Cell Surface Protein Labeling for Chromium Fixed RNA Profiling *for Singleplexed Samples with Feature Barcode technology*

Getting Started

Cell surface proteins can be labeled using a specific protein binding molecule, such as an antibody conjugated to a Feature Barcode oligonucleotide. This protocol outlines cell surface protein labeling for use with Chromium Fixed RNA Profiling with Feature Barcode 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.

Consult Demonstrated Protocol Thawing Dissociated Tumor Cells for Single RNA Sequencing (CG000233) for guidance on thawing dissociated tumor cells.

Pre-read and have available Demonstrated Protocol Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling (CG000478) before starting the cell surface protein labeling protocol.

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
For Cell Surface Protein Labeling		
BioLegend	Human TruStain FcX (Fc Receptor Blocking Solution)	422301
	True-Stain Monocyte Blocker	426101
	TotalSeq™-B Antibody-Oligonucleotide Conjugates	-
	Cell Staining Buffer	420201
Thermo Fisher Scientific	UltraPure Bovine Serum Albumin (BSA, 50 mg/ml)	AM2616
	Fetal Bovine Serum, qualified, heat inactivated	16140071
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) *Alternative to Thermo Fisher product*	97068-085
For Cell Counting		
Thermo Fisher Scientific	Countess II FL Automated Cell Counter	AMQAF1000
	Countess II FL Automated Cell Counting Chamber Slides	C10228
	Trypan Blue Stain (0.4%)	T10282

This list may not include some standard laboratory equipment.

Tips & Best Practices

Cell Viability

- Determine sample viability before starting the cell surface protein labeling protocol.

Labeling & Wash Buffer

- Labeling or washing buffer for most sample types is PBS + 1% BSA.
- For samples containing <70% viable cells, PBS + 10% FBS can be used.
- BioLegend's Cell Staining Buffer can also be used for labeling cells. However, this buffer may not be optimal for all sample types. Cell Staining Buffer should only be used for the labeling step. PBS + 1% BSA should be used for the washing steps.
- If using lyophilized antibody panel/cocktails, please follow BioLegend's instructions for use regarding reconstitution and labeling volumes.
- The addition of True-Stain Monocyte Blocker to Human TruStain FcX during labeling can generally help to reduce staining background (e.g. monocytes and T cells).

Centrifugation Conditions

- Centrifugation speed and time depend upon the sample type.
- Larger or fragile cell types may require slower centrifugation speeds.

Table 1. Sample type specific centrifugation conditions

Sample Type	Centrifugation Conditions
Samples containing >70% viable cells, e.g., PBMCs	400 rcf for 5 min
Samples containing <70% viable cells, e.g., tumor cells	150 rcf for 10 min

Optimal Antibody Concentration

- Consult Technical Note Quality Control of Cell Surface Protein Labeling using Flow Cytometry (CG000231) for guidance on how to titrate antibodies using flow cytometry for determining optimal antibody concentrations.

Sample Washing

- This protocol provides three different wash options after incubation with the antibody-oligonucleotide conjugate. See the following page for guidance on choosing the appropriate wash option.

Sample Fixation

- Consult Demonstrated Protocol Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling (CG000478) for guidance on fixing single cell or nuclei suspensions following cell surface protein labeling.

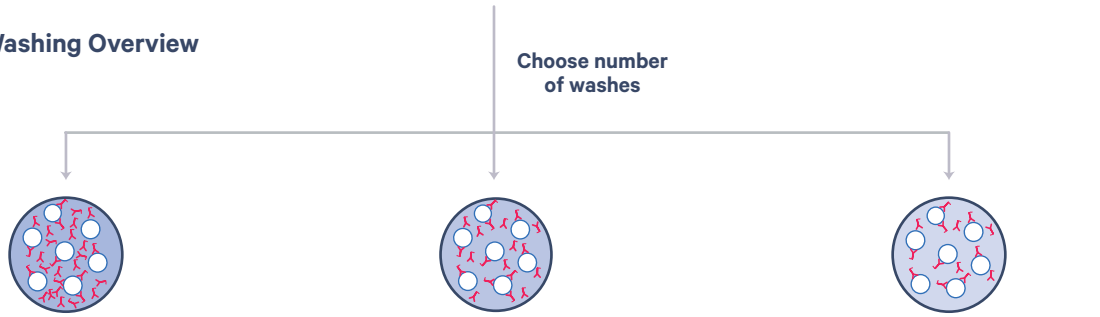
Protocol Overview

Below is a general illustrative overview of the cell surface protein labeling protocol and wash options. Using the appropriate wash option is critical for obtaining high quality data and minimizing background signal. Choose a washing protocol based on the guidance outlined in the following diagram. Refer to the Appendix for supplemental data on the different wash options.

Protocol & Sample Washing Overview

Protocol Overview

Sample Washing Overview



No-Wash Option
(Proceed directly to Sample Fixation, Step c (CG000478)

- For samples with low viability or input counts or time-sensitive samples.
- Antibody background is expected to be high.
- Compatible with antibodies that have clear distinction between populations.

1-Wash Option
(Includes option for FACS enrichment)

- For when a reduced number of wash steps is desired.
- Antibody background is expected to be moderate.
- Compatible with most antibodies that have a clear distinction between populations.

2-Wash Option
(recommended)

- The recommended wash protocol for most sample types.
- Antibody background is expected to be low.
- For best quality Feature Barcode data.

* Refer to Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478).

Cell Surface Protein Labeling Protocol

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

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

Buffers – Preparation

For Labeling Cells

- Chilled (**4°C**): PBS + 1% BSA
- For samples containing <70% viable cells, chilled (**4°C**) PBS + 10% FBS can be used.

This protocol was optimized using 300,000–2 x 10^6 cells.

Prepare Antibody Mix Supernatant

- Add appropriate/manufacturer's recommended amount of TotalSeq-B antibody-oligonucleotide conjugates to a 1.5-ml microcentrifuge tube.
- If using lyophilized antibody panel/cocktails, rehydrate lyophilized panel in the recommended volume of Cell Staining Buffer as directed from BioLegend. Follow manufacturer's instructions for use for cell labeling. Perform cell wash steps as described in this Demonstrated Protocol.
- 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 FACS Antibody Pool

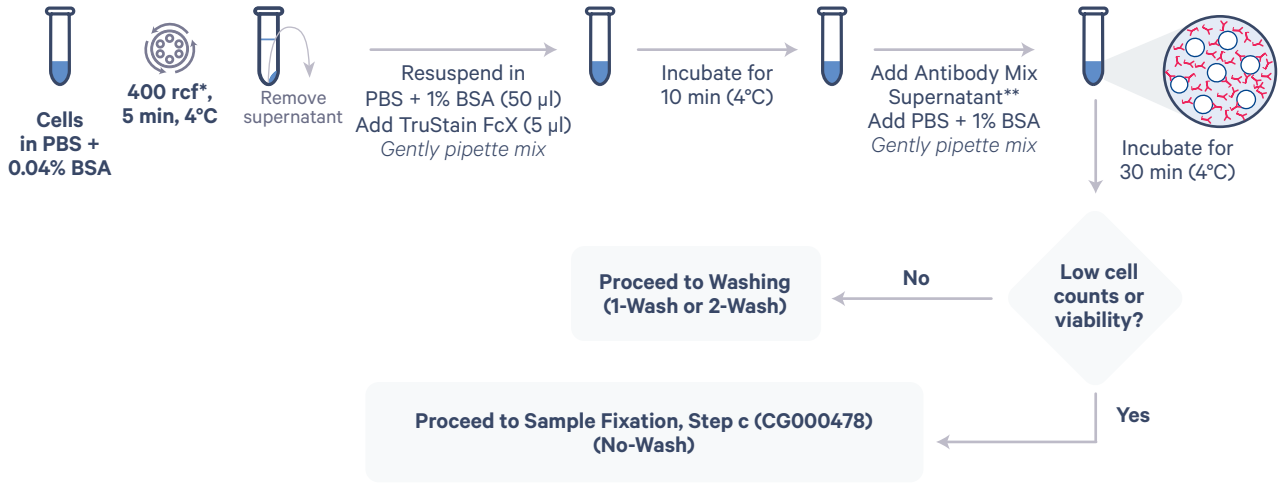
- Add appropriate/manufacturer's recommended amount of fluorophore antibodies to a 1.5-ml microcentrifuge tube on ice.
- Gently pipette mix and maintain at **4°C**. Avoid light exposure.

Labeling

When using custom conjugated antibodies, be sure to follow manufacturer's instructions. Refer to Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols (Document CG000149) for details. For preconjugated antibodies, we recommend BioLegend TotalSeq™-B.

Label Cells

Prepare Antibody Mix Supernatant and FACS Antibody Pool (if performing FACS enrichment) as described in Buffers – Preparation.



*For dissociated tumor cells, centrifuge at **150 rcf** for **10 min** at **4°C**.
**If performing FACS, add FACS Antibody Pool.

Protocol Steps:

a. Transfer ≤2 x 10^6 cells to a new 1.5-ml microcentrifuge tube.

b. Centrifuge cells at **400 rcf** for **5 min** (PBMCs) or at **150 rcf** for **10 min** (dissociated tumor cells) at **4°C**. Use of a swinging-bucket rotor is recommended for higher cell recovery. Centrifugation speed and time depend upon the sample type. Larger or fragile cell types may require slower centrifugation speeds.

c. Remove supernatant without disturbing pellet.

d. Resuspend pellet in **50 µl** chilled PBS + 1% BSA. For samples containing <70% viable cells, chilled PBS + 10% FBS can be used.

e. Add **5 µl** Human TruStain FcX. Gently pipette mix. **OPTIONAL: 5 µl** True-Stain Monocyte Blocker can also be added at this step in addition to TruStain FcX. See Tips & Best Practices for more information.

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

g. Add prepared Antibody Mix Supernatant. If performing FACS, 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). For samples containing <70% viable cells, chilled PBS + 10% FBS can be used.

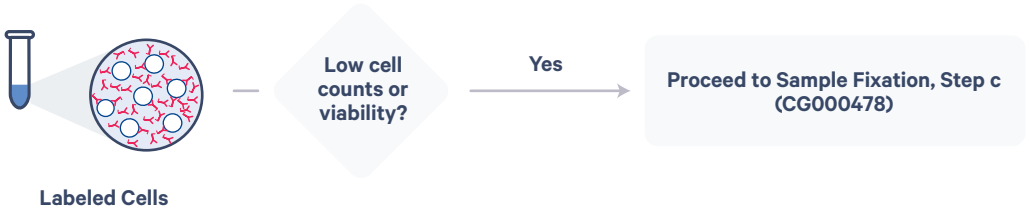
i. Incubate for **30 min** at **4°C**. If using FACS antibodies, incubate without light exposure.

j. Proceed to appropriate **Washing** section for **1-Wash or 2-Wash Options**
OR
Proceed directly to **Sample Fixation, Step c** of the Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478) (**No-Wash Option**).

TIPS *Sample fixation without washing is recommended for samples with low inputs, low viability, or time-sensitive samples where increased background is acceptable.*

Washing

No-Wash Option



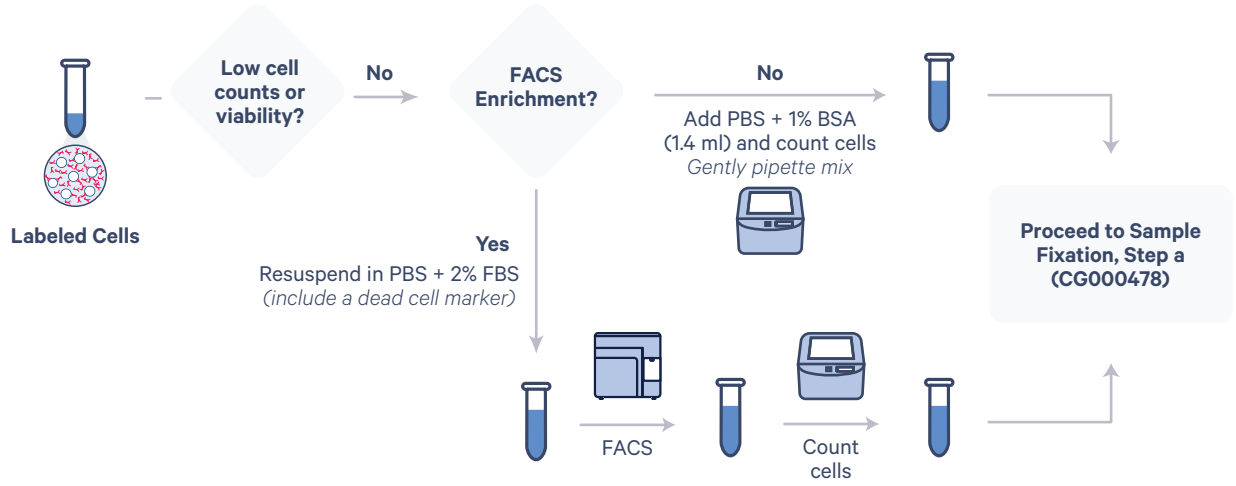
Protocol Steps:

a. Proceed **immediately** to **Sample Fixation, Step c** of the Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478).

Step c: *Add* ***1 ml*** *Fixation Buffer to the labeled cells and pipette mix 5x. Fixation Buffer preparation and fixation protocol are listed in Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478).*

Washing

1-Wash Option



Protocol Steps:

a. OPTIONAL: For enrichment of labeled and viable 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.
- Proceed to **FACS (see FACS Guidance)**. After FACS, determine cell concentration and viability using a Countess II Automated Cell Counter or a hemocytometer.

If not performing FACS enrichment:

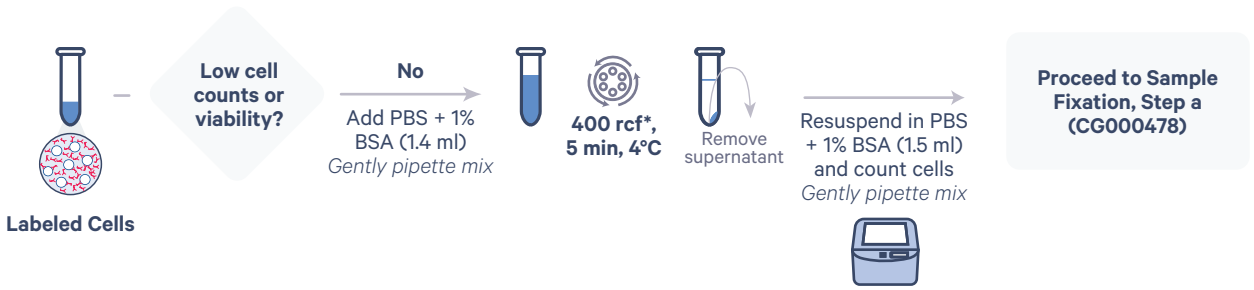
- Add **1.4 ml** chilled PBS + 1% BSA to the labeled cells. Gently pipette mix. For samples containing <70% viable cells, chilled PBS + 10% FBS can be used.

b. After resuspension, take an aliquot and determine cell concentration and viability using a Countess II Automated Cell Counter or a hemocytometer.

c. Proceed **immediately** to **Sample Fixation, Step a** of the Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478).

Washing

2-Wash Option



*For dissociated tumor cells, centrifuge at **150 rcf** for **10 min** at **4°C**.

Protocol Steps:

a. Add **1.4 ml** chilled PBS + 1% BSA to the labeled cells. Gently pipette mix. For samples containing <70% viable cells, chilled PBS + 10% FBS can be used.

b. Centrifuge cells at **400 rcf** for **5 min** (PBMCs) or at **150 rcf** for **10 min** (dissociated tumor cells) at **4°C**. Centrifugation speed and time depends upon the sample type.

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

Leaving behind excess supernatant (>30 µl) may cause non-specific binding, which may result in increased background reads during sequencing.

d. Resuspend the cell pellet in **1.5 ml** chilled PBS + 1% BSA and place on ice. For samples containing <70% viable cells, chilled PBS + 10% FBS can be used.

e. After resuspension, take an aliquot and determine cell concentration and viability using a Countess II Automated Cell Counter or a hemocytometer.

f. Proceed **immediately** to **Sample Fixation, Step a** of the Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478).

Appendix

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 300 µl volume in a 5-ml tube. 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 should be counted and viability measured before proceeding to the Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478).

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

*Once sorting is complete, proceed **immediately** to **Sample Fixation, Step a** of the Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478).*

Antibody-Oligonucleotide Conjugate Capture

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

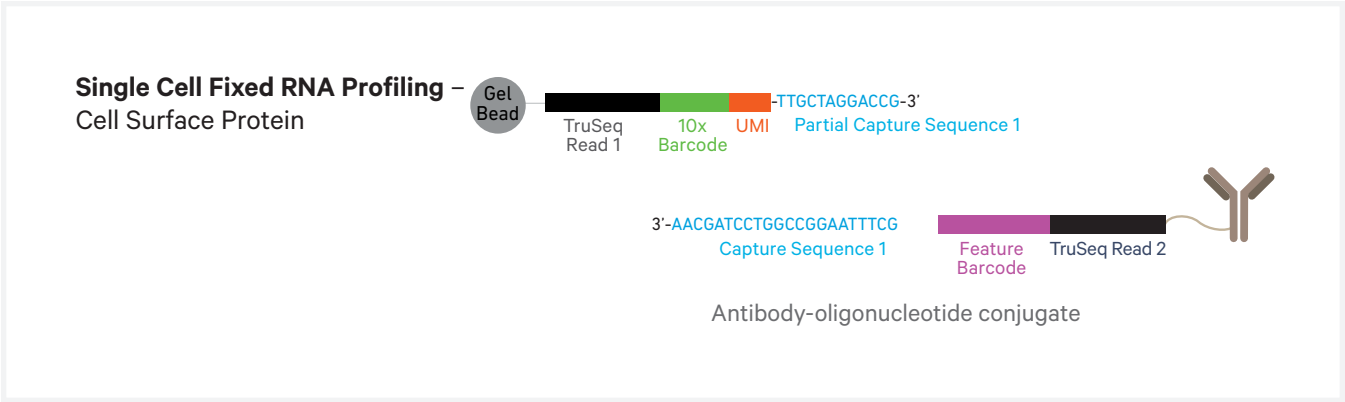


Figure 1. Illustrative overview of antibody-oligonucleotide conjugate capture.

Appendix, *contd.*

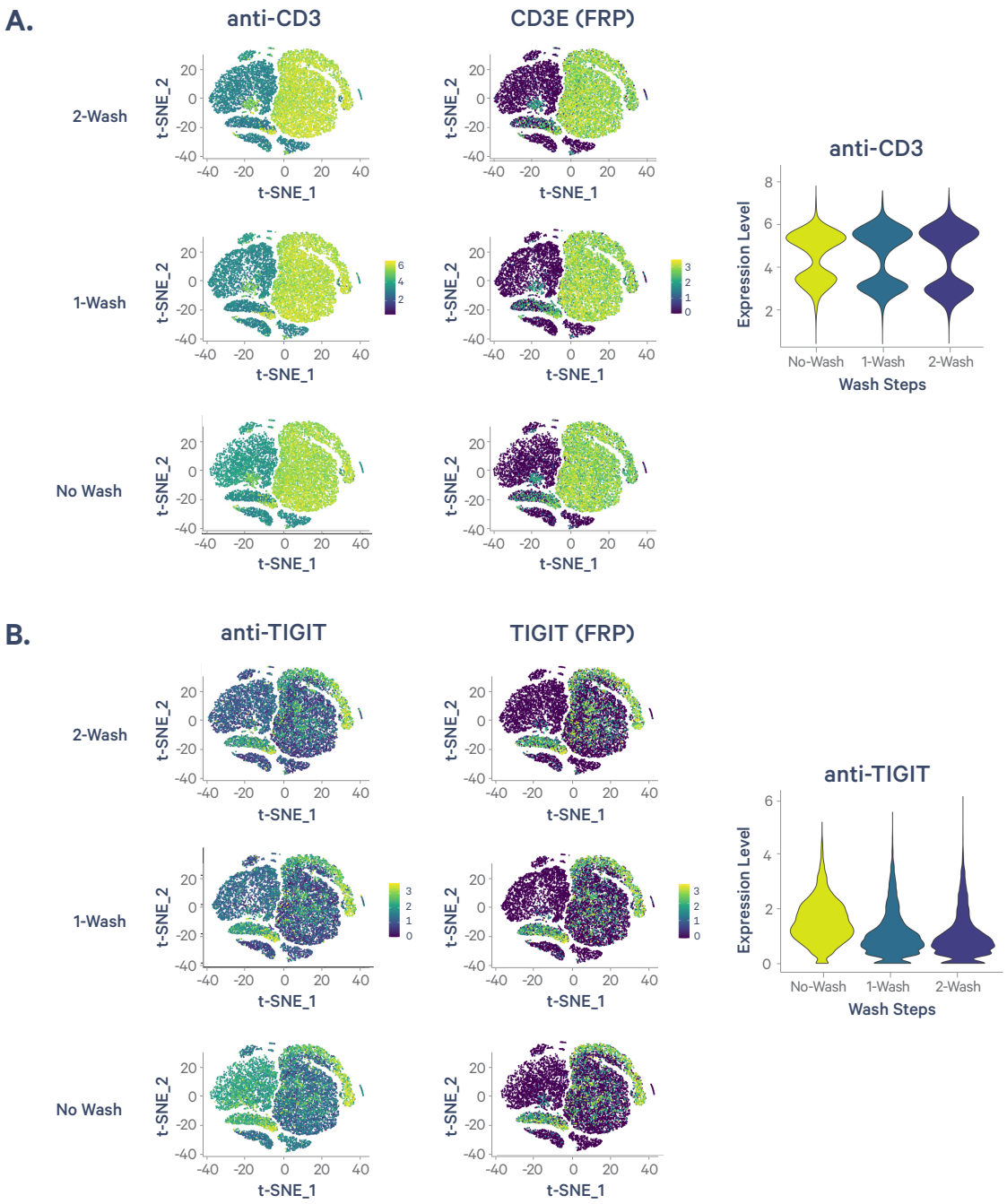


Figure 2. A. t-SNE plots of human PBMCs comparing Feature Barcode staining for T cell marker CD3 across differing number of washes following antibody incubation (left). Corresponding CD3E gene expression measured with Fixed RNA Profiling (FRP). Scale adjusted for better visualization with top 1% of cells removed. Violin plot showing the expression of the anti-CD3 antibody Feature Barcode (right). **B.** t-SNE plots of human PBMCs comparing Feature Barcode staining for Natural Killer and T cell marker TIGIT across differing number of washes following antibody incubation (left). Corresponding TIGIT gene expression measured with Fixed RNA Profiling (FRP). Scale adjusted for better visualization with top 1% of cells removed for each feature. Violin plot showing the expression of the anti-TIGIT antibody Feature Barcode (right).

Appendix, *contd.*

Table 2. Signal to noise for select antibodies

Antibody	Comparison	Signal to Noise* (On-target cells / all other cells)		
		No-Wash	1-Wash	2-Wash
anti-CD3	T cells / Other	6.24	8.88	10.15
anti-CD4	T cells / Other	4.51	4.23	3.86
anti-CD8	T cells / Other	4.43	7.67	7.33
anti-TIGIT	T & NK cells / Other	1.04	1.77	2.04
anti-CD19	B cells / Other	53.84	92.20	112.16
anti-CD15	Monocytes / Other	8.84	11.91	12.80
anti-CD14	Monocytes / Other	14.62	16.94	19.88
anti-CD11c	DCs & Monocytes / Other	22.96	27.93	28.33

*Ratio of mean expression level

Signal to noise was generated by calculating the ratio of an expected group comprised of cell type(s) expected to display antibody signal (e.g. CD3 on T cells) and a background group (those cell types that would not be expected to display antibody signal, e.g. CD3 on B cells).

Conclusions

Choosing an appropriate wash protocol following cell surface protein labeling is critical for experimental success in the Chromium Fixed RNA Profiling assay. The data presented in this Demonstrated Protocol show that a lower number of washes following cell labeling can be used for antibodies with distinct positive and negative populations (Figure 2A, CD3 data). However, lower number of washes cause a reduction in separation between the positive and negative populations (Figure 2A, violin plot; Table 2, lower signal to noise for CD3, CD14, CD19, etc). Feature Barcode data from antibodies with poorer separation between the positive and negative populations is adequate with the 1-Wash protocol, but the No-Wash protocol is not typically recommended due to poor signal to noise (Figure 2B, TIGIT; Table 2, TIGIT).

References

1. Chromium Fixed RNA Profiling Reagent Kits for Singleplexed Samples with Feature Barcode technology for Cell Surface Protein User Guide (CG000477)
2. Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples User Guide (CG000527)
3. Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478)

Document Revision Summary

Document Number	CG000529
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Contact:
support@10xgenomics.com
10x Genomics
6230 Stoneridge Mall Road
Pleasanton, CA 94588 USA