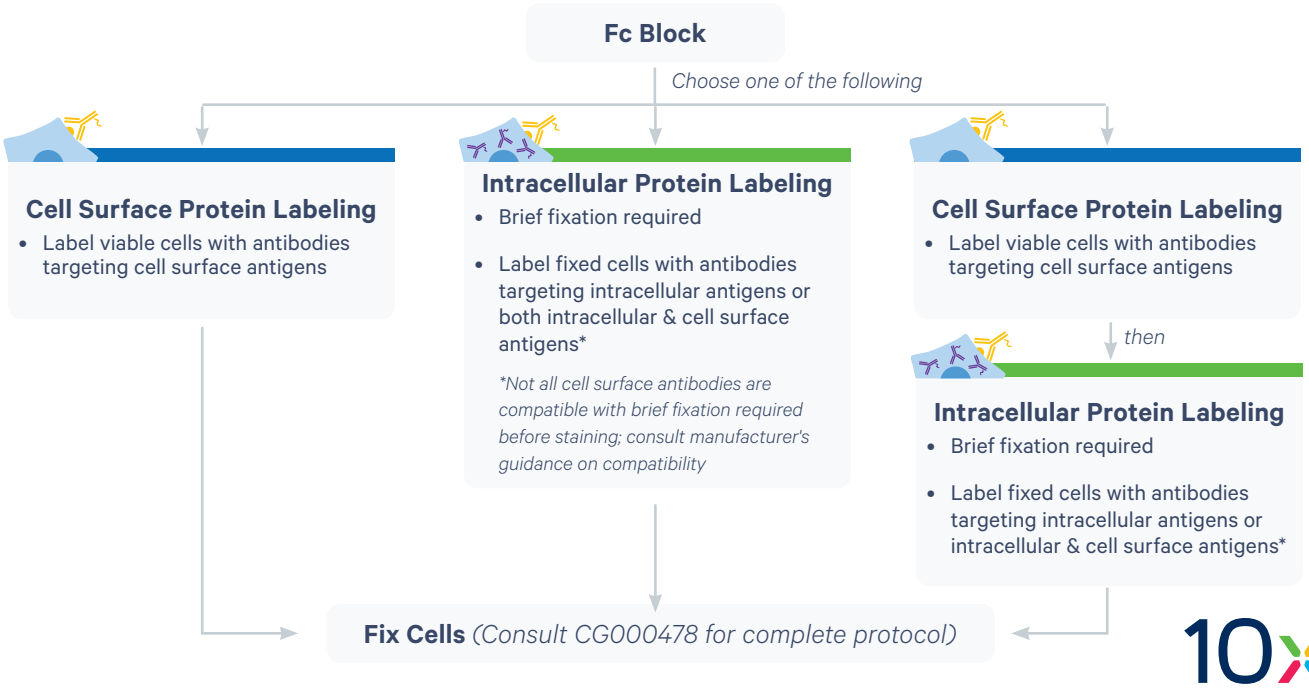
Cell Surface & Intracellular Protein Labeling for Chromium Fixed RNA Profiling

Introduction

Chromium Fixed RNA Profiling (Gene Expression Flex) with Feature Barcode technology offers comprehensive, scalable solutions to measure gene and protein expression in formaldehyde fixed samples. To measure protein expression, cell surface and intracellular proteins are labeled using an antibody conjugated to a Feature Barcode oligonucleotide, followed by a fixation with formaldehyde to ensure 1) the cells are properly fixed and permeabilized for probe hybridization and 2) the antibodies firmly bind to the cells, preventing their loss during probe hybridization.

This document outlines cell surface and intracellular protein labeling protocols for use with Chromium Fixed RNA Profiling. General guidance on antibody-oligonucleotide conjugation, evaluating antibodies with flow cytometry, and best practices when staining cells is also provided here.

Both the cell surface and intracellular protein labeling protocols described in this document require a Fc receptor blocking step, after which the appropriate labeling protocol should be followed depending on the type of antibodies used. The Intracellular Protein Labeling Protocol, which includes a brief fixation before staining to allow cell permeabilization, can also be used with certain fixation compatible cell surface protein antibodies.



Additional Guidance

Preread and have available Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478) before starting the protocols provided in this document. Consult the Cell Preparation Guide Handbook (CG00053) for Tips & Best Practices on handling and counting cells. Consult Cell Thawing Protocols for Single Cell Assays (CG000447) for guidance on thawing dissociated tumor cells.

Consult Chromium Fixed RNA Profiling - Protocol Planner (CG000528) for details on workflow overview, document resources, and guidance on selecting the appropriate sample preparation and library construction protocols for different Chromium Fixed RNA workflows.

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

For Cell Surface Protein Labeling

Vendor	Item	Part Number
BioLegend	Human TruStain FcX (Fc Receptor Blocking Solution)	422301
	TruStain FcX PLUS (anti-mouse CD16/32) Antibody	156604
	True-Stain Monocyte Blocker	426101
	TotalSeq™-C Human Universal Cocktail, V1.0	399905
	TotalSeq™-B Human Universal Cocktail, V1.0	399904
	Choose appropriate antibodies based on the Chromium Fixed RNA Profiling workflow. See TotalSeq™ 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*	A1595
Miltenyi Biotec	MACS BSA Stock Solution *Alternative to Thermo Fisher*	130-091-376
Corning	Phosphate-Buffered Saline, *1X without Calcium and Magnesium*	21-040-CV
VWR	Fetal Bovine Serum (FBS) *Alternative to Thermo Fisher*	97068-085
For Cell Counting		
Thermo Fisher Scientific	Countess II FL Automated Cell Counter	AMQAF1000
	Countess 3 FL Automated Cell Counter	A49866
	Countess Cell Counting Chamber Slides	C10228
	Trypan Blue Stain (0.4%)	T10282
Nexcelom Bioscience	Celleca MX High-throughput Automated Cell Counter	MX-112-0127
	ViaStain AOPI Staining Solution	CS2-0106-5mL
	Cellometer K2 Bundle w/ Matrix Software	CMT-K2-MX-150
	VS Cellometer AOPI Staining Solution	CS2-0106-25ML
	PD100 Counting Chambers 1 case	CHT4-PD100-003

This list may not include some standard laboratory equipment.

TotalSeq™ Antibody-Oligonucleotide Conjugates

Chromium Fixed RNA Profiling Workflows	TotalSeq™ Antibody-Oligonucleotide Conjugates
Gene & Protein Expression using Barcode Oligo Capture – Singleplex & Multiplex Workflows	TotalSeq™-C antibody-oligonucleotide conjugates
Gene & Protein Expression – Singleplex Workflow	TotalSeq™-B antibody-oligonucleotide conjugates

Choose appropriate TotalSeq™ antibody-oligonucleotide conjugates based on the Chromium Fixed RNA Profiling workflow.

For Intracellular Protein Labeling

Vendor	Item	Part Number
10x Genomics	Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit*	1000414
BioLegend	Human TruStain FcX (Fc Receptor Blocking Solution)	422301
	TruStain FcX PLUS (anti-mouse CD16/32) Antibody	156604
	True-Stain Monocyte Blocker	426101
	TotalSeq™ intracellular conjugates	-
Proteintech Genomics	MultiPro Human Fixed Cell Immune Profiling Antibody cocktail	G900004
Corning	Phosphate-Buffered Saline, *1X without Calcium and Magnesium*	21-040-CV
Thermo Fisher Scientific	Tween 20	28320
	NP-40 Surfact-Amps Detergent Solution (10%)	85124
	Nonidet P40 Substitute, Ultrapure, Thermo Scientific Chemicals *Alternative to NP-40; consult manufacturer's instructions for preparing 10% stock solution*	J19628.AP
	Formaldehyde (37% by Weight/Molecular Biology), Fisher BioReagents	BP531-25
	Nuclease-free Water (not DEPC-Treated)	AM9937
Millipore Sigma	Albumin, Bovine Serum, Fraction V, Fatty Acid-Free, Nuclease- and Protease-Free	126609
	Dextran Sulfate Sodium Salt 8 KDa	RES2029D-A7
Invitrogen	UltraPure Salmon Sperm DNA Solution	15-632-011
	Phosphate-Buffered Saline (10X) pH 7.4, RNase-free	AM9625
Avantor	PBS 1X	K812-500ML
Roche	Protector RNase inhibitor	3335399001
Additional Materials		
Eppendorf	DNA LoBind Tubes 2.0 ml	022431048
	1.5mL Protein LoBind tubes	022431081
	1.5mL DNA LoBind tubes	022431021
	ThermoMixer C	5382000023
VWR	Vortex Mixer	10153-838
Corning	Phosphate-Buffered Saline, *1X without Calcium and Magnesium*	21-040-CV
New England Biolabs	T4 Gene 32 Protein**	M0300S/ M0300L
For Cell Counting		
Thermo Fisher Scientific	Countess II FL Automated Cell Counter	AMQAF1000
	Countess Cell Counting Chamber Slides	C10228
	Trypan Blue Stain (0.4%)	T10282
Nexcelom	Celleca MX High-throughput Automated Cell Counter	MX-112-0127
	ViaStain AOPI Staining Solution	CS2-0106-5mL

***Optional: Only required if adding to the antibody pool + buffer to reduce background signal.*

*Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit, 16 rxns PN-1000414

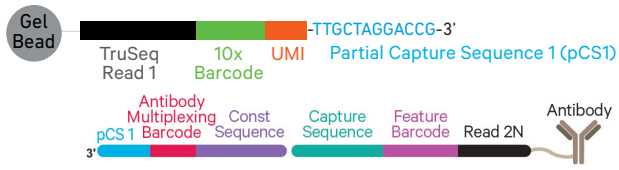
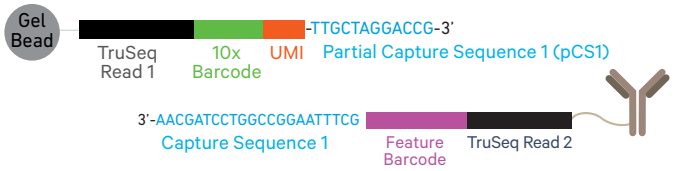
Chromium
Single Cell Fixed RNA Sample Preparation Kit
16 rxns, PN-1000414
Store at -20°C

	#	PN
Conc. Fix & Perm Buffer	3	2000517
Conc. Quench Buffer	6	2000516
Enhancer	1	2000482

Antibody-Oligonucleotide Conjugates

- The Cell Surface Protein Labeling protocol described in this document was optimized using preconjugated TotalSeq™-C or TotalSeq™-B antibodies from BioLegend. Choose appropriate antibody-oligonucleotide conjugates based on the Chromium Fixed RNA Profiling workflow (see the table below).
- The Intracellular Protein Labeling protocol was optimized using preconjugated TotalSeq™ intracellular conjugates from Biolegend & MultiPro Fixed Cell Immune Profiling Antibody Cocktail, a pool of cell surface, intracellular, & isotype control antibodies, from Proteintech Genomics.
- Antibodies conjugated to appropriate Feature Barcode oligonucleotide from other vendors can also be used. See the table below for antibody-oligonucleotide conjugate capture by protocol-specific Gel Bead primers.
- Both the labeling protocols are also compatible with custom conjugated antibody panels.

Antibody-Oligonucleotide Conjugate Capture

Chromium RNA Fixed RNA Profiling Workflows	Antibody-Oligonucleotide Conjugates
Gene & Protein Expression using Barcode Oligo Capture* - Singleplex & Multiplex Workflows Consult the following User Guides for more information: • Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture User Guide (CG000673). • Chromium Fixed RNA Profiling Reagent Kits for Singleplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture User Guide (CG000674).	/5AmMC12/CGGAGATGTGTATAAGAGACAG-N10-N15-N9-CCCATATAAGAAA Nextera partial Read 2 Feature Barcode (15 nt) Capture Sequence **TotalSeq™-C antibody-oligonucleotide conjugates** /5AmMC12/CGGAGATGTGTATAAGAGACAG-N15-CCCATATAAGAAA Nextera partial Read 2 Feature Barcode (15 nt) Capture Sequence **MultiPro Fixed Cell Immune Profiling Antibody Cocktail** 
Gene & Protein Expression - Singleplex Workflow Consult the following User Guide for more information: • Chromium Fixed RNA Profiling Reagent Kits for Singleplexed Samples with Feature Barcode technology for Protein User Guide (CG000477).	/5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-N10-N15-N9-GCTTTAAGGCCGGTCCTAGCAA TruSeq Read 2 Feature Barcode (15 nt) Capture Sequence 1 **TotalSeq™-B antibody-oligonucleotide conjugates** 

**Barcode Oligo Capture can be achieved by using either Totalseq™-C or MultiPro Fixed Cell Immune Profiling Antibody Cocktail. If using both the antibodies in a single experiment, an alternative sequencing configuration will be required. Consult 10x Genomics support website for details.*

Conjugation Guidance

- Follow manufacturer's instructions (Antibody Conjugation Kit from Abcam and oYo-Link Antibody Labeling Reagents from AlphaThera) for antibody-oligonucleotide conjugation.
- Antibody conjugation services from Abcam and AlphaThera can also be utilized.
- Consult Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology Demonstrated Protocol (CG000149) for guidance on antibody-oligonucleotide conjugation and verification of conjugation.

Fc Receptor Blocking

Both cell surface and intracellular labeling protocols require a Fc receptor blocking step. After this step, follow the appropriate labeling protocol based on the type of antibodies used.

Buffers – Preparation

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

a. Resuspend cells in PBS + 1% BSA.

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

b. Transfer ≤1 x 10^6 cells to a new 1.5-ml microcentrifuge tube.

c. Centrifuge cells at **400 rcf** for **5 min** (PBMCs) 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.

d. Remove the supernatant without disturbing the pellet.

e. Resuspend pellet in **50 µl** chilled PBS + 1% BSA or chilled PBS + 10% FBS (for <70% viable cells).

TIPS *If using >1 x 10^6cells, scale up the buffer volumes accordingly.*

f. Add **5 µl** Human TruStain FcX or **2 µl** TrueStain FcX PLUS (anti-mouse CD16/32 Antibody). Gently pipette mix.

OPTIONAL: If performing cell surface protein labeling, **5 µl** True-Stain Monocyte Blocker can also be added at this step in addition to TruStain FcX to reduce staining background.

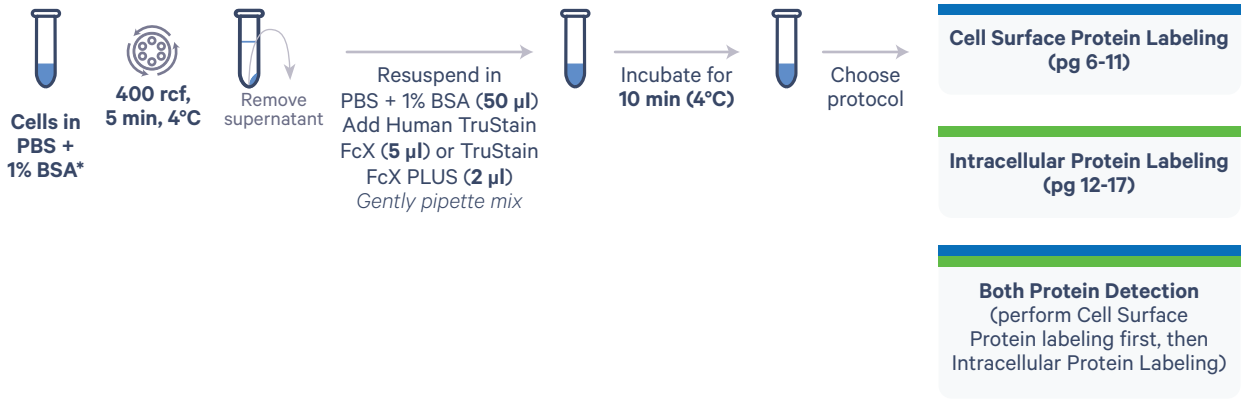
TIPS *Consult manufacturer's instruction for the recommended volume of Fc receptor blocking solutions.*

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

h. Immediately proceed to the appropriate step:

- Cell surface protein detection only: proceed to Cell Surface Protein Labeling
- Intracellular protein detection only and/or using cell surface antibodies that are compatible with brief fixation: proceed to Intracellular Protein Labeling
- Both cell surface protein and intracellular protein detection: proceed to Cell Surface Protein Labeling followed by Intracellular Protein Labeling

Block Fc receptor



*For samples containing <70% viable cells, PBS + 10% FBS can be used.

Cell Surface Protein Labeling Protocol

Cell Surface Protein Labeling Protocol Overview

Label Cells

Wash Cells
(no wash, 1-wash, or 2-wash options)

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

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

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

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 protocol. See Appendix for supplemental data on the different wash options.

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.

Prepare Antibody Mix Supernatant

Add the appropriate/manufacturer's recommended amount of antibody-oligonucleotide conjugates to a 1.5-ml Protein LoBind tube.

- If using lyophilized antibody panel/cocktails, rehydrate the lyophilized panel in the recommended volume of Cell Staining Buffer as directed from BioLegend. Follow the 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 protein Lobind tube. Leave residual volume so the antibody aggregates are not carried over. Maintain at 4°C.

Prepare Flow Sorting Antibody Pool

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

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.
- Follow BioLegend's instructions if using lyophilized antibody panel/cocktails regarding reconstitution and labeling volumes.

Centrifugation Conditions

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

Optimal Antibody Concentration

- The optimal concentration is 0.1 µg to 0.5 µg.
- Consult Quality Control of Cell Surface Protein Labeling using Flow Cytometry Technical Note (CG000231) for guidance on how to titrate antibodies using flow cytometry for determining optimal antibody concentrations.
- It is highly recommended to titrate antibodies before use for optimal performance. Consult the manufacturer's instructions for recommended dilutions for each antibody. A dilution of 0.5 µg per antibody for up to 1 x 10^6 cells is suggested as a starting point.

Sample Washing

- This protocol provides three wash options after incubation with the antibody-oligonucleotide conjugate. See Cell Surface Protein Labeling: Wash Cells for guidance on choosing the appropriate wash option.

Sample Fixation

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

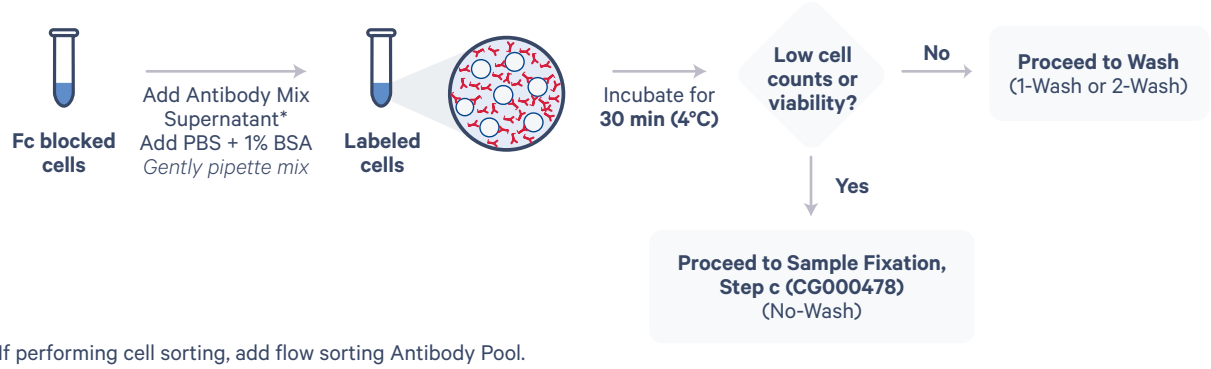
Sample Storage

- After labeling, samples may be fixed in smaller batches and stored. If batching samples together, it is recommended that samples with different fixation times in one experiment are not mixed.
- Fixed samples can be stored either at -80°C for up to 6 months or at 4°C for up to 7 days with appropriate storage reagents.
- Consult Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478) for details on storage conditions and reagents.

Cell Surface Protein Labeling: Label Cells

Label Cells

Prepare Antibody Mix Supernatant and Flow Sorting Antibody Pool (if performing flow sorting) as described in Buffers – Preparation.



*If performing cell sorting, add flow sorting Antibody Pool.

Follow manufacturer's instructions if using custom conjugated antibodies. Consult Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols Demonstrated Protocol (CG000149) for details.

a. Add prepared Antibody Mix Supernatant to the Fc blocked sample. If performing flow sorting, add Flow Sorting Antibody Pool.

b. 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.

c. Incubate for **30 min** at **4°C**. If using fluorophore-conjugated antibodies, incubate without light exposure.

d. Proceed to appropriate **Cell Surface Protein Labeling: Wash Cells** 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.*

Cell Surface Protein Labeling: Wash Cells

Using the appropriate wash option is critical for obtaining high-quality data and minimizing background signal. Choose a protocol based on the following guidance. See the Appendix for supplemental data on the different wash options.

No Wash Option

- 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

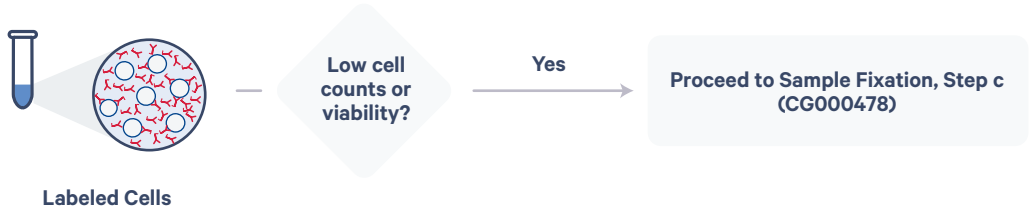
- When a reduced number of wash steps is desired
- Antibody background is expected to be moderate
- Compatible with most antibodies that have a clear distinction between populations
- Includes option for flow sorting enrichment

2-Wash Option (Recommended)

- Recommended for most sample types
- Antibody background is expected to be low
- Best quality Feature Barcode data

No Wash Option Protocol

No-Wash Option



a. Proceed **immediately** to:

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

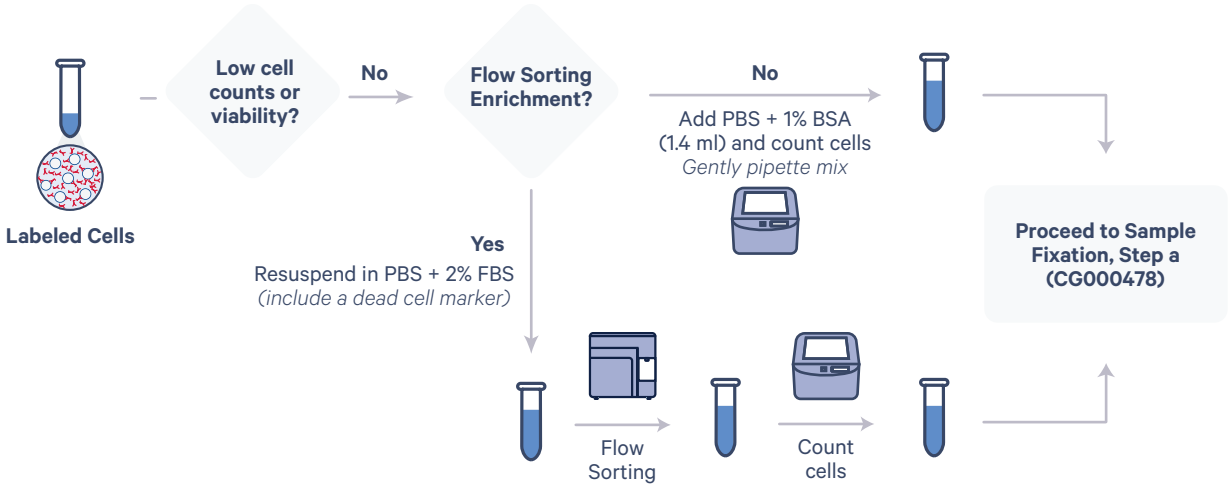
  Step c: *Add* ***1 ml*** *Fixation Buffer to the labeled cells and pipette mix 5x. Fixation Buffer preparation and fixation protocol are listed in Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478).*

OR

- Intracellular Protein Labeling

1-Wash Option Protocol

1-Wash Option



a. OPTIONAL: For enrichment of labeled and viable cells by flow sorting:

- 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 sorting (see Appendix for Flow Sorting Guidance). After sorting, determine cell concentration and viability using an automated cell counter or a hemocytometer.

If not performing flow sorting 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 an automated cell counter or a hemocytometer.

c. Proceed **immediately** to:

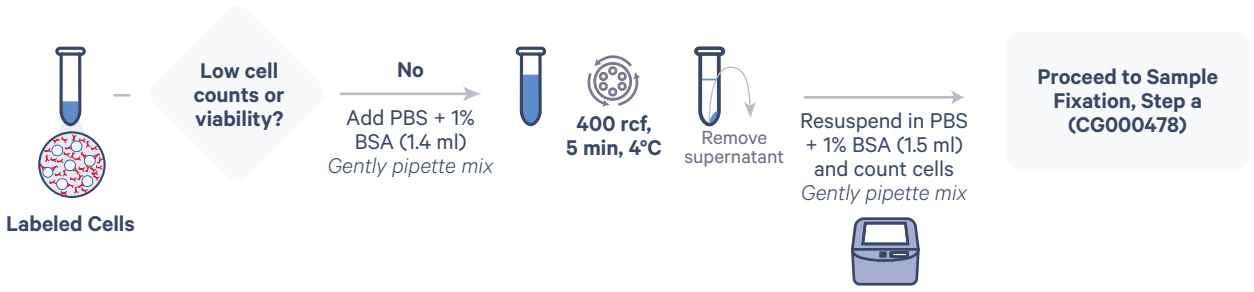
- **Sample Fixation**, **Step a** of the Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478)

OR

- Intracellular Protein Labeling

2-Wash Option Protocol

2-Wash Option



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) at **4°C**. Centrifugation speed and time depend 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 nonspecific 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 an 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)

OR

- Intracellular Protein Labeling

Intracellular Protein Labeling

Intracellular Protein Labeling Protocol Overview

Fix Cells
Brief fixation

Label Cells

Fix Cells
CG000478

This protocol was optimized using human PBMCs and the TotalSeq™ intracellular conjugates from Biolegend and MultiPro Fixed Cell Immune Profiling Antibody Cocktail, a pool of cell surface, intracellular, and isotype control antibodies, from Proteintech Genomics.

This protocol involves a short fixation before staining and can be also be used with other cell surface protein antibodies, provided they are compatible with the brief fixation step. The labeled cells can be enriched by flow sorting (see Appendix for Flow Sorting Guidance).

Use distinct and compatible antibody clones for sorting. Optimize the working concentration of each antibody used.

Buffers – Preparation

For Fixing Cells

Buffers for Fixation - Prepare fresh			
Fixation Buffer *Maintain at room temperature*	**Stock**	**Final**	**Per Sample + 10% (µl)**
Nuclease-free Water	-	-	871.1
Conc. Fix & Perm Buffer (10x Genomics PN-2000517) *Thaw at room temperature. Vortex, check for precipitation, and centrifuge briefly.*	10X	1X	110.0
Formaldehyde*	37%	4%	118.9
Total	**-**	**-**	**1,100.0**

**Formaldehyde should always be used with adequate ventilation, preferably in a fume hood. Follow appropriate regulations.*

Buffers for Fixation - Prepare fresh			
Quenching Buffer *Maintain at 4°C*	**Stock**	**Final**	**Per Sample + 10% (µl)**
Nuclease-free Water	-	-	962.5
Conc. Quench Buffer (10x Genomics PN-2000516) *Thaw at room temperature. Vortex and centrifuge briefly.*	8X	1X	137.5
Total	**-**	**-**	**1,100.0**

For Labeling Cells

Flow Sorting Antibody Pool Preparation

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

Intracellular Antibody Mix Supernatant (Antibody + Buffer) Preparation

Follow the manufacturer's recommendations for resuspension and aggregate removal. General guidance is provided below.

If using a lyophilized antibody panel:

- Equilibrate the lyophilized panel vial(s) to **room temperature** for **5 min**.
- Place the lyophilized panel vial in an empty 2-ml tube and centrifuge the vial at **10,000 rcf** for **30 sec** at **room temperature**.

a. Prepare Antibody Buffer in a Protein LoBind tube.

Buffer for Labeling - Prepare fresh			
Antibody Buffer *Maintain at 4°C*	**Stock**	**Final**	**For 1 Sample + 10% (µl)**
RNase Inhibitor	40 U/µl	0.4 U/µl	1.1
Nuclease-free BSA	30%	7.55%	27.68
Tween 20	10%	0.2%	2.2
NP-40 or Nonidet P40 Substitute	10%	0.5%	5.5
Salmon Sperm DNA	10 mg/ml	0.5 mg/ml	5.5
Dextran Sulfate 8 KDa	1%	0.025%	2.75
Monocyte Blocker	-	5 µl	5.5
FcX	-	5 µl	5.5
10X PBS	10X	1X	11.0
Nuclease-free Water* *If using lyophillized antibody*	-	-	43.27*
Total	**-**	**-**	**110.0**

**Adjust water volume if:*

- *adding reconstituted antibody pool derived from non-lyophilized antibodies*
- *adding fluorophore-conjugated antibodies for flow sorting*

Final buffer volume should not exceed 110 µl per sample.

b. In case of lyophilized antibody panel: Add **110 µl** Antibody Buffer to rehydrate the lyophilized antibody panel. If using non-lyophilized antibody, directly proceed to step g.

c. Vortex the rehydrated antibody pool for **15 sec** and centrifuge at **10,000 rcf** for **30 sec** at **room temperature**.

d. Incubate for **5 min** at **room temperature**.

e. Vortex for **15 sec** and centrifuge at **10,000 rcf** for **30 sec** at **room temperature**.

f. Transfer the entire volume of the reconstituted antibody pool to a new Protein LoBind tube and maintain at **4°C**.

g. Centrifuge at **14,000 rcf** for **10 min** at **4°C**.

h. Transfer **100 µl** supernatant (containing antibody + buffer) to a new Protein LoBind tube and maintain at **4°C**.

For Washing Cells

Buffer for Washing - Prepare fresh			
Intracellular Wash Buffer *Maintain at 4°C*	**Stock**	**Final**	**For 1 Sample + 10% (µl)**
RNase Inhibitor	40 U/µl	0.2 U/µl	11.0
Tween 20	10%	0.1%	22.0
Nuclease-free BSA	30%	1%	73.33
10X PBS	10X	1X	220.0
Nuclease-free Water	-	-	1873.67
Total	**-**	**-**	**2,200.0**

Tips & Best Practices

Brief Fixation & Antibody Compatibility

- The intracellular labeling protocol includes a brief fixation (20 min) step.
- Before starting the protocol, it is recommended to test the antibodies by flow cytometry to ensure compatibility with the fixation.

Optimal Antibody Concentration

- It is highly recommended to titrate antibodies before use for optimal performance. Consult the manufacturer's instructions for recommended dilutions for each antibody.
- A dilution of 0.2 µg per antibody for up to 1 x 10^6 cells is suggested as a starting point.
- Consult Quality Control of Cell Surface Protein Labeling using Flow Cytometry Technical Note (CG000231) for guidance on how to titrate antibodies using flow cytometry for determining optimal antibody concentrations.

Antibody-Oligonucleotide Conjugation

- Custom conjugated antibodies can also be used with this protocol. See Conjugation Guidance for details.
- Consult the Demonstrated Protocol Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (CG000149) for guidance on antibody-oligonucleotide conjugation.

Antibody Quality

- The specific antibody clones used needs to be of high-quality and validated by flow cytometry.
- It may be helpful to use antibodies compatible with flow cytometry.

Sample Fixation

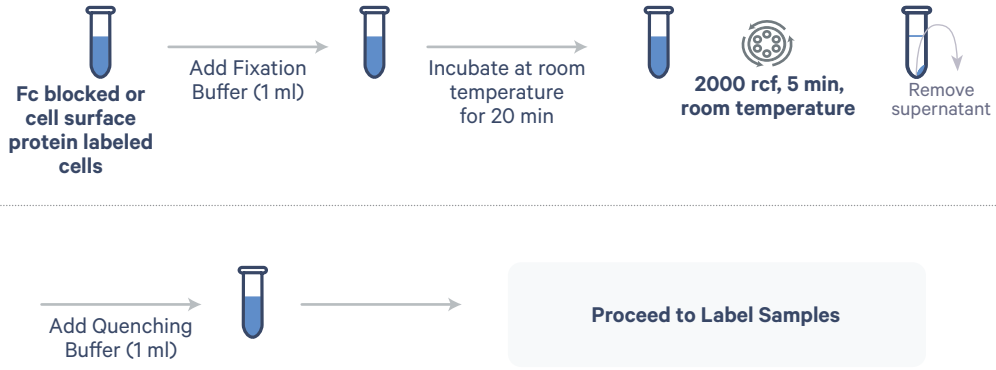
- Consult Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478) regarding fixing single cell suspensions following intracellular protein labeling.

Sample Storage

- After labeling, samples may be fixed in smaller batches and stored. If batching samples together, it is recommended that samples with different fixation times in one experiment are not mixed.
- Fixed samples can be stored either at -80°C for up to 6 months or at 4°C for up to 7 days with appropriate storage reagents.
- Consult Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478) for details on storage conditions and reagents.

Intracellular Protein Labeling: Fix Samples

Fix Sample



a. Optional Step Cell Surface Protein Labeling
Skip this step and directly proceed to step b if not utilizing the cell surface protein labeling section in this document.

- Perform cell surface protein labeling and washing as described in the Cell Surface Protein Labeling section in this document.
- Centrifuge labeled & washed sample at 300-400 rcf for 5 min (PBMCs/cell lines) at 4°C.
- Remove the supernatant without disturbing the pellet.
- Proceed immediately to step b.

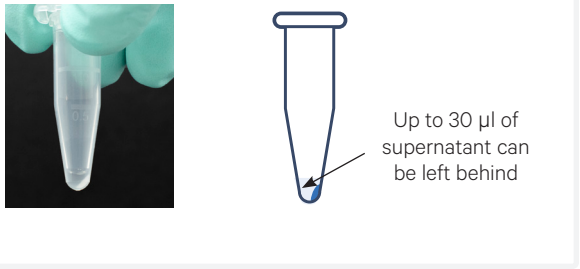
b. Add **1.0 ml room temperature** Fixation Buffer to the sample (Fc blocked or cell surface protein labeled) and pipette mix 5x.

c. Incubate for **20 min** at **room temperature (20°C)**.

DO NOT agitate or mix the sample during incubation. To minimize variability for room temperature fixations, incubation at controlled 20°C temperature (e.g. with a thermomixer, heat block, or water bath) is recommended.

d. Centrifuge at **2000 rcf** for **5 min** at **room temperature**.

e. Remove the supernatant without disturbing the pellet.

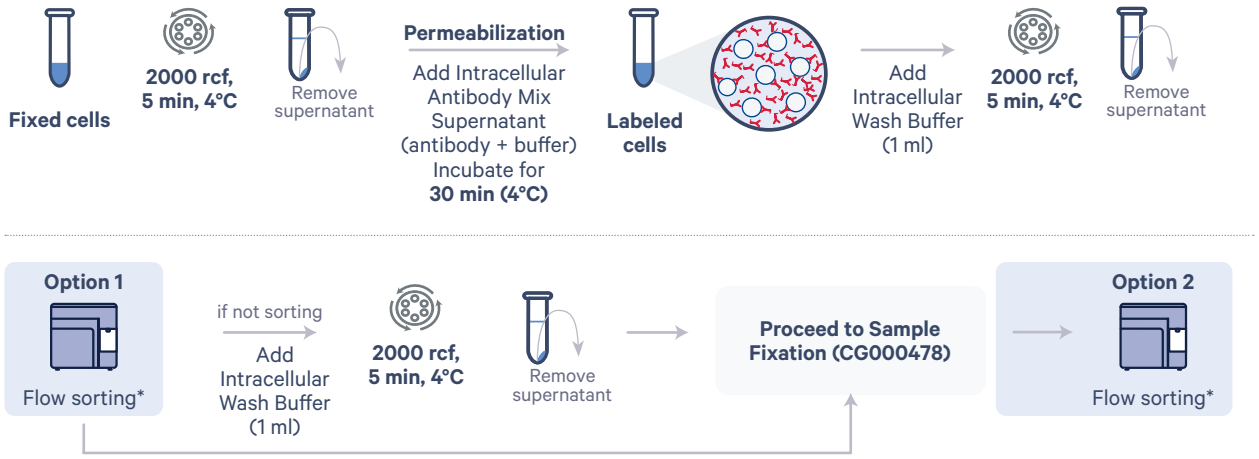


f. Add **1 ml chilled** Quenching Buffer to the sample pellet and pipette mix 5x and keep on ice.

g. Proceed to the next step (Label Samples).

Intracellular Protein Labeling: Label Samples

Label Samples



*Flow sorting can be performed after the first wash OR after Sample Fixation (CG000478) depending on sample and antibody.

a. Centrifuge at **2000 rcf** for **5 min** at **4°C**.

b. Remove the supernatant without disturbing the pellet.

c. Add **100 µl** Intracellular Antibody Mix Supernatant (antibody + buffer) to the pellet and gently pipette mix 10x.

d. Incubate for **30 min** at **4°C**. If using fluorophore-conjugated antibodies, incubate without light exposure.

e. Add **1.0 ml** chilled Intracellular Wash Buffer to the labeled cells. Gently pipette mix.

f. Centrifuge cells at **2000 rcf** for **5 min** at **4°C**.

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

OPTIONAL: For enrichment of cells by flow sorting:

- Proceed to flow sorting. See Post-Fixation Flow Sorting for details. For collection of sorted cells, use RNase-free reagents and supplement all buffers with RNase inhibitors.
- Flow sorting can also be performed after step i (second fixation following the Demonstrated Protocol CG000478). See Post-Fixation Flow Sorting for additional information on collection.

h. **Repeat** e-g one more time for a total of 2 washes.

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

Intracellular Protein Labeling – Troubleshooting

Problem	Solution
Low cell viability when beginning the protocol	Perform dead cell removal (flow sorting or bead based)
High background	Remove as much residual buffer as possible during the centrifugation steps, leaving no more than 30 µl buffer
	Add single-stranded DNA binding protein (SSB) from bacteriophage T4 (T4 Gene 32 Protein) to the antibody pool + antibody buffer if the background signal is an issue. Use 8 µg SSB per µg of antibody and add $MgCl_2$ at 4 mM final concentration. It is recommended to use flow cytometry to ensure that the SSB addition is not leading to a reduction in antibody signal.
Poor intracellular protein labeling performance	Modify fixation time ranging from 15 to 30 min; 1 h fixation may be compatible as well, but will require testing with flow cytometry. Lowering the fixation time may increase clumping while increasing the time may reduce the antibody signal. Modify NP-40 concentration ranging from 0.25% to 1%. Note that ≤0.25% NP-40 may not be enough for permeabilization, while >0.5% may induce clumping. Determine optimal antibody concentration using flow cytometry Remove dextran sulfate in the blocking buffer, substitute with BSA Increase antibody incubation time to >30 min
Poor Flex assay performance	Ensure that all buffers are made fresh and RNase-free Ensure RNase inhibitors have been added to the Antibody Buffer and the Intracellular Wash Buffer
Cell clumping	Perform dead cell removal (flow sorting or bead based) Increase fixation time to up to 1 hr (testing antibody compatibility with flow cytometry is recommended) Reduce NP-40 concentration to <0.5%. Note that ≤0.25% NP-40 may not be enough for permeabilization. Optimization and testing with flow cytometry is recommended. Perform additional filtering steps, including before hybridization
Reads derived from antibodies targeting cell surface antigens are a high percentage of the antibody library	Titrate surface antibodies to lower the amount of antibody added Consult Quality Control of Cell Surface Protein Labeling using Flow Cytometry Technical Note (CG000231) for guidance on how to titrate antibodies using flow cytometry for determining optimal antibody concentrations.

Appendix

Flow Sorting Guidance

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

Pre-Fixation Flow Sorting

It is recommended to collect 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.

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 flow sorting 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).*

Post-Fixation Flow Sorting

Post fixation samples can be flow sorted for advanced sample clean-up, as well as enrichment of specific populations. For samples undergoing intracellular protein labeling, sorting can be performed either after the first wash post labeling or after second fixation using the guidance provided in Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478). Samples should be collected in PBS + 1% nuclease-free BSA supplemented with RNase inhibitor (Protector RNase inhibitor from Sigma, PN-3335399001). A final concentration of 0.2 U/µl RNase inhibitor is recommended.

Consult 10x Genomics support website for more information on postfixation cell sorting.

Supplemental Data on Different Wash Options – Cell Surface Protein Labeling

The Cell Surface Protein Labeling protocol provides three wash options after incubation with the antibody. Using the appropriate wash option is critical for obtaining high-quality data and minimizing background signal. Supplemental data on the different wash options is provided in the following sections.

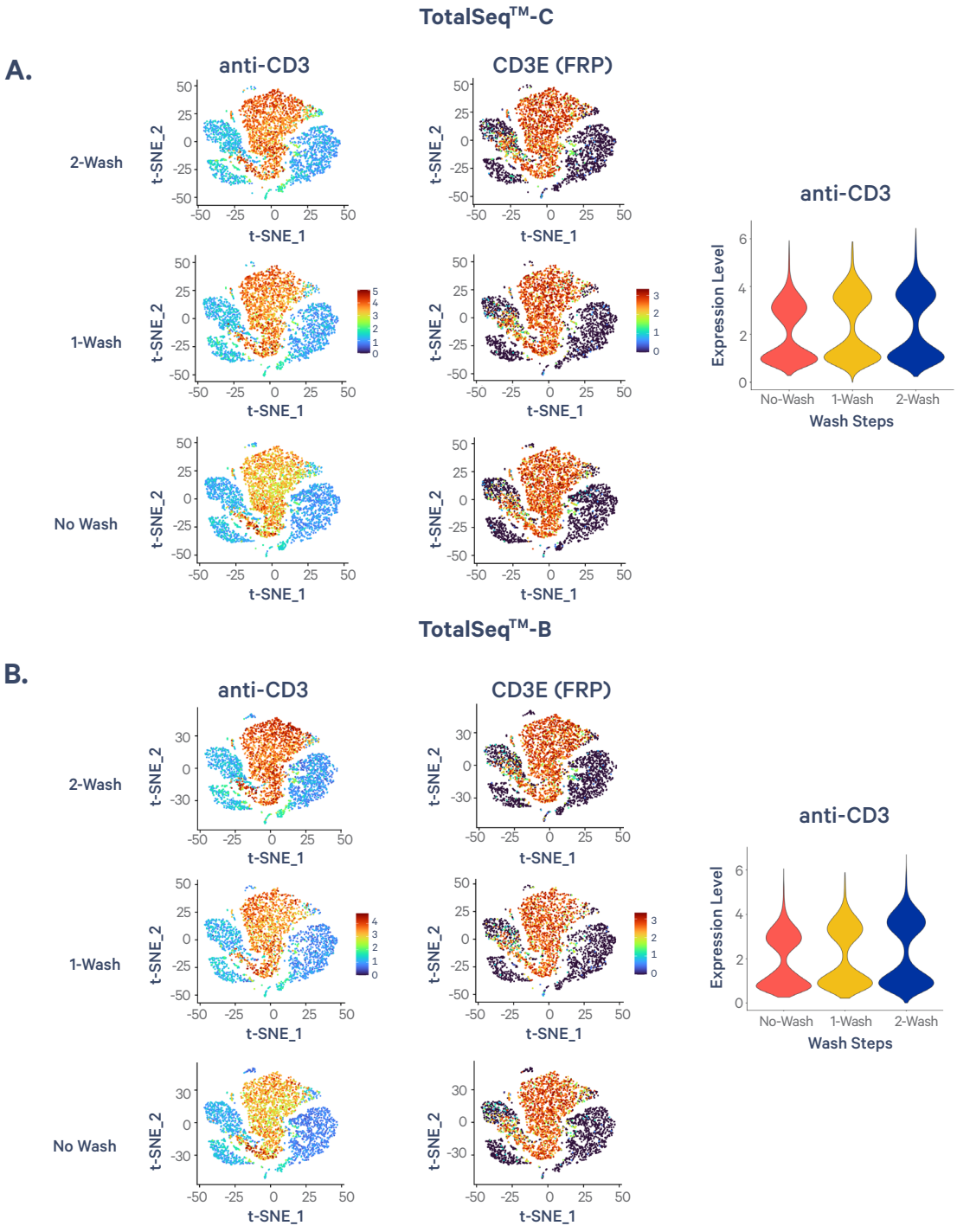


Figure 2. t-SNE plots of human PBMCs comparing staining for T cell marker CD3 across differing number of washes following antibody incubation (left), showing equivalent data quality between TotalSeq™-C **(A)** and TotalSeq™-B **(B)** configuration of the same antibody clone. Corresponding CD3E gene expression 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).

Supplemental Data on Different Wash Options – Cell Surface Protein Labeling *contd.*

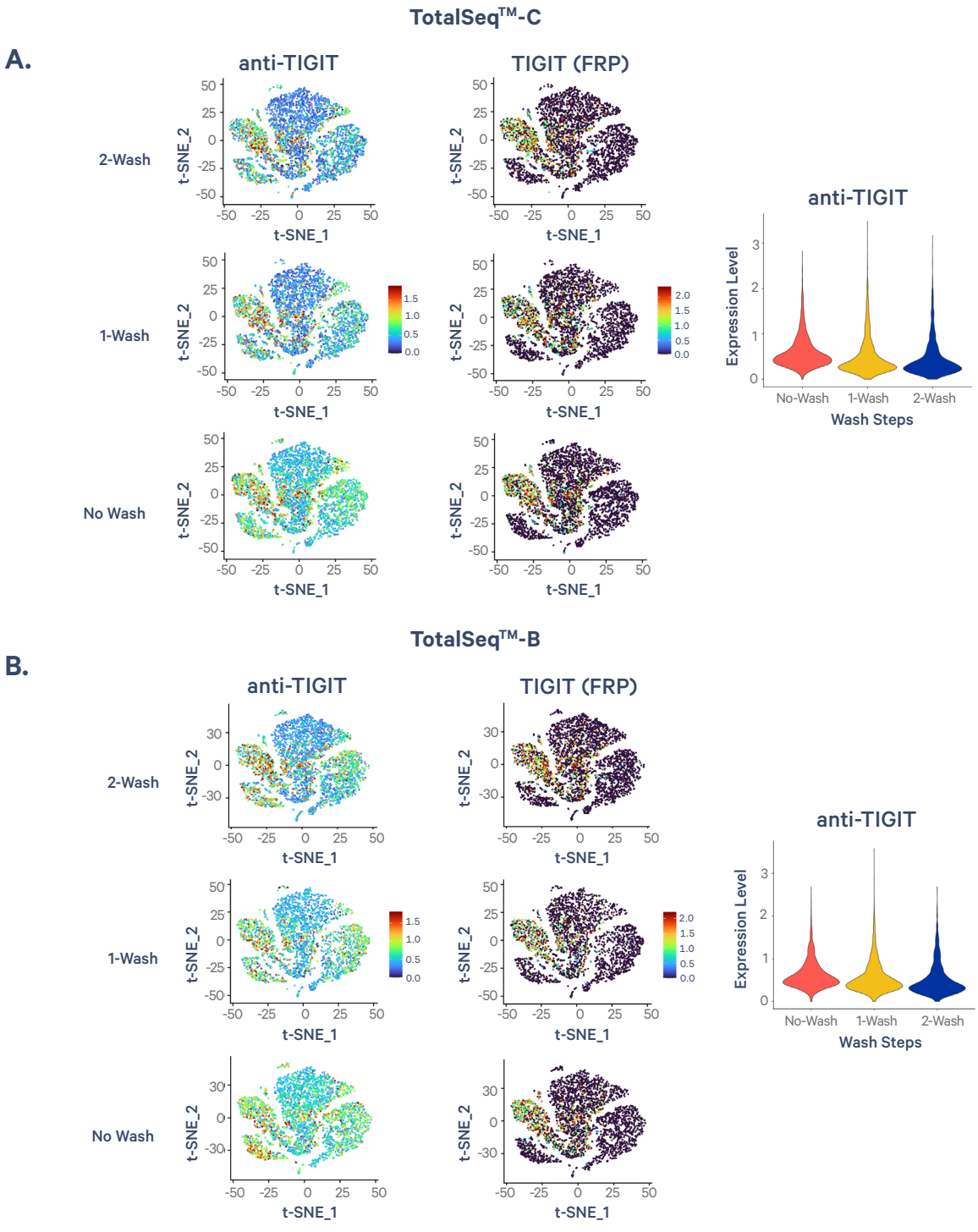


Figure 3. t-SNE plots of human PBMCs comparing staining for Natural Killer and T cell marker TIGIT across differing number of washes following antibody incubation (left), showing equivalent data quality between TotalSeq™-C **(A)** and TotalSeq™-B **(B)** configuration of the same antibody clone. Corresponding 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).

Supplemental Data on Different Wash Options – Cell Surface Protein Labeling *contd.*

		TotalSeq™-C			TotalSeq™-B		
		Signal to Noise* (On-target cells / all other cells)			Signal to Noise* (On-target cells / all other cells)		
Antibody	Comparison	No-Wash	1-Wash	2-Wash	No-Wash	1-Wash	2-Wash
anti-CD3	T cells / Other	12.44	15.72	16.99	13.09	14.89	17.58
anti-CD4	T cells / Other	9.92	10.71	9.36	7.61	7.17	8.05
anti-CD8	T cells / Other	3.2	5.81	6.32	3.11	4.94	5.93
anti-TIGIT	T & NK cells / Other	1.14	1.4	1.54	0.85	0.89	1.1
anti-CD19	B cells / Other	27.11	39.67	45.61	16.94	23.26	35.48
anti-CD15	Monocytes / Other	2.9	4.51	4.61	1.94	2.24	2.88
anti-CD14	Monocytes / Other	6.62	7.4	8.26	6.45	7.53	9.77
anti-CD11c	DCs & Monocytes / Other	13.5	12.89	13.07	14.07	12.1	14.81

*Ratio of mean expression level

Table 1. Signal to noise was generated by calculating the ratio of counts from a target group comprised of cell type(s) expected to display antibody signal (e.g. CD3 on T cells) to counts from a background group (e.g. CD3 on non-T cells).

Conclusion on Different Wash Options – Cell Surface Protein Labeling

Equivalent antibody Feature Barcode data quality can be obtained regardless of format, either with TotalSeq™-C or TotalSeq™-B antibody oligonucleotide conjugates. However, only TotalSeq™-C enables sample multiplexing.

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 (Figures 2A and 2B, CD3 data). However, a lower number of washes cause a reduction in separation between the positive and negative populations (Figures 2A and 2B, violin plot; Table 1, 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 (Figures 3A and 3B, TIGIT; Table 1, TIGIT).

References

1. Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (CG000478)
2. Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology Demonstrated Protocol (CG000149)
3. Chromium Fixed RNA Profiling - Protocol Planner (CG000528)
4. Chromium Fixed RNA Profiling Reagent Kits for Singleplexed Samples with Feature Barcode technology for Cell Surface Protein User Guide (CG000477)
5. Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture User Guide (CG000673)
6. Chromium Fixed RNA Profiling Reagent Kits for Singleplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture User Guide (CG000674)

Document Revision Summary

Document Number	CG000529
Title	Cell Surface & Intracellular Protein Labeling for Chromium Fixed RNA Profiling Demonstrated Protocol
Revision	Rev C
Revision Date	July 2024
Specific Changes	Added guidance on choosing the appropriate labeling protocol Added Specific Reagents & Consumables list for Intracellular Protein Labeling protocol Added guidance on Intracellular Protein Labeling protocol
General Changes	Updated for general minor consistency of language and terms throughout.

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