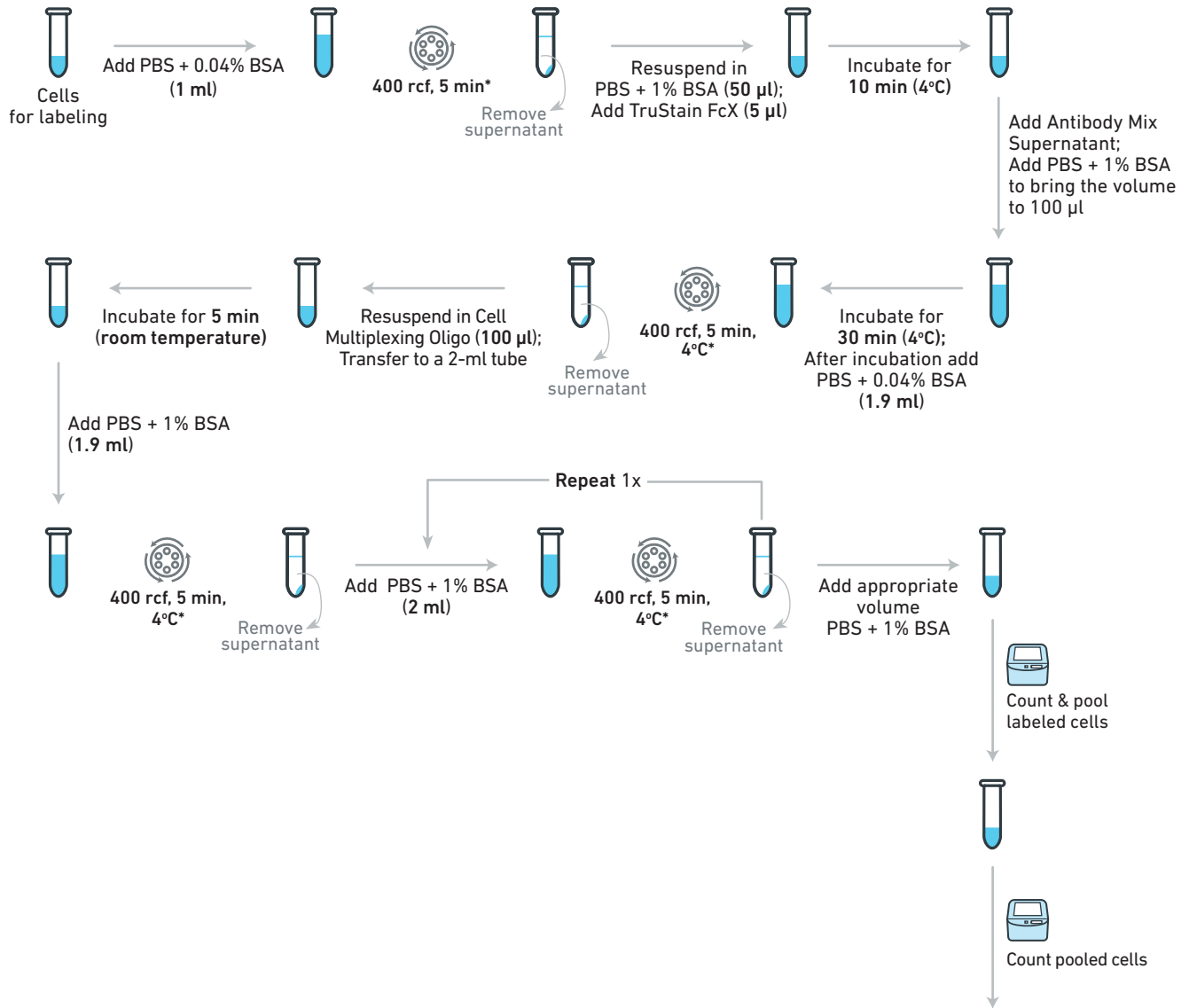
Sample Types	Wash & Resuspension Buffer (maintain at $4^{\circ}\text{C}$ )
PBMCs/Cell Lines/ Dissociated Tumor Cells	PBS + 1% BSA
Dissociated Brain Tissue	NbActiv-1 + 1% BSA
Nuclei	PBS + 1% BSA + RNase Inhibitor ( $0.2 \text{ U}/\mu\text{l}$ )

- Centrifuge cells/nuclei at  $4^{\circ}\text{C}$ . Centrifugation speed and time depends upon the sample type. See Table 1 for guidance.
  - Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet.  
  
Leaving behind excess supernatant may cause non-specific binding, which may result in increased background reads during sequencing.
- 
- Resuspend the pellet in **2 ml** chilled Wash & Resuspension Buffer. Gently pipette mix.
  - Centrifuge at  $4^{\circ}\text{C}$ . Centrifugation speed and time depends upon the sample type. See Table 1 for guidance.
  - Remove the supernatant without disturbing the pellet
  - Repeat i-k** for a total of two washes.
  - Based on starting concentration and assuming  $\sim 50\%$  cell loss, add an appropriate volume chilled Wash & Resuspension buffer to obtain a final concentration of:
    - $700\text{-}1,200 \text{ cells}/\mu\text{l}$  for targeted cell recovery of  $500\text{-}10,000$  cells or
    - $1,300\text{-}1,600 \text{ cells}/\mu\text{l}$  for targeted cell recovery of  $10,000\text{-}30,000$  cells.  
Gently pipette mix 10-15x.
  - Determine cell/nuclei concentration and viability using a Countess II Automated Cell Counter or a hemocytometer.
  - Pool labeled cells/nuclei using the calculation provided in the Appendix.
  - 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).

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.

## Protocol Overview : Cell Surface Protein & Cell Multiplexing Oligo Labeling

### 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)

## 2. Cell Surface Protein and Cell Multiplexing Oligo Labeling Protocol

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.

**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**.

**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  $0.2\text{-}2 \times 10^6$  PBMCs. Use  $0.5\text{-}2 \times 10^6$  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.

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

- Remove the supernatant without disturbing the pellet
- Resuspend cell pellet in **50 µl** chilled PBS + 1% BSA.
- Add **5 µl** Human TruStain FcX. Gently pipette mix.
- Incubate for **10 min** at **4°C**.
- Add the prepared Antibody Mix supernatant.
- 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).
- 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.
- Wash by adding **1.9 ml** chilled PBS + 0.04% BSA (for a total volume of **2.0 ml**).

- Centrifuge at **4°C**. Larger or fragile cell types may require slower centrifugation speeds. See Table 3 for guidance.
- Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet.

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



- 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**.
  - 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.
  - Wash by adding **1.9 ml** chilled PBS + 1% BSA (for a total **2 ml** volume) to the sample.
  - Centrifuge at **4°C**. Centrifugation speed and time depends upon the sample type. See Table 3 for guidance.
  - Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet.
  - Wash by adding **2.0 ml** chilled PBS + 1% BSA to the sample.
  - Centrifuge at **4°C**. Centrifugation speed and time depends upon the sample type. See Table 3 for guidance.
  - Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet.
  - Repeat r-t** for a total of two washes.
  - Based on starting concentration and assuming ~50% cell loss, add an appropriate volume chilled PBS + 1% BSA to obtain a final concentration of:
    - 700-1,200 cells/µl for targeted cell recovery of 500-10,000 cells or
    - 1,300-1,600 cells/µl for targeted cell recovery of 10,000-30,000 cells.
 Gently pipette mix 10-15x.
  - Determine cell concentration and viability of the sample using a Countess II Automated Cell Counter or a hemocytometer
  - Pool labeled cells using the calculation provided in the Appendix.
  - Determine cell concentration and viability of the pooled sample using a Countess II Automated Cell Counter or a hemocytometer.
  - 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.

## Appendix

### Pooling Labeled Samples

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 populations 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:**

	Chromium Next GEM Chip G Wells							
	1	2	3	4	5	6	7	8
Targeted Cell Recovery	1,000	1,000	10,000	10,000	20,000	20,000	30,000	30,000
Number of Cells to be Loaded	1,650	1,650	16,500	16,500	33,000	33,000	49,500	49,500

Total number of cells to be loaded in a full chip (all 8 wells) with 50% pipetting coverage = 301,950

Pooling ratio =	1/Number of samples to be pooled 1/4 <b>0.25</b>
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Cell concentration sample 1 = 1,500 cells/ $\mu$ l

Cell concentration sample 2 = 1,400 cells/ $\mu$ l

Cell concentration sample 3 = 1,300 cells/ $\mu$ l

Cell concentration sample 4 = 1,200 cells/ $\mu$ l

Sample volume ( $\mu$ l) =	$\frac{\text{Total number of cells to be loaded} \times \text{Pooling ratio}}{\text{Cell concentration of sample}}$
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Sample 1 volume =  $(301,950 \times 0.25) / 1500 = 50.3 \mu\text{l}$

Sample 2 volume =  $(301,950 \times 0.25) / 1400 = 53.9 \mu\text{l}$

Sample 3 volume =  $(301,950 \times 0.25) / 1300 = 58.1 \mu\text{l}$

Sample 4 volume =  $(301,950 \times 0.25) / 1200 = 62.9 \mu\text{l}$

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

Pooled cell volumes from samples 1-4 =  $50.3 \mu\text{l} + 53.9 \mu\text{l} + 58.1 \mu\text{l} + 62.9 \mu\text{l} = 225.2 \mu\text{l}$

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).

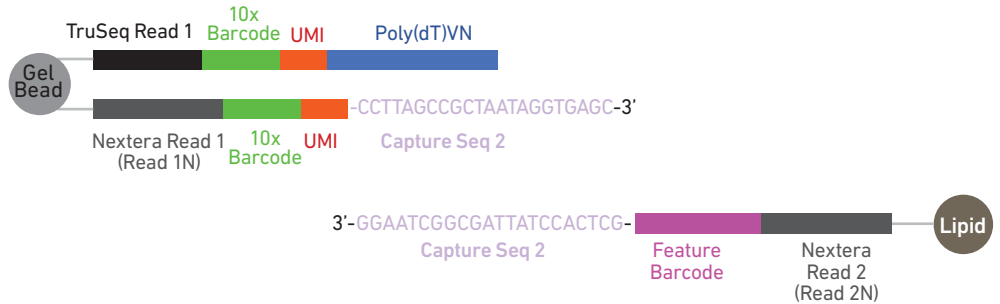
## Illustrative Overview of Cell Multiplexing Oligo Capture



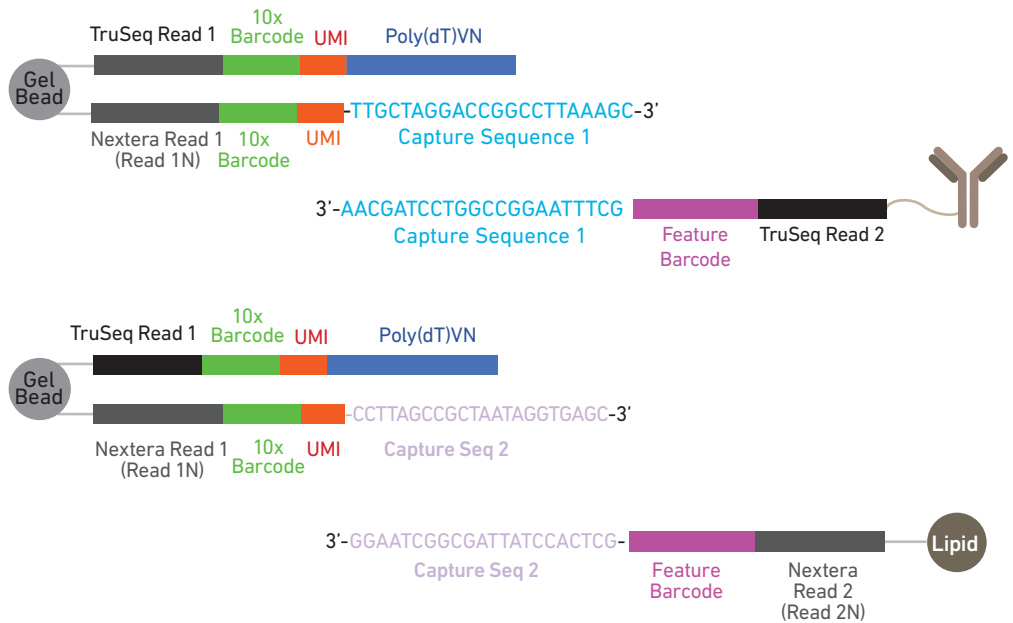
Illustrative overview of Cell Multiplexing Oligo capture by protocol specific Gel Bead primers is illustrated below.

### Illustrative Overview of Cell Multiplexing Oligo Capture

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



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



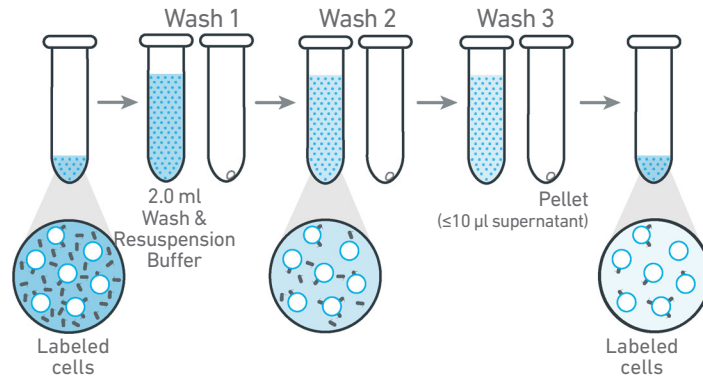


## Illustrative Overview of Wash Steps

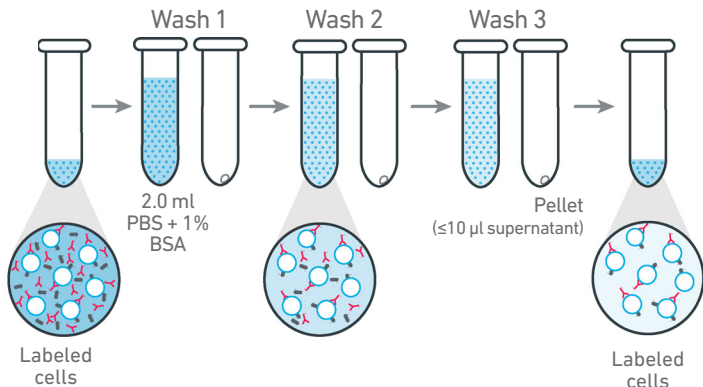
To eliminate non-specific binding with comparable efficiency, wash steps should be performed in 2-ml microcentrifuge tubes using indicated buffer volumes. Non-specific binding contributes to increased background reads during sequencing.

### Wash Steps Overview

#### Cells labeled with Cell Multiplexing Oligo Only



#### Cells labeled with Cell Surface Protein & Cell Multiplexing Oligo



## References

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

- 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)

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

- 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)

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