DEMONSTRATED PROTOCOL

Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols

with Feature Barcode technology

Overview

Cell surface proteins can be labeled using a specific protein binding molecule, such as an antibody conjugated to a Feature Barcode oligonucleotide. This protocol provides guidance for antibody-oligonucleotide conjugation and outlines cell surface protein labeling for use with Single Cell RNA sequencing protocols with Feature Barcode technology for cell surface protein. This protocol also provides guidance for enriching labeled cells using Fluorescence Activated Cell Sorting (FACS).

Consult Demonstrated Protocol Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (CG000391) for guidance on cell surface protein and Cell Multiplexing Oligo labeling,

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, maintain at 4°C
PBS + 1% BSA
Alternatively, Cell Staining Buffer from BioLegend can be used for labeling.
PBS + 0.04% BSA
PBS + 2% FBS (Only if performing FACS enrichment of labeled cells)

Specific Reagents & Consumables

For Antibody-Oligonucleotide Conjugation		
Vendor	**Item**	**Part Number**
Abcam	Oligonucleotide Conjugation Kit	ab218260
IDT	Custom DNA Oligos (see Table 1)	-
-	100 µg Purified Azide-free Antibody (1 mg/ml)	-

For Cell Surface Protein Labeling		
Vendor	**Item**	**Part Number**
BioLegend	Human TruStain FcX (Fc Receptor Blocking Solution)	422301
	TotalSeq™ Antibody-Oligonucleotide Conjugates*	-
	Cell Staining Buffer	420201
	Antibodies (Fluorophore)†	-
	If using FACS for enriching labeled cells	
Thermo Fisher Scientific	UltraPure Bovine Serum Albumin (BSA, 50 mg/ml)	AM2616
	Trypan Blue Stain (0.4%)	T10282
Millipore Sigma	Bovine Serum Albumin In DPBS (10%) (alternative to Thermo Fisher product)	A1595
Miltenyi Biotec	MACS BSA Stock Solution (alternative to Thermo Fisher product)	130-091-376
Corning	Phosphate-Buffered Saline, 1X without Calcium and Magnesium	21-040-CV
VWR	Fetal Bovine Serum (FBS)	97068-085
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.

*TotalSeq™-B for: Single Cell 3' v3 and v3.1 protocol with Feature Barcode technology for Cell Surface Protein

*TotalSeq™-C for: Single Cell 5' v1, v1.1 & v2 protocol with Feature Barcode technology for Cell Surface Protein & Antigen Specificity.

†Choose different clones than antibody-oligonucleotide conjugates

Protocol Overview

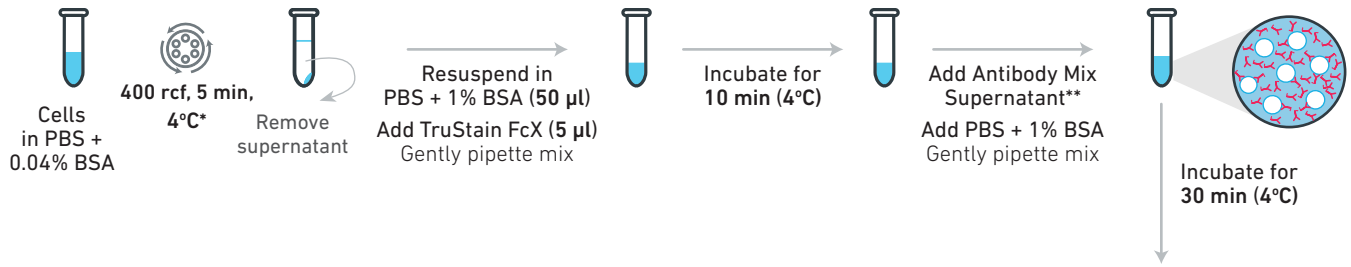
Option A. Custom Conjugated Antibodies

Follow manufacturer's instructions
(Abcam Oligonucleotide Conjugation Kit)
for conjugation and purification

Option B. Preconjugated Antibodies

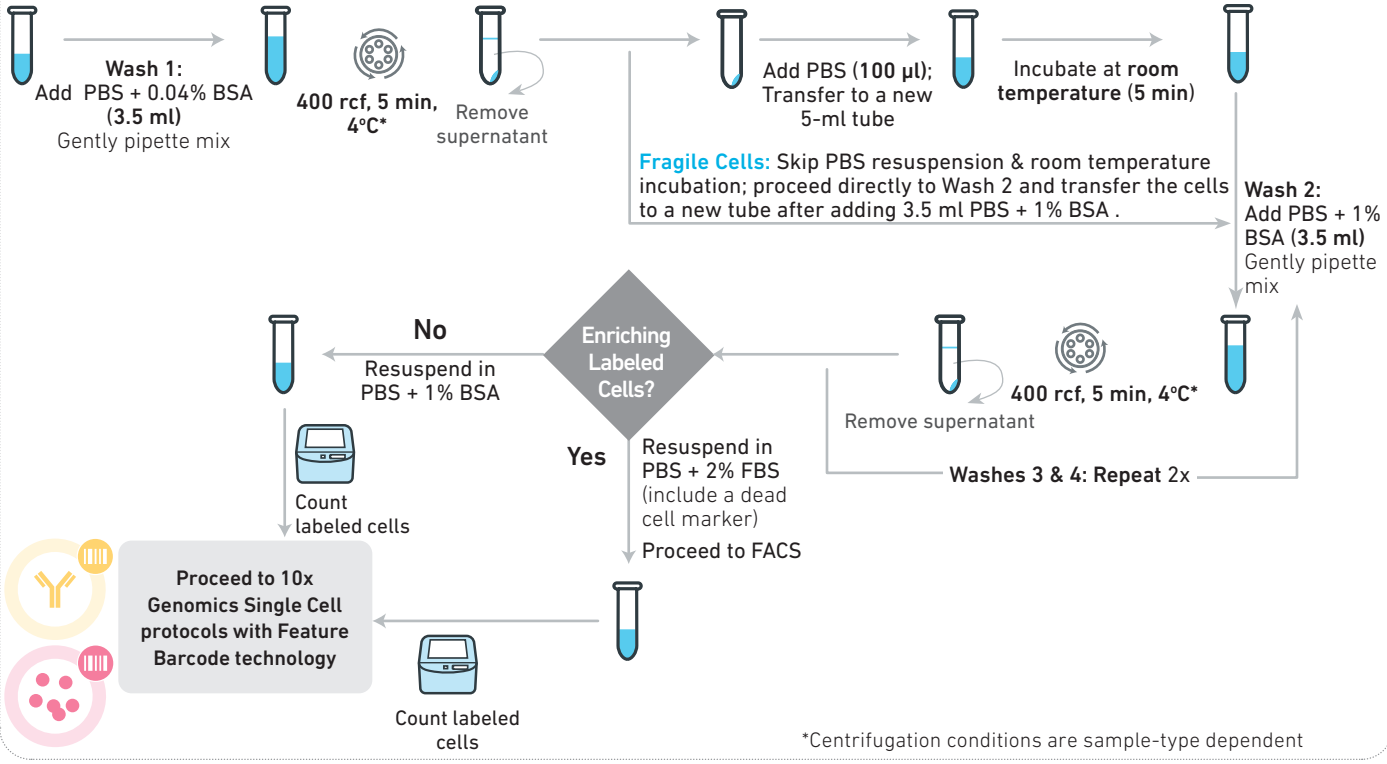
BioLegend TotalSeq™-B
OR
BioLegend TotalSeq™-C

1. Label Cells Prepare Antibody Mix & FACS Antibody Pool (if performing FACS enrichment) as described in the Labeling Protocol



*Centrifugation conditions are sample-type dependent
**If performing FACS enrichment, add prepared FACS Antibody Pool

2. Wash Cells



*Centrifugation conditions are sample-type dependent

Antibody-Oligonucleotide Conjugation Guidance

Choose antibody based on cell surface protein/s being labeled and a Feature Barcode oligonucleotide compatible with specific 10x Genomics protocol (Table 1) for conjugation. Alternatively, use compatible preconjugated antibodies from BioLegend or other vendors for labeling cells.

Specific Reagents for Conjugation

Vendor	Item	Part Number
Abcam	Oligonucleotide Conjugation Kit	ab218260
IDT	Custom DNA Oligos (see Table 1)	-
-	100 µg Purified Azide-free Antibody (1 mg/ml)	-
Corning	Phosphate-Buffered Saline, 1X without Calcium and Magnesium	21-040-CV

Oligonucleotide:

- Use ≥10 nmole HPLC-purified and lyophilized Feature Barcode oligonucleotide for conjugation. The oligonucleotide must contain an amine group at 5'-end (5' amine modified; IDT code /5AmMC12/).
- Resuspend lyophilized oligonucleotide in PBS or other compatible buffer (see Oligonucleotide Conjugation Kit) at **100 µM,** i.e. 10 nmole dissolved in **100 µl** buffer.

DO NOT use Tris buffers for resuspension as they are not compatible with conjugation.

Conjugation:

Follow manufacturer's instructions (Oligonucleotide Conjugation Kit from Abcam) for antibody-oligonucleotide conjugation. Abcam Oligonucleotide Conjugation Kit is compatible with many purified antibodies.

Antibody-Oligonucleotide Ratio:

This protocol was demonstrated using 1:3 antibody-oligonucleotide ratio for conjugation. Optimization may be needed depending on the antibodies used.

Conjugate Purification:

The antibody-oligonucleotide conjugate purification is recommended to remove any unbound oligonucleotides. Follow the Conjugate Purification protocol (Abcam).

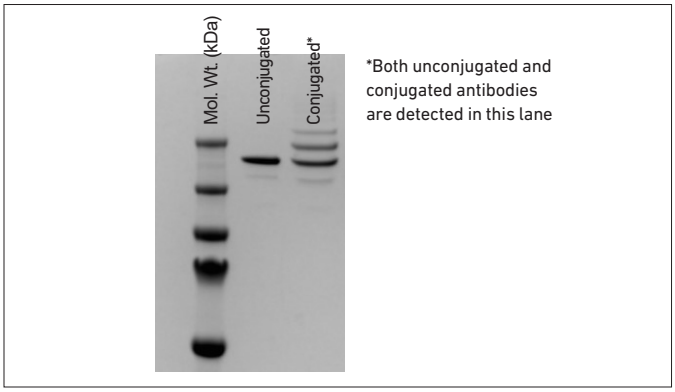
Verification of Conjugation:

Verify the conjugation by comparing the antibody-oligonucleotide conjugate with an unconjugated antibody control resolved on a non-reducing, gradient SDS-PAGE gel.

Run a known volume and concentration of unconjugated antibody next to a known volume of antibody-oligonucleotide conjugate on SDS-PAGE gel.

Estimate the conjugate concentration and calculate the degree of conjugation by comparing the respective band intensities.

Figure 1. Verification of conjugation on a 4-12% gradient SDS-PAGE gel under non-reducing conditions.



OPTIONAL Use a BCA or Bradford Protein Assay Kit to calculate the final antibody concentration.

Table 1. Feature Barcode Oligonucleotide Sequence for Antibody Conjugation.

10x Genomics Protocol	Feature Barcode Oligonucleotide Sequence
Single Cell 3' v3 & v3.1* - Cell Surface Protein (CG000185, CG000206 & CG000317) *single & dual index libraries	/5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNNNNNNNN-NNNNNNNNNNNNNNN-NNNNNNNNN-GCTTTAAGGCCGGTCCTAGCAA TruSeq Read 2 · 10 nt · Feature Barcode (15 nt) · 9 nt · Capture Sequence 1
Single Cell 5' v1, v1.1 & v2 – Cell Surface Protein (CG000186, CG000208 & CG000330)	/5AmMC12/CGGAGATGTGTATAAGAGACAGNNNNNNNNNN-NNNNNNNNNNNNNNN-NNNNNNNNNCCCATATAAGAAA Nextera partial Read 2 · 10 nt · Feature Barcode (15 nt) · 9 nt · Capture Sequence

See Appendix for an illustrative overview of antibody-oligonucleotide conjugate capture by 10x Gel Bead primers. Consult Barcode Whitelist for Custom Feature Barcode conjugates (Document CG000193), for more information.

Cell Surface Protein Labeling Protocol

This protocol was optimized using TotalSeq-B/C antibody-oligonucleotide conjugates from BioLegend. The labeled cells were enriched by FACS (see Appendix).

Use distinct antibody clones for FACS and cell surface protein labeling. Optimize working concentration of each of the antibodies used.

1. Label Cells

This protocol was demonstrated using 0.2-1 x 10^6 cells. Wash cells according to the appropriate 10x Genomics Demonstrated Protocol for the cell type being prepared.

All steps can be performed in 5-ml centrifuge 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**.

Use TotalSeq-B for Single Cell 3' v3 and v3.1 (single & dual index) protocol and TotalSeq-C for Single Cell 5' v1, v1.1 & v2 protocol with Feature Barcode technology for Cell Surface Protein.

Prepare FACS Antibody Pool:

- Add appropriate/manufacturer's recommended amount of fluorophore antibodies to a 1.5-ml microcentrifuge tube on ice.
- Gently pipette mix and maintain at **4°C**. Avoid light exposure.

a. Transfer cells to a new 5-ml 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. Larger or fragile cell types may require slower centrifugation speeds. Use the following table for guidance.

Table 2. 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. If also performing FACS enrichment, add FACS antibody pool.

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**. If using FACS antibodies, incubate without light exposure.

Recommended incubation temperature for most sample types is **4°C**. However, incubation temperature is sample type dependent and should be chosen accordingly.

2. Wash Cells

Thorough washing of cells post labeling is critical to obtain high-quality data. Optimization of centrifugation speed/time may be needed based on cell type.

a. <u>Wash 1:</u> Wash by adding **3.5 ml** chilled PBS + 0.04% BSA to the cells from step 1i. Gently pipette mix.

b. Centrifuge at **4°C** . Centrifugation speed and time depends upon the sample type. Use Table 2 for guidance.

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

Pellet (may not be visible)

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

d. Resuspend the pellet in **100 µl** room temperature PBS and transfer to a new 5-ml tube. Incubate for **5 min** at **room temperature**. Proceed to step e (Wash 2).

Step d can be excluded for the fragile cells (see Cell Washing for Fragile Cell Types in Appendix) to maximize cell health and recovery. If not performing step d, proceed directly to step e (Wash 2) and transfer the cells to a new tube after adding **3.5 ml** PBS + 1% BSA in step e.

e. <u>Wash 2:</u> Using a pipette tip, resuspend the pellet or cells in **3.5 ml** chilled PBS + 1% BSA.

f. Centrifuge at **4°C**. Centrifugation speed and time depends upon the sample type. Use Table 2 for guidance.

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

h. <u>Washes 3 & 4:</u> **Repeat e - g** 2x for a total of three washes.

i. **OPTIONAL For enrichment of labeled cells by FACS**: Based on starting concentration and assuming ~50% cell loss, add an appropriate volume chilled PBS + 2% FBS (including a dead cell marker) to obtain a final cell concentration of 5-10 x 10^6 cells/ml and proceed to FACS (see FACS Guidance).

After FACS, determine cell concentration and viability using a Countess II Automated Cell Counter or a hemocytometer and proceed **immediately** to relevant Chromium Single Cell RNA Sequencing protocols with Feature Barcode technology (see References).

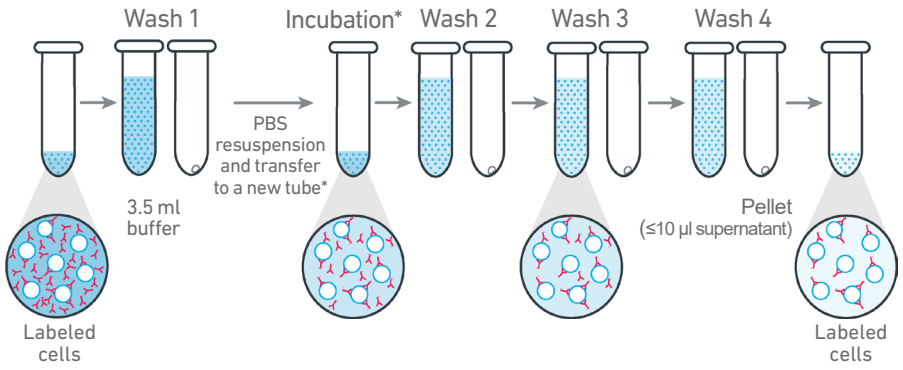
j. **If not performing FACS:** Based on starting concentration and assuming ~50% cell loss, add an appropriate volume chilled PBS + 1% BSA to obtain a concentration of 700-1,200 cells/µl. Determine cell concentration and viability using a Countess II Automated Cell Counter or a hemocytometer and proceed **immediately** to relevant Chromium Single Cell RNA Sequencing protocols with Feature Barcode technology (see References).

Appendix

Illustrative Overview of Wash Steps

To eliminate non-specific binding with comparable efficiency, wash steps may be performed in 5-ml microcentrifuge tubes using indicated buffer volumes. Non-specific binding contributes to increased background reads during sequencing.

Wash Steps in 5-ml Microcentrifuge Tubes



*Exclude these steps for fragile cells (See Cell Washing for Fragile Cell Types) and proceed directly to Wash 2 and transfer the cells to a new tube after adding 3.5 ml buffer.

Cell Washing for Fragile Cell Types

PBS resuspension and room temperature incubation (step 2d) during cell wash can be excluded for fragile cell types. The exclusion of this step may yield slightly lower Cell Surface Protein library metrics, but will preserve cell health and recovery. Step 2d can be excluded in the following sample types:

- Cell types with low viability (<70%)
- Sample types that are known to degrade quickly
- Samples with very low cell input (<200,000 cells)

FACS Guidance

Enrich labeled cells using FACS prior to library generation to enable identification of rare subpopulations.

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 **200 µl** volume in a 1.5-ml tube.

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 **350 rcf** 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).

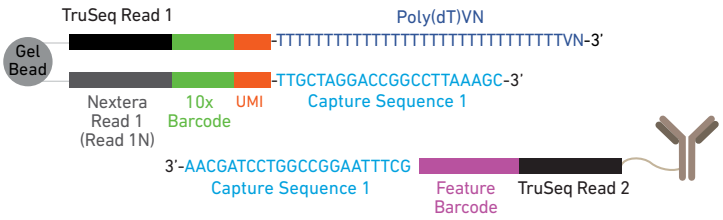
Illustrative Overview of Antibody-Oligonucleotide Conjugate Capture

Antibody-oligonucleotide conjugate capture by protocol specific Gel Bead primers is illustrated below.

Illustrative Overview of Antibody-Oligonucleotide Conjugate Capture

Single Cell 3' v3 & v3.1* - Cell Surface Protein (CG000185, CG000206 & CG000317)

*single & dual index libraries



TotalSeq-B Antibody-oligonucleotide conjugate

Single Cell 5' v1, v1.1 & v2 - Cell Surface Protein (CG000186, CG000208 & CG000330)

TotalSeq-C Antibody-oligonucleotide conjugate

Compatible Primers

To generate Cell Surface Protein libraries with TotalSeq-B/C, use the primers indicated below.

Antibody-Oligonucleotide Conjugate	Compatible Primers	10x PN
TotalSeq-B	Feature cDNA Primers 2	2000097
TotalSeq-C	SC5' Feature cDNA Primer/Feature cDNA Primer 4	2000119/ 2000277

If generating libraries with TotalSeq™-A, an additive primer is required for successful amplification (not provided by 10x Genomics). See Cite Seq Protocols and BioLegend for details.

References

This protocol provides guidance for antibody-oligonucleotide conjugation and outlines cell surface protein labeling for use with:

- Chromium Single Cell V(D)J Reagent Kits with Feature Barcode technology for Cell Surface Protein User Guide (CG000186)
- Chromium Next GEM Single Cell V(D)J Reagent Kits v1.1 with Feature Barcode technology for Cell Surface Protein User Guide (CG000208)
- Chromium Next GEM Single Cell 5' v2 (Dual Index) with Feature Barcode technology for Cell Surface Protein & Antigen Specificity User Guide (CG000330)
- Chromium Single Cell 3' Reagent Kits v3 with Feature Barcode technology for Cell Surface Protein User Guide (CG000185)
- Chromium Next GEM Single Cell 3' Reagent Kits v3.1 with Feature Barcode technology for Cell Surface Protein User Guide (CG000206)
- Chromium Next GEM Single Cell 3' v3.1 (Dual Index) with Feature Barcode technology for Cell Surface Protein User Guide (CG000317)

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