DEMONSTRATED PROTOCOL

Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols

with Feature Barcoding 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 Barcoding technology. This protocol also provides guidance for enriching labeled cells using Fluorescence Activated Cell Sorting (FACS).

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 Maintain at 4°C	Composition
Labeling Buffer	PBS + 1% BSA
Resuspension Buffer	PBS + 0.04% BSA
Dextran Sulfate Solution Only if adding Dextran Sulfate in step 1	1% w/v (10 mg/ml) Dextran Sulfate Sodium Salt in Nuclease-free Water
PBS + 2% FBS (maintain at 4°C)	

Specific Reagents & Consumables

For Antibody-Oligonucleotide Conjugation		
Vendor	**Item**	**Part Number**
Expedeon	Thunder-Link PLUS Conjugation Kit	425-0300
IDT	Custom DNA Oligos (see Table 1)	-
-	100 µg Purified Azide-free Antibody (1 mg/ml)	-

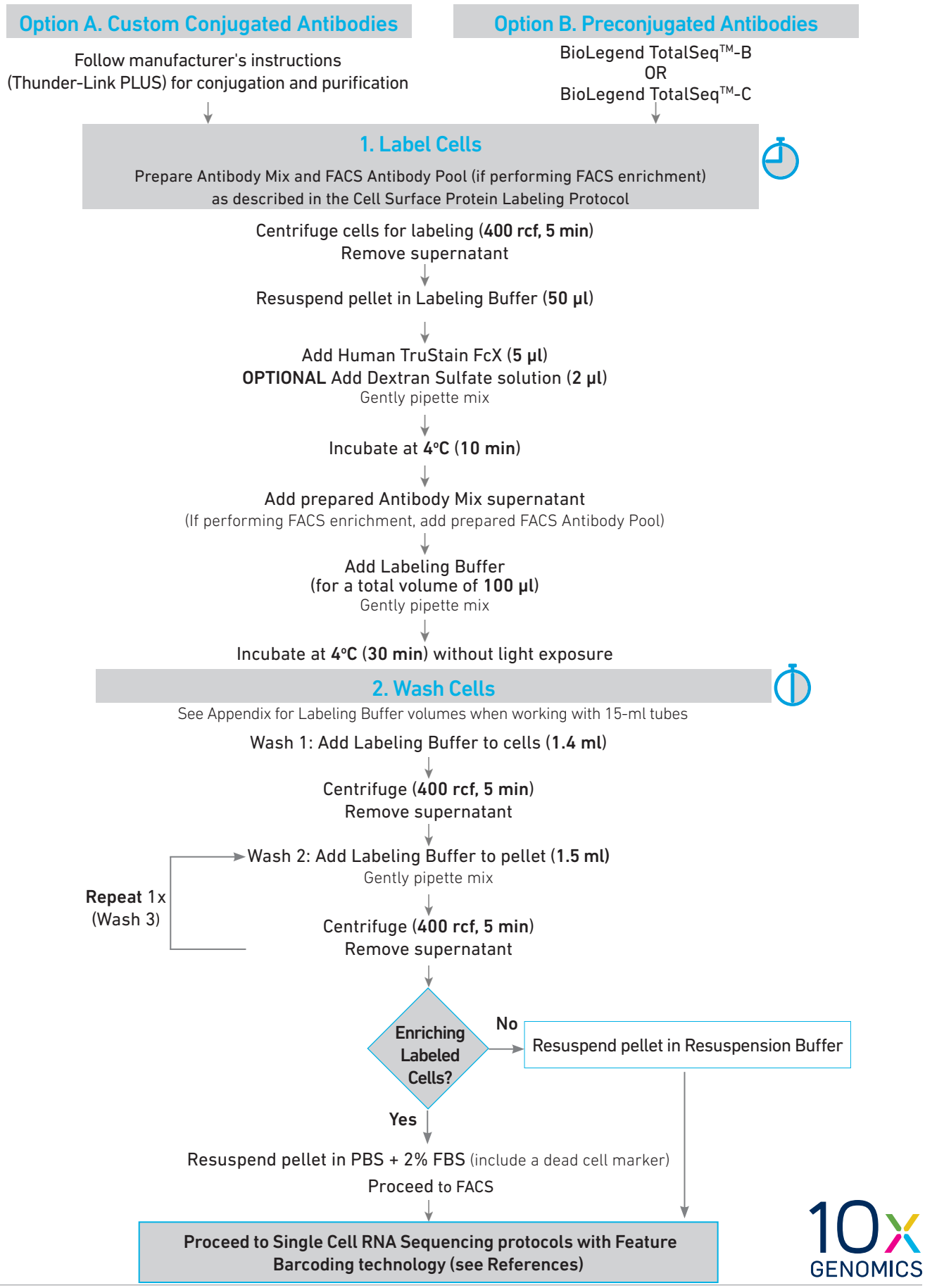
For Cell Surface Protein Labeling		
Vendor	**Item**	**Part Number**
BioLegend	Human TruStain FcX (Fc Receptor Blocking Solution)	422301
	TotalSeq™ Antibody-Oligonucleotide Conjugates*	-
	Antibodies (Fluorophore)† If using FACS for enriching labeled cells	-
MP Biomedicals	Dextran Sulfate Sodium Salt (Optional)	101516
Thermo Fisher Scientific	Dextran Sulfate Sodium Salt (Optional; alternative to MP Biomedicals product)	AC441490050
	UltraPure Bovine Serum Albumin (BSA, 50 mg/ml)	AM2616
Millipore Sigma	Phosphate-Buffered Saline (PBS) with 10% Bovine Albumin (alternative to Thermo Fisher product)	SRE0036
Corning	Phosphate-Buffered Saline, 1X without Calcium and Magnesium	21-040-CV
VWR	Fetal Bovine Serum (FBS)	97068-085

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

***TotalSeq™-C for:** Single Cell V(D)J and v1.1 protocol with Feature Barcoding technology for Cell Surface Protein

†Choose different clones than antibody-oligonucleotide conjugates

Protocol Overview



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
Expedeon	Thunder-Link PLUS Conjugation Kit	425-0300
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 Thunder-Link PLUS) 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 (Thunder-Link PLUS Oligo Conjugation Kit from Expedeon) for antibody-oligonucleotide conjugation. Thunder-Link PLUS 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 Thunder-link PLUS conjugate purification protocol.

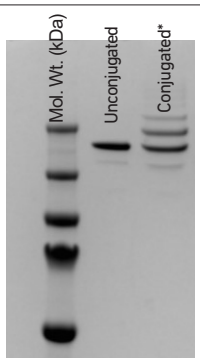
Verification of Conjugation:

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.



*Both unconjugated and conjugated antibodies are detected in this lane

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)	/5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNN-NNNNNNNNNNNNNNN-NNNNNNNNNGCTTTAAGGCCGGTCCTAGCAA TruSeq Read 2 — 10 nt — Feature Barcode (15 nt) — 9 nt — Capture Sequence 1
Single Cell V(D)J & V(D)J v1.1 – Cell Surface Protein (CG000186 & CG000208)	/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 Barcoding 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

Prepare Antibody Mix:

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 **room temperature**. 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 protocol and TotalSeq-C for Single Cell V(D)J and v1.1 protocol with Feature Barcoding 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**.

This protocol was demonstrated using 0.2-1 x 10^6 PBMCs.

a. Centrifuge cells at **400 rcf** for **5 min** at **4°C.** Use of swinging-bucket rotor is recommended for higher cell recovery.

b. Remove the supernatant without disturbing the pellet

c. Resuspend cell pellet in **50 µl** Labeling Buffer.

d. Add **5 µl** Human TruStain FcX. Gently pipette mix.

OPTIONAL

To reduce non-specific background, Dextran Sulfate Solution (1:50 dilution of **100 µl** reaction) may be added at this step.
The use of Dextran Sulfate can be omitted, if concerned about its affect on cell viability.

e. Incubate for **10 min** at **4°C**.

f. Add the prepared Antibody Mix supernatant. If also performing FACS enrichment, add FACS antibody pool.

g. Add Labeling Buffer to the cell suspension to bring the total volume to **100 µl**. Gently pipette mix 10x (pipette set to 90 µl).

h. Incubate for **30 min** at **4°C** without light exposure.

2. Wash Cells

To eliminate non-specific binding with comparable efficiency, wash steps may be performed either in 1.5-ml microcentrifuge tubes or 15-ml tubes using indicated Labeling Buffer volumes. If concerned about dislodging the pellet during supernatant removal, perform wash steps in 15-ml tubes (see Appendix).

Optimization of centrifugation speed/time may be needed based on cell type.

a. Wash by adding **1.4 ml** Labeling Buffer (for a total volume of **1.5 ml**) to the cells from step 1h.

b. Centrifuge at **400 rcf** for **5 min** at **4°C** . Larger or fragile cell types may require slower centrifugation speeds.

c. 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.

Pellet (may not be visible)

d. Using a **wide-bore** pipette tip, resuspend the pellet in **1.5 ml** Labeling Buffer.

e. Centrifuge at **400 rcf** for **5 min** at **4°C**.

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

g. Repeat d - f.

h. OPTIONAL For enrichment of labeled cells by FACS:
Based on starting concentration and assuming ~50% cell loss, add an appropriate volume 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, proceed **immediately** to relevant Chromium Single Cell RNA Sequencing protocols with Feature Barcoding technology (see References).

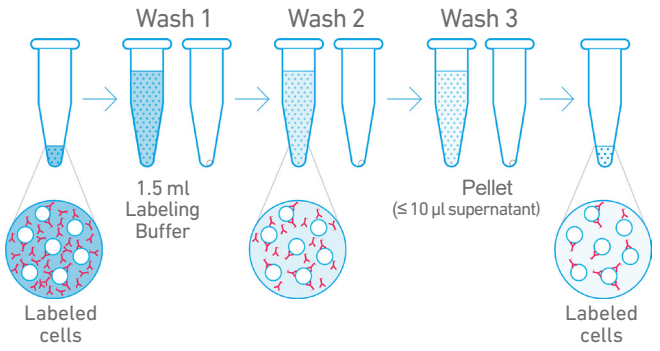
i. If not performing FACS:
Based on starting concentration and assuming ~50% cell loss, add an appropriate volume Resuspension Buffer to obtain a concentration of 700-1,200 cells/µl. Determine cell concentration and viability using a Countess II Automated Cell Counter and proceed **immediately** to relevant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

Appendix

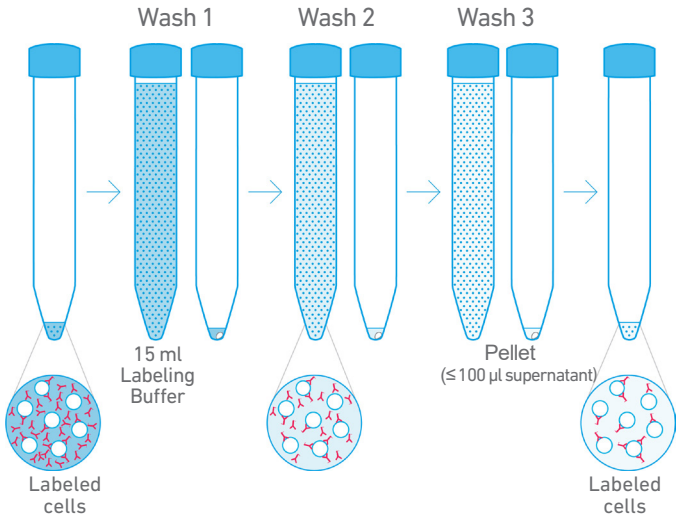
Illustrative Overview of Wash Steps

To eliminate non-specific binding with comparable efficiency, wash steps may be performed either in 1.5-ml microcentrifuge tubes or 15-ml tubes using indicated Labeling Buffer volumes. Non-specific binding contributes to increased background reads during sequencing. If concerned about dislodging the pellet during supernatant removal, perform wash steps in 15-ml tubes.

Wash Steps in 1.5 ml Microcentrifuge Tubes



Wash Steps in 15 ml Tubes



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 100% 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.

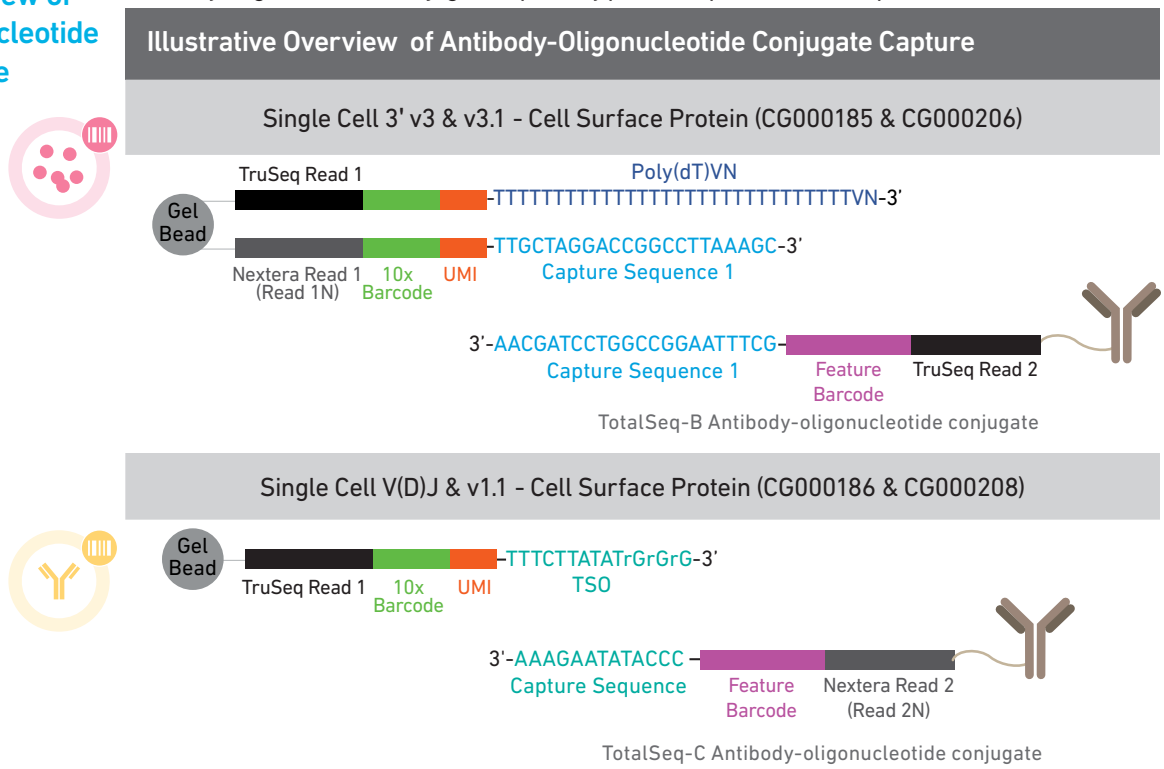
The sort stream should be adjusted so that the cell-droplet falls into the collection buffer. Sorted cells must be counted and viability measured before proceeding to the 10x Genomics Single Cell protocols. If necessary, the collected cells may be concentrated by centrifugation at **350 rcf** and removing 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 Barcoding technology (see References).

Illustrative Overview of Antibody-Oligonucleotide Conjugate Capture

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



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	2000119

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 3' Reagent Kits v3 with Feature Barcoding technology for Cell Surface Protein User Guide (CG000185)
- Chromium Next GEM Single Cell 3' Reagent Kits v3.1 with Feature Barcoding technology for Cell Surface Protein User Guide (CG000206)
- Chromium Single Cell V(D)J Reagent Kits with Feature Barcoding technology for Cell Surface Protein User Guide (CG000186)
- Chromium Next GEM Single Cell V(D)J Reagent Kits v1.1 with Feature Barcoding technology for Cell Surface Protein User Guide (CG000208)

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