Cell Fixation Protocol for GEM-X Single Cell 3' & 5' Assays

Introduction

GEM-X Single Cell 3' and 5' assays with the advanced GEM-X microfluidics offer unmatched sensitivity and cell recovery efficiency for transcriptomic profiling. This document provides a fixation protocol for both freshly isolated and cryopreserved peripheral blood mononuclear cells (human and mouse PBMCs) that can be used as input for compatible GEM-X 3' and 5' assays, including on-chip multiplexing workflows. Additionally, as described in 10x Genomics Demonstrated Protocol CG000149, cell surface protein labeling and/or flow sorting may be performed before the cells are fixed using this fixation protocol. Fixation streamlines the workflow and enhances consistency.

Storage recommendations for the fixed cells and post-storage processing conditions are also provided. An overview of data derived from fixed human PBMCs processed using the GEM-X Single Cell 5' v3 workflow is shown in the Data Highlights section. See the Reference section for compatible user guides.

Additional Guidance

Isolate PBMCs as described in Demonstrated Protocol for Isolation of Leukocytes, Bone Marrow, and Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing (CG000392 Rev B or later). Consult the Cell Preparation Guide (CG000053) for Tips & Best Practices during sample preparation and guidance on determining accurate cell counts.

Tissue and 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 Fixation		
Thermo Fisher Scientific	DSP *Premium grade*	PG82081
	Pierce DSP *No-Weigh Format (1 g/ 10 mg/ 50 mg) (alternative to DSP PG82081)*	22585/ A35393/ 22586
	Sucrose	S5-500
	10X DPBS	14-200-075
	1M MgCl2	AM9530G
	Nuclease-free Water	AM9906
Millipore Sigma	Methanol *≥99.9%*	34860-1L-R
Thermo Fisher Scientific	Methyl sulfoxide (DMSO) *99.7+%, Extra Dry*	127790025
	Tris-HCL pH 7.5	BP1757-100

For Rehydration		
10x Genomics	RNase Inhibitor 40X	2001488 Kit PN-1000887
Thermo Fisher Scientific	Sodium Citrate pH 6	J61815-AK
	Nuclease-free Water	AM9906
Millipore Sigma	Albumin, Bovine Serum, *10% Aqueous Solution, Nuclease-Free*	126615
	Sodium Chloride Solution	71386
	RNase Inhibitor *(alternative to 10x product)*	3335402001
Vendor	**Item**	**Part Number**
For Cell Counting		
Nexcelom Biosciences	ViaStain PI Staining Solution	CS1-0109-5mL
	ViaStain AOPI Staining Solution	CS2-0106-5mL
-	Automated Cell Counter *See recommendations in Appendix*	-
Biotium	NucSpot 470	40083
Additional Materials		
-	Syringes (3-10 ml) and Needles (18-21 gauge) *(for aspirating Methyl Sulfoxide from bottle)*	- -
-	Pipette tips *(including wide-bore tips)*	-
Vendor	**Item**	**Part Number**
-	Disposable transfer pipette *(optional)*	-
Eppendorf	DNA LoBind Tubes 2.0 ml	022431048
VWR	Vortex Mixer *(or equivalent)*	10153-838
Centrifuge		
Eppendorf	Centrifuge 5427 R *(or equivalent microcentrifuge)*	5429000133
Beckman Coulter	Avanti J-15R Refrigerated Centrifuge or Allegra X-14 Centrifuge *(or equivalent centrifuge for 50/15/5 ml tubes)*	

This list may not include some standard reagents and laboratory equipment.

Buffer Preparation

Stock Solutions

DSP Stock Solution *(example for ~150 rxns; scale accordingly)*

For 100 mM DSP stock sol., add 24.73 µl DMSO per 1 mg DSP

- Weigh ~100 mg DSP in a 1.5-ml tube. Note the exact DSP mass (mg).
- Calculate DMSO volume (ml)= Exact DSP mass (mg) X 24.73
- Add calculated DMSO volume to tube with DSP (negligible impact of DSP mass on final volume & concentration)
- Aliquot 65 µl/tube & store -80°C (each aliquot sufficient for 4 samples).
- Prepared stock can be stored at -80°C for up to 6 months.
- Thaw at room temperature before use. Once thawed, DO NOT refreeze.

Reagents	Stock	Final	Volume* (1 rxn)
Dehydration Buffer *Maintain on ice* • *Prepared buffer can be stored at 4°C for up to 1 week or store aliquots at -80°C for up to 3 months (thaw before use, do not refreeze)*			
Sucrose	1,000 mM	300 mM	300 µl
10X DPBS	10X	1X	100 µl
MgCl2	1,000 mM	3 mM	3 µl
Water (RNase free)	-	-	597 µl
Total			**1,000 µl**

Reagents	Stock	Final	Volume* (1 rxn)
Rehydration Buffer/Wash Buffer *Maintain on ice***			
Sodium Citrate pH6	500 mM	45 mM	135 µl
Sodium Chloride	5,000 mM	450 mM	135 µl
BSA	10 %	1 %	150 µl
RNase Inhibitor	40X	0.2X	7.5 µl
Water	-	-	1,072.5 µl
Total			**1,500 µl**
Resuspension Buffer *Maintain on ice***			
10x DPBS	10X	2X	100 µl
BSA	10 %	1 %	50 µl
RNase Inhibitor	40X	1X	12.5 µl
Water (RNase free)	-	-	337.5 µl
Total			**500 µl**

**When calculating volumes for >1 rxn, add 10% overage.*

***Buffer prepared without RNase inhibitor can be stored at 4°C for 1 week. Add RNase Inhibitor when ready to use. Alternatively, prepare aliquots after adding RNase inhibitor and store at -80°C for up to 3 months. Thaw before use. Do not refreeze.*

Vortex Speed for Rehydration

- Optimize vortex speed as described in the Vortex Speed Optimization section.
- Once the vortex speed is optimized, note the setting for future experiments.

Tips & Best Practices

Follow recommendations for optimal performance.

- Use wide-bore pipette tips when indicated (mixing cells in suspension).
- A regular-bore pipette tip should be used when resuspending a cell pellet.

Fixation

- Before adding Dehydration Buffer, remove all the supernatant without disturbing the pellet. Residual BSA can cause DSP precipitation.
- Always prepare fresh Fixation Buffer.
- Dispense Fixation Buffer slowly by placing the pipette tip in the cell suspension and dispensing directly into the cell suspension while gently swirling the tip in the tube to mix.
- After fixation, remove all the supernatant without disturbing the pellet. If precipitate is observed after rehydration, filter the sample. Filtration may result in some cell loss. Consult the Cell Preparation Handbook for guidance on sample filtration (CG000053).

  Recommended filters

  Sysmex: Sysmex Sterile Single-Pack CellTrics Filters (30 µm), 04-004-2326

  OR

  Miltenyi Biotec: MACS SmartStrainers (30 µm) 130-098-458/ Pre-Separation Filters (30 µm) 130-041-407

Centrifugation

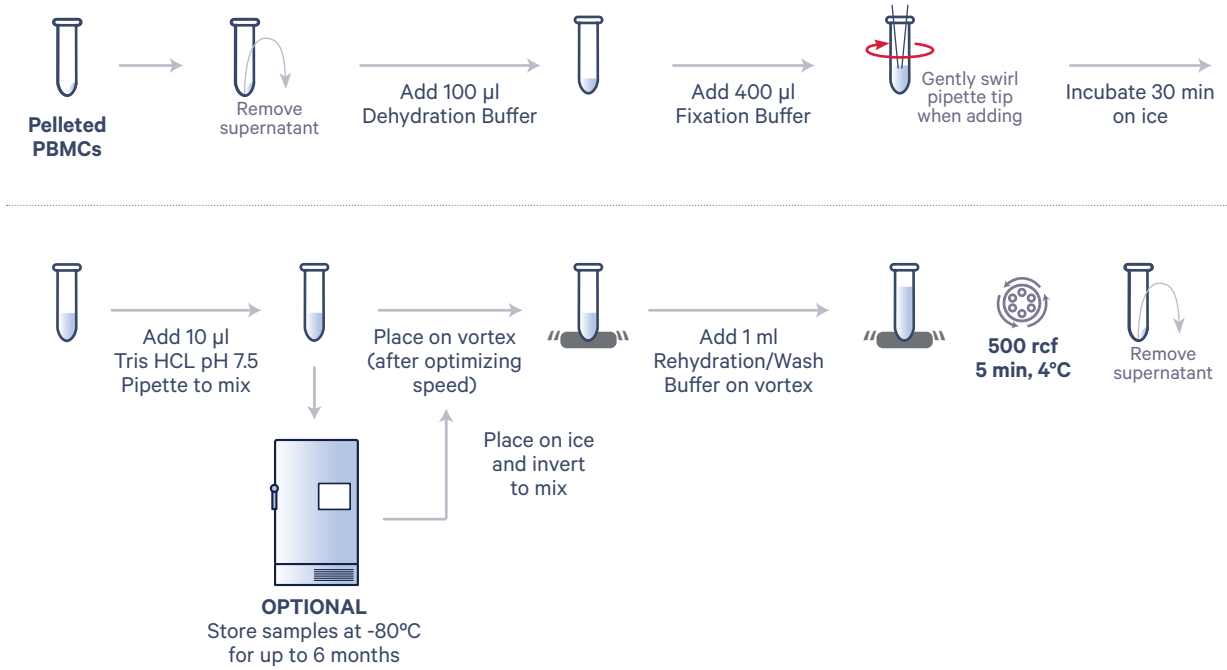
- Use a swinging-bucket rotor for higher cell recovery. A refrigerated centrifuge with a fixed angle rotor may be used as an alternative but that may result in higher cell loss.
- When centrifuging 2-ml tubes, always use a compatible microcentrifuge. Using adaptors in a centrifuge designed for larger volume tubes is not recommended as it can alter speed and impact cell pellet formation.
- If pellet is not clearly visible while removing the supernatant, initially draw supernatant from the center of the liquid volume without touching the pipette tip to the tube wall.
- When ~100 µl volume is left, tilt the tube at an angle and touch the tip to the side of the tube where no visible particulate material is present and remove the remaining supernatant.

Cell Counting

- Samples should have minimal debris for best results; debris can have associated RNA that can contribute to non-cell background.
- Accurate sample counting is critical for optimal assay performance.
- Sample should be stained with a fluorescent nucleic acid dye and counted using an automated cell counter. See Appendix for details.
- When counting fixed cells, small debris/crystals may be visible. See protocol for guidance on sample filtration recommendations based on presence of debris (representative images shown).
- DO NOT use trypan blue for counting (not supported).

Protocol Overview

Fixation Protocol



Cell Fixation & Rehydration Protocol

This protocol used both freshly isolated and cryopreserved human and mouse PBMCs, isolated as described in CG000392 (Rev B & higher). For thawing guidance, consult the Cell Thawing Protocol CG000447 (Rev B & higher).

Prepare the buffers specified in the Buffer Preparation section. Pre-chill Methanol on ice or at 4°C prior to Fixation Buffer preparation (step c).

Prepare Cells

TIPS *Use wide-bore pipette tips where indicated.*

a. Prepare PBMC single cell suspension (as described in CG000392 & CG000447 - Rev B & higher) and determine cell concentration. Transfer 100,000 - 1x10^6 cells to a 2-ml Eppendorf tube.

b. Centrifuge cell suspension at **500 rcf** for **10 min** at **4°C**.

c. Using a pipette tip (or disposable transfer pipette), remove the supernatant without disturbing the pellet. Maintain pellet on ice.

Remove all the supernatant without disturbing the pellet, as residual BSA can cause DSP precipitation.

d. Prepare Fixation Buffer and maintain on ice.

Fixation Buffer should be prepared fresh right before cell fixation. Processing more than 8 samples at one time is not recommended.

Fixation Buffer- Prepare fresh				
Reagents	**Stock**	**Final***	**1 Sample* (µl)**	**8 Samples + 10% (µl)**
DSP Stock Solution	100 mM	3.125 mM	12.5	110
Methanol *Pre-chilled*	100%	-	387.5	3,410
Total			**400**	**3,520**

**When calculating volumes for >1 rxn, add 10% overage.*

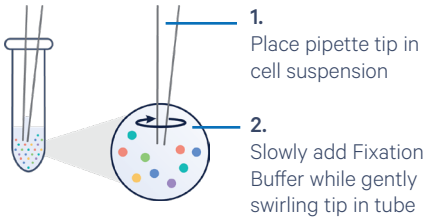
e. Resuspend the cell pellet in **100 µl** Dehydration Buffer (1x10^5 - 1x10^6 cells/100 µl recommended) and **immediately** proceed to Fixation.

For multiple samples, process one cell pellet at a time. Resuspend the pellet from the first sample and immediately add Fixation Buffer. Then process the pellet from the second sample and so on. Leaving cells in Dehydration Buffer for longer can result in precipitation.

Fixation

f. Add **400 µl** Fixation Buffer (over ~10-15 sec) by placing the pipette tip in the cell suspension and dispensing while swirling the tip in the tube to mix.

Adding Fixation Buffer to Cells



g. Using a wide-bore pipette tip (pipette set at 400 µl), gently pipette mix until the suspension looks uniform. Once the buffer is fully mixed, the solution will appear clear.

h. Incubate for **30 min** on **ice**.
OPTIONAL: Optimize vortex speed for Rehydration during this incubation (see next page).

i. Add **10 µl** 1M Tris-HCl (pH 7.5) per 500 µl reaction volume. Pipette mix.

j. Proceed either directly to Rehydration or store cells at **−80°C**.

STOP *Samples can be stored at −80°C for up to 6 months.*

If storing cells at **−80°C**, when ready to use, retrieve cells and place on ice. Invert the tube a few times to mix and proceed directly to Rehydration.

Vortex Speed Optimization for Rehydration

Use an empty 2-ml Eppendorf practice tube

k. Mark the 1 ml volume line on the outside of an empty 2-ml Eppendorf practice tube

l. Add 500 µl Methanol (80%) to tube.

m. Turn the vortex mixer "ON" and set the speed to 1 (lowest setting).

n. With the tube open, firmly grasp the top of the filled 2-ml practice tube and set on the vortex. Slowly increase the vortex speed until the liquid reaches up to the 0.75 - 1 ml mark but not over it (typically ~ 2-3 setting or ~700 rpm for most vortex mixers). Note/keep this setting. It will be used during rehydration.

Rehydration

o. With one hand, firmly hold the top of the tube with the fixed cells from step j (cap open) on top of the vortex mixer that has been turned on at the setting described above. With the other hand, slowly (over ~10-15 sec) pipette **1 ml** Rehydration/Wash Buffer directly to the fixed cells while vortexing the tube dropwise.

TIPS *Dispense buffer directly on to the cells and not to the tube walls.*

p. After vortexing, invert the tube to mix completely

TIPS *The pellet may not be fully resuspended. A wide-bore tip may be used to gently mix the suspension. DSP precipitate may be observed after rehydration due to residual BSA from sample preparation. The solution may appear cloudy but should be clear after vortexing and centrifugation.*

q. Centrifuge at **500 rcf** for **5 min** at **4°C.**

TIPS *Use of swinging-bucket rotor refrigerated microcentrifuge is recommended. A refrigerated microcentrifuge with a fixed angle rotor may be used but may result in higher cell loss.*

r. Using a pipette (or disposable transfer pipette), remove the supernatant without disturbing the pellet.

s. Add **500 µl** Rehydration/Wash buffer and gently pipette mix to resuspend the pellet.

TIPS *If the solution appears cloudy, fully resuspend the pellet and filter the sample into a new tube using a 30 µm filter.*

t. Centrifuge at **500 rcf** for **5 min** at **4°C.**

u. Using a pipette (or disposable transfer pipette), remove the supernatant without disturbing the pellet.

v. Based on starting cell concentration and assuming ~40-50% cell loss, add an appropriate volume (~100*-500 µl) of chilled Resuspension Buffer. Pipette mix to resuspend the pellet, aiming for a final cell concentration of 700-1,600 cells/µl.

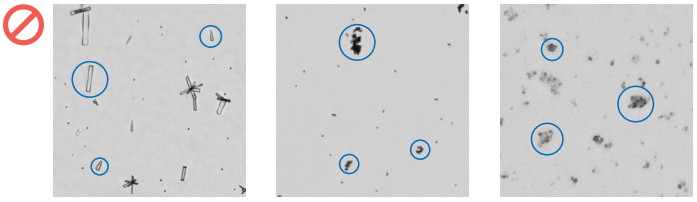
TIPS *Always assess the sample for presence of debris (see examples below). See Appendix for counting guidance.*

w. Proceed **immediately** to compatible GEM-X Single Cell 3' or 5' protocol (see References).

**If less than 100 µl is used for resuspension, use 12.5 µl instead of 25 µl for counting.*

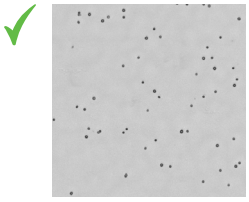
Assessing Sample Quality - Examples

Samples with Debris - Filtering Recommended
Check for debris or cell aggregates. They may cause microfluidic chip clogs. Additional pipetting or filtering may be required.



Crystals | Cell aggregates | BSA aggregates

Clean Sample - Filtering Not Needed
Filtering is not needed for samples that show little to no debris



Data Highlights

The representative Data Highlights demonstrate that fixed human PBMCs (with or without storage at −80°C prior to processing), retain the single cell transcriptome information when processed using the GEM-X Single Cell 5' v3 workflow (Figs. 1-2). It is important to note that while there are differences in data quality for fixed samples compared to fresh samples, both gene expression and V(D)J information is maintained in fixed samples.

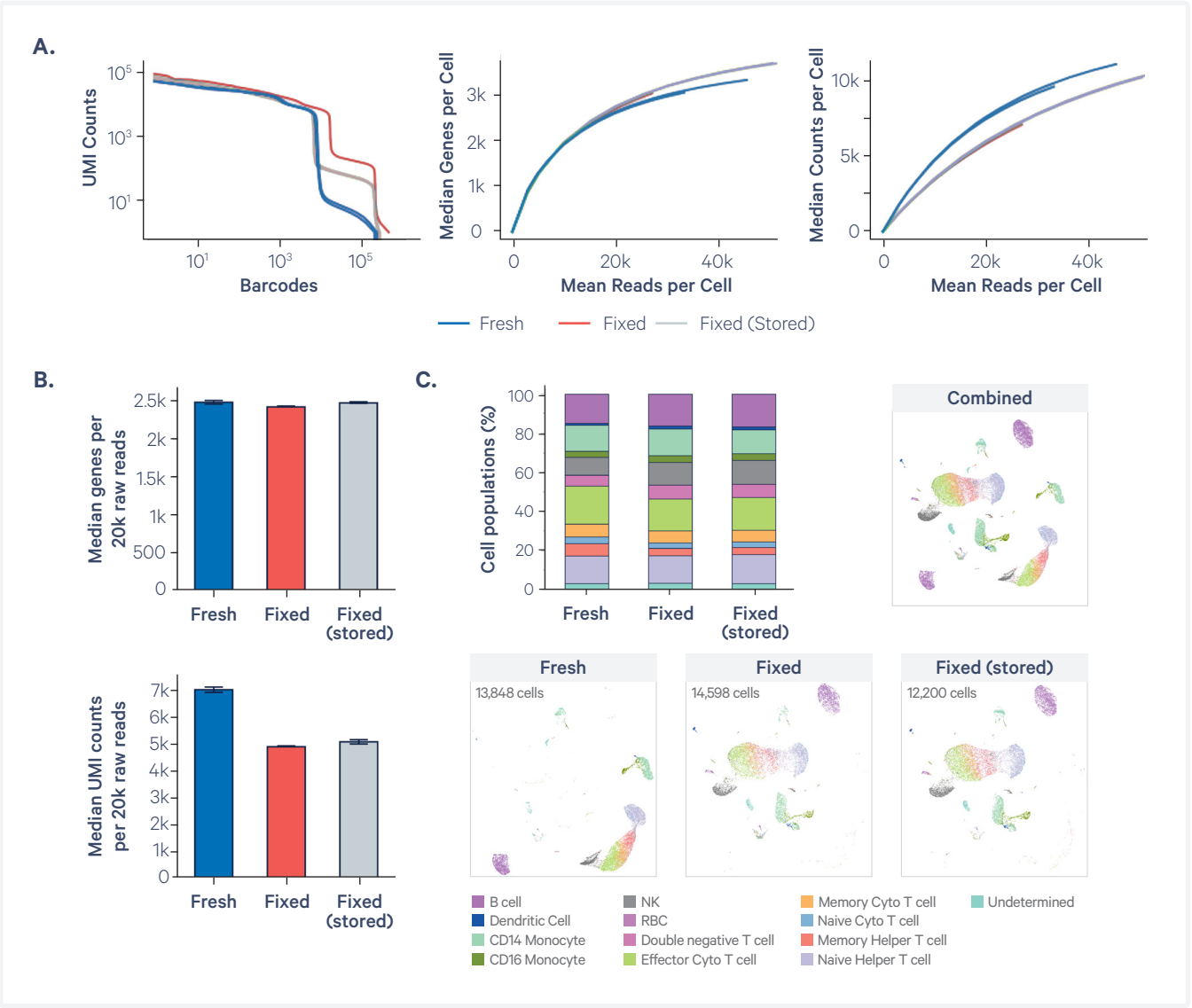


Figure 1. Analysis of fixed human PBMCs using the GEM-X Single Cell 5' v3 workflow demonstrates that the transcriptome is preserved post fixation. **(A)** Barcode rank plot & assay complexity or sensitivity plots of PBMCs isolated from whole blood which were either directly processed (fresh) or fixed and processed or fixed and stored for 1 month and then processed. 10,000 cells were processed directly while 5,000 fixed cells were stored and then processed. **(B)** Quantification of assay complexity and sensitivity at 20k raw reads per cell (RRPC). **(C)** Cell population frequency and representative UMAP plots (no batch correction) across the three conditions.

Data Highlights

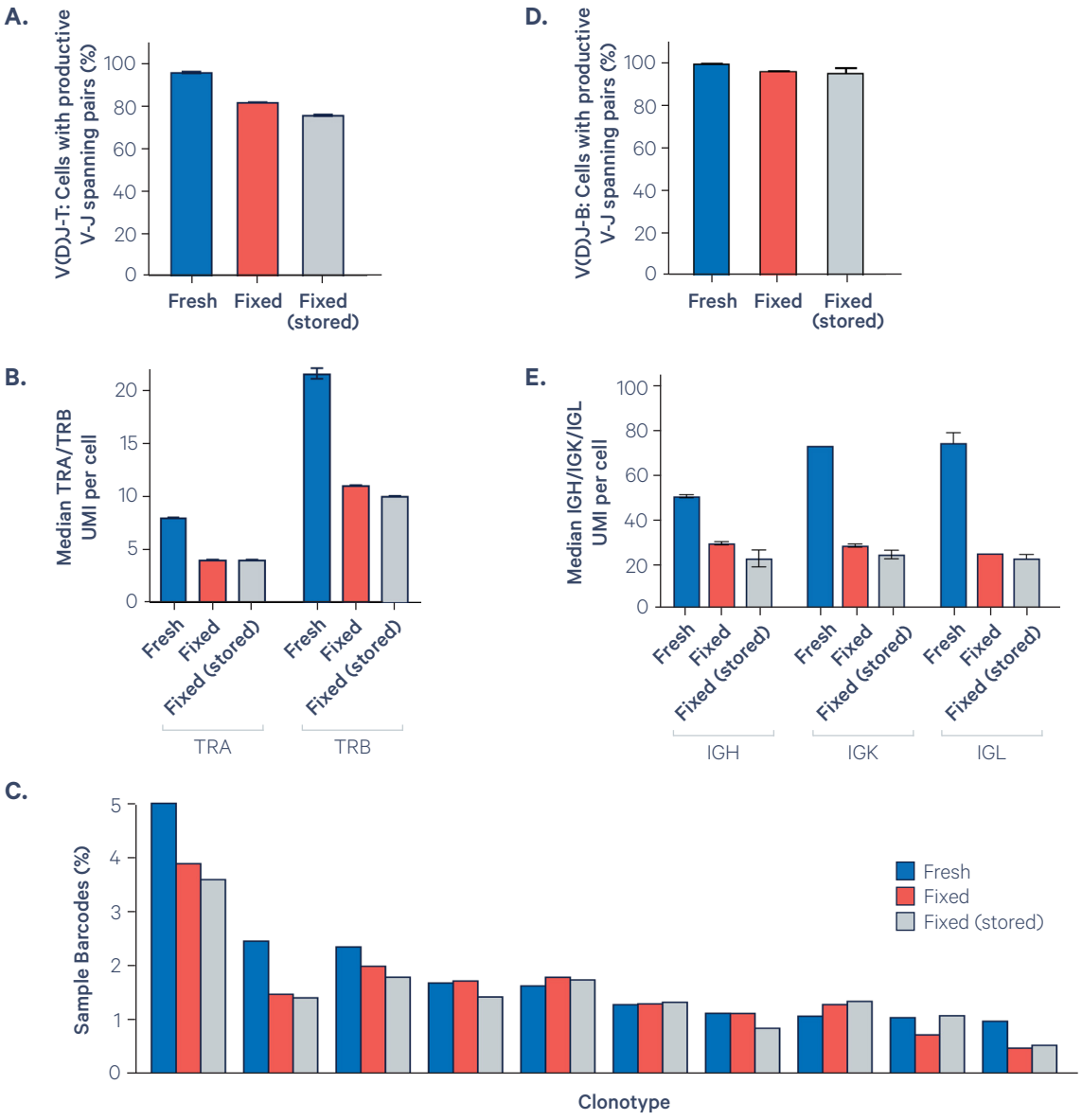


Figure 2. Analysis of fixed human PBMCs using the GEM-X Single Cell 5' v3 workflow demonstrates that V(D)J transcripts are preserved post fixation. Data derived from PBMCs isolated from whole blood which were either directly processed (fresh) or fixed and processed or fixed and stored for 1 month and then processed. **(A-B)** V(D)J paired sequences and T-cell receptor distribution. **(C)** Sample barcode frequency for each T-cell clonotype across the three conditions. **(D-E)** V(D)J B-cell receptor distribution and paired sequences.

Appendix

Post-Storage Processing of Fixed Cells

- When ready to use samples stored at **−80°C**, retrieve the cells from **−80°C** and place on ice.
- Invert/flick the tube a few times to mix and proceed to Rehydration.

Cell Counting

- Accurate sample counting is critical for optimal assay performance.
- The cells should be stained with an appropriate dye and counted using an automated cell counter. See below for the dye recommendation for a specific counter.

Counter	Dye Recommended
Cellaca MX	AO/PI staining solution
Countess 3 FL	PI staining solution
Cellometer K2	**Nucspot 470

*** Dilute the stock to 1:100 and mix 1:1 with the sample. For example, add 10 µl diluted dye to 10 µl sample.*

The following section provides counting guidance using AO/PI staining solution and the Cellaca counter. For counting guidance using other dyes/counters, refer to manufacturer's instructions.

- **Counting using AO/PI Staining Solution:** This protocol provides instructions for counting samples using AO/PI staining solution and the Cellaca counter to enable accurate quantification even in the presence of subcellular debris. The optimal cell concentration for the Cellaca counter is 100-10,000 cells/µl. Refer to manufacturer's instructions for details on operations.
  - Add **25 µl** AO/PI staining solution into Mixing Row of Cellaca plate
  - Gently mix the sample. If the sample is too concentrated, a 1:1 dilution in PBS can also be prepared. For example, add 15 µl fixed cell suspension to 15 µl PBS.

    If starting cell input is 100,000 cells, dilute sample prior to counting (For example, dilute the single cell suspension with 20 µl Resuspension Buffer, mix with 25 µl AO/PI). If the cell input is less than 100,000 cells, contact support@10xgenomics.com for guidance.
  - Add **25 µl** sample to Mixing Row of plate containing AO/PI staining solution. Gently pipette mix 8x.
  - Transfer stained sample to Loading Row of Cellaca plate.
  - For counting fixed samples, **only** use the PI (Propidium Iodide) channel.

    Refer to manufacturer's instructions for details.

References

1. Chromium GEM-X Single Cell 3' v4 Gene Expression (CG000731)
2. Chromium GEM-X Single Cell 3' v4 Gene Expression with Feature Barcoding technology for Cell Surface Protein (CG000732)
3. Chromium GEM-X Single Cell 5' v3 Gene Expression (CG000733)
4. Chromium GEM-X Single Cell 5' v3 Gene Expression with Feature Barcoding technology for Cell Surface Protein (CG000734)
5. GEM-X Universal 3' Gene Expression v4 4-plex On-chip Multiplexing (CG000768)
6. GEM-X Universal 3' Gene Expression v4 4-plex On-chip Multiplexing with Feature Barcode technology for Cell Surface Protein(CG000769)
7. GEM-X Universal 5' Gene Expression v3 4-plex On-chip Multiplexing (CG000770)
8. GEM-X Universal 5' Gene Expression v3 4-plex On-chip Multiplexing with Feature Barcode technology for Cell Surface Protein (CG000771)

Consult the 10x Genomics Support Website for the most current information regarding compatible user guides and associated datasets.

Take 1 minute to evaluate this protocol. Scan this code or click here.

Document Revision Summary

Document Number	CG000776
Title	Cell Fixation Protocol for GEM-X Single Cell 3' & 5' Assays
Revision	Rev B to Rev C
Revision Date	June 2025
Description of Changes	Included information regarding cell surface protein labeling protocol on page 1 Added 10x Genomics RNase Inhibitor (PN-2001488) on page 1 Alternate DSP options on page 1 Additional filtration step after addition of rehydration buffer on pages 2 and 6 Updated post-fixation storage guidance on pages 2 and 6 Updated fixation buffer addition guidance along with image placement Added sample quality guidance on page 6 Minor updates in guidance, language, and format throughout

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