Blood Fixation and Cell Isolation for GEM-X Flex Gene Expression

Introduction

GEM-X Flex Gene Expression offers comprehensive scalable solutions to measure gene expression in single cell and nuclei suspensions that are fixed with formaldehyde. This protocol outlines fixation of blood and isolation of peripheral blood mononuclear cells (PBMCs) and leukocytes from the fixed blood for use with the GEM-X Flex Gene Expression workflow. Storage recommendations for the PBMCs isolated from fixed blood and post-storage processing conditions are also provided. An overview of data derived from fixed PBMCs and leukocytes used with the GEM-X Flex Gene Expression is also shown.

Additional Guidance

Consult the Handbook – Cell Preparation Guide (CG000053) for Tips & Best Practices during sample preparation and for more information 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 Collection		
BD	BD Vacutainer EDTA Tubes	366643*
	*Choose any size	
For Fixation		
Millipore Sigma	Formaldehyde (37% by Weight/Molecular Biology), Fisher BioReagents	BP531-25
	Nuclease-free Water (not DEPC-Treated)	AM9937
Corning	Phosphate-Buffered Saline, 1X *without Calcium and Magnesium*	21-040-CV
For Cell Isolation		
Thermo Fisher Scientific	Invitrogen UltraPure 0.5M EDTA, pH 8.0	15575020
STEMCELL Technologies	EasySep Direct Human PBMC Isolation Kit *Includes:* • *EasySep Direct Human PBMC Isolation Cocktail, 2 x 2.5 mL* • *EasySep Direct RapidSpheres, 4 x 2.5 mL*	19654
	EasySep RBC Depletion Reagent Kit *Includes:* *EasySep RBC Depletion Reagent, 10 mL*	18170
	EasyEights EasySep Magnet Or "The Big Easy" EasySep Magnet	18103 18001
10x Genomics	GEM-X Flex Sample Preparation v2 Kit	1000781
For Cell Counting		
Nexcelom Biosciences	*ViaStain PI Staining Solution	CS1-0109-5mL
	*ViaStain AOPI Staining Solution	CS2-0106-5mL
	†Cellaca MX High-throughput Automated Cell Counter	MX-112-0127
	Cellometer K2 Fluorescent Cell Counter	CMT-K2-MX-150
	PD100 Counting Chambers 1 case	CHT4-PD100-003
Biotium	*NucSpot 470	40083
	Dilute 1:100 in PBS and use at 1:1 ratio with sample. Do not incubate before imaging/counting.	

Thermo Fisher Scientific	†Countess II FL Automated Cell Counter *Discontinued*	AMAQAF1000
	Countess Automated Cell Counting Chamber Slides	C10228
	†Countess 3 FL Automated Cell Counter	AMQAF2000
	*DAPI solution, 1 mg/mL	62248

†Choose Countess II/3, Cellaca, or equivalent fluorescent counter.
**Choose either AOPI, NucSpot, PI, or DAPI solution.*

For Storage		
Thermo Fisher Scientific	UltraPure BSA (50 mg/mL) *Alternative to Millipore Sigma*	AM2616
Millipore Sigma	Glycerol for molecular biology, ≥99.0%	G5516-100ML
	Albumin, Bovine Serum, 10% Aqueous Solution, Nuclease Free	126615
	Protector RNase Inhibitor	3335402001
Additional Materials		
Fisher Scientific	Falcon Round-Bottom Polypropylene Test Tubes With Cap	14-959-10B
Eppendorf	DNA LoBind Tubes 1.5 ml	022431021
Corning	Corning 50-ml centrifuge tube	CLS430829
VWR	Vortex Mixer	10153-838

GEM-X Flex Sample Preparation v2 Kit, 48 rxns PN-1000781

This protocol uses Conc. Quench Buffer B (PN 2001300) for cell Isolation and Enhancer (PN 2000482) for storage of fixed cells.

GEM-X Flex Sample Preparation v2 Kit
48 rxns, PN-1000781
Shipped on dry ice
Store at -20°C

	#	PN
Conc. Fix & Perm Buffer B	2	2001301
Conc. Quench Buffer B	6	2001300
Enhancer	3	2000482
Additive C	4	2001332

10x GENOMICS

The sample preparation kit provides sufficient reagents to process 48 samples.

Tips & Best Practices

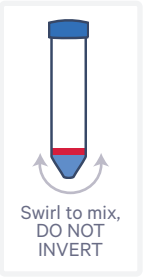
The recommendations are critical for optimal performance.

Blood Collection

- Use K2 EDTA blood collection tubes. DO NOT use any other anticoagulant vacutainer to prevent loss of monocytes.

Blood Fixation

- If fixing more than one sample, prepare the Fixation Buffer in bulk and aliquot into individual tubes.
- Before adding blood, ensure that the blood tube is inverted 3x if kept for longer than 5 min.
- Swirl the 50-ml tube containing blood and Fixation Buffer to mix for 2-3 sec.



Post-Fixation Processing

- During vortexing steps, foam is expected. This is normal and does not affect the performance.

Cell Isolation

- During magnet incubation steps, place the tube uncapped and flush with the back and the bottom of the magnet.
- After magnet incubation, transfer supernatant from the top of the liquid without touching the magnetic beads on the side of the tube.
- With each separation step, the supernatant will become clear.

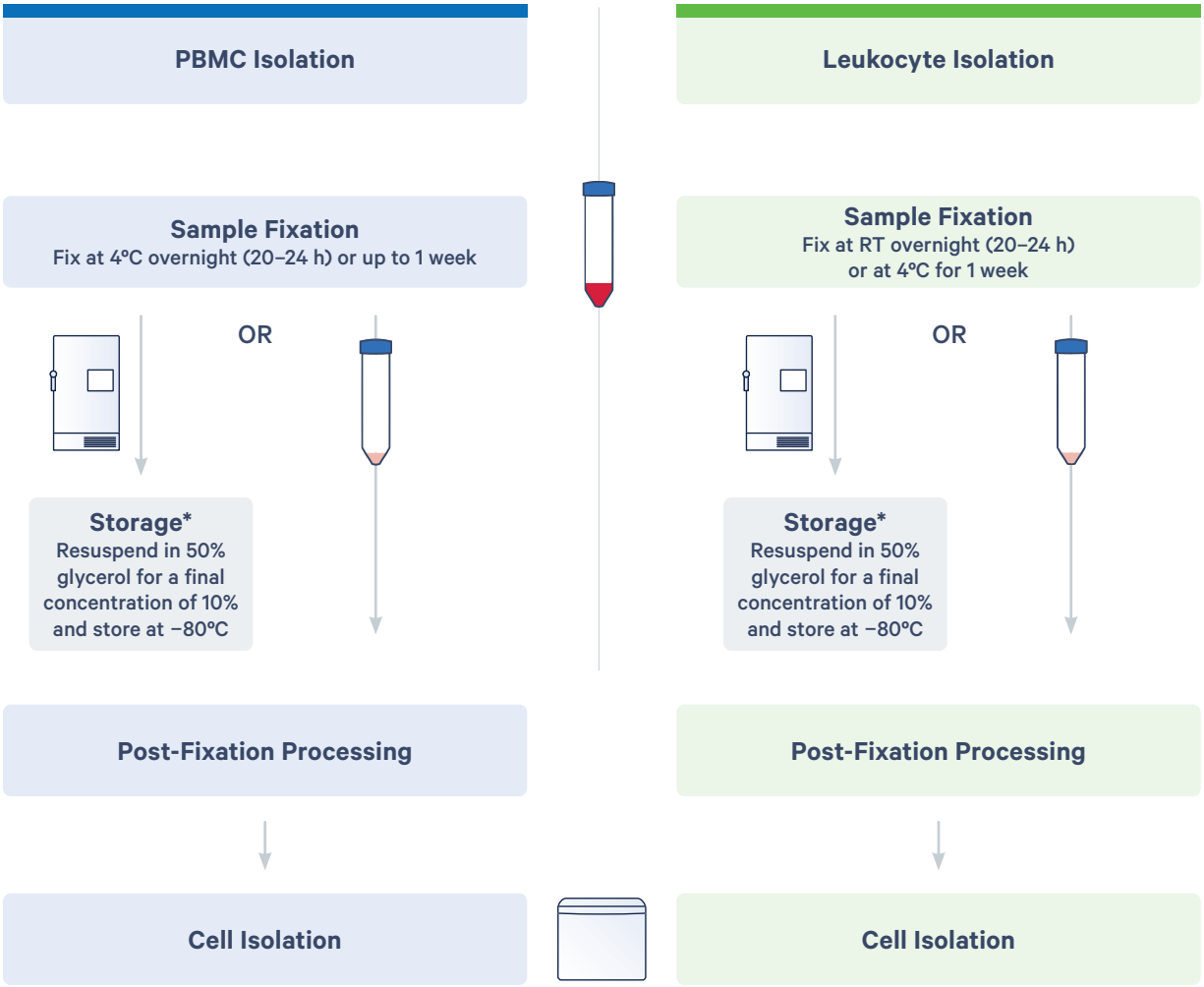
Centrifugation Guidelines

- Use a swinging-bucket rotor for higher cell recovery.

Fixed Cell Counting

- Samples should have minimal debris for best results; debris can have associated RNA that can contribute to noncell background.
- Accurate sample counting is critical for achieving desired cell recovery.
- Sample should be stained with a fluorescent nucleic acid dye and counted using an automated cell counter. See Appendix for details.
- DO NOT use trypan blue for counting as it will count RBCs and thus lead to incorrect counts.

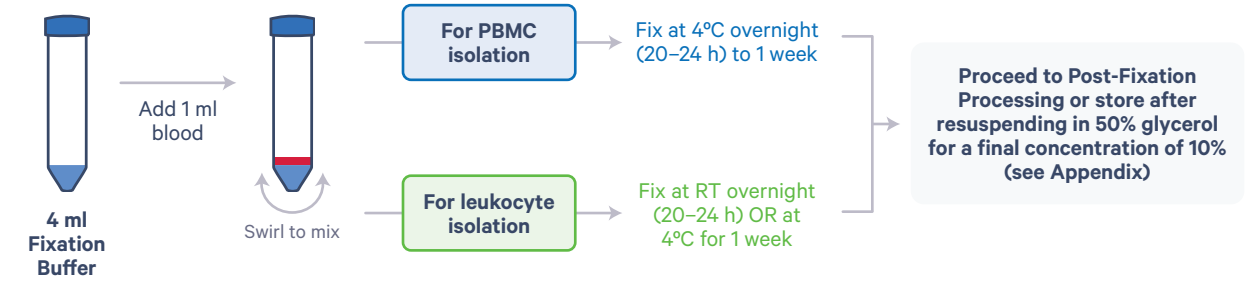
Protocol Overview



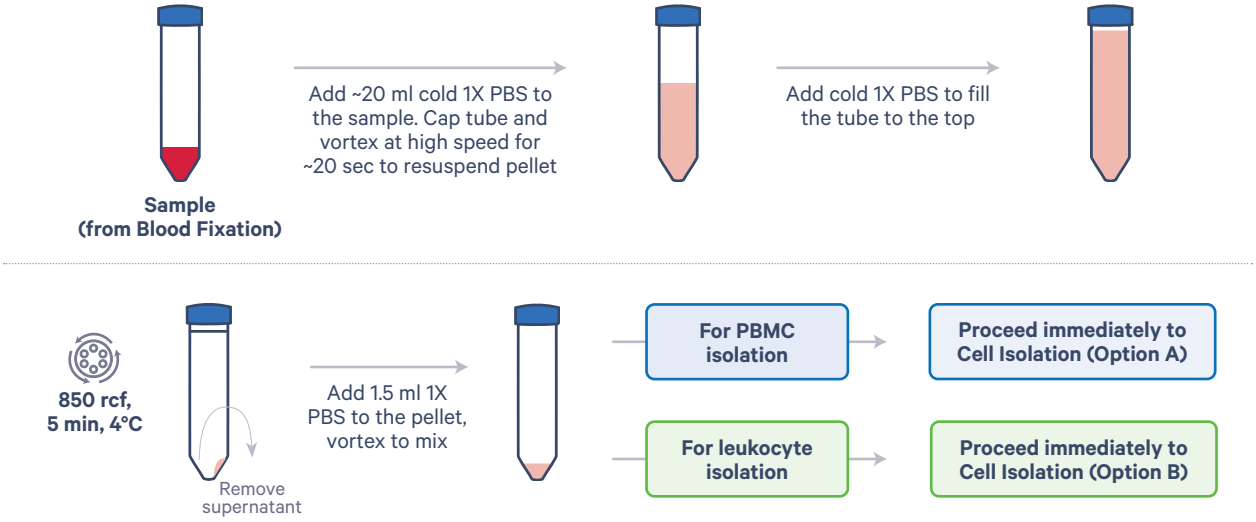
** See Appendix for more details*

Protocol Overview

1. Blood Fixation

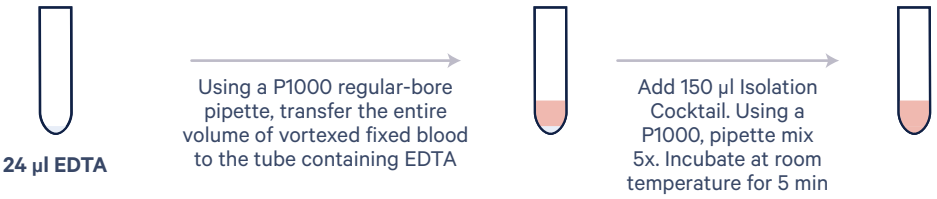


2. Post-Fixation Processing

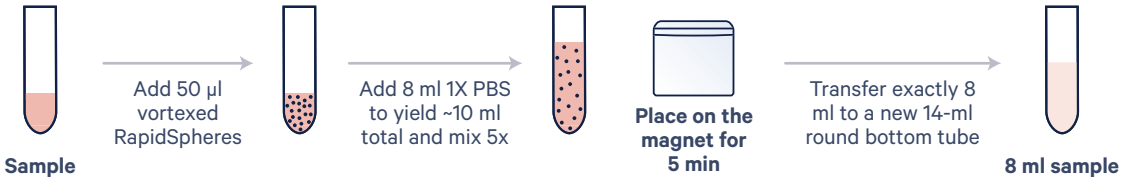


3. Cell Isolation
Option A. PBMC Isolation

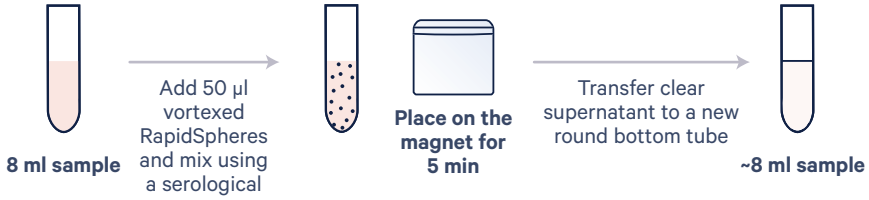
All separation steps were performed in 14-ml round bottom tubes



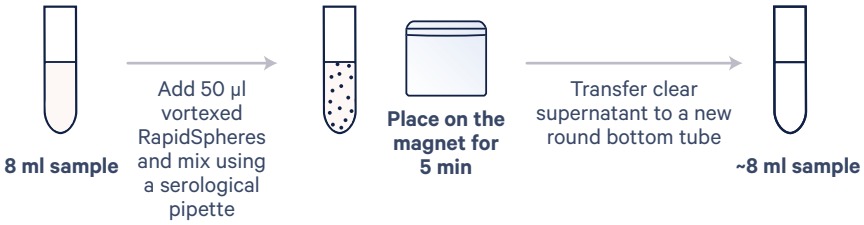
First Separation



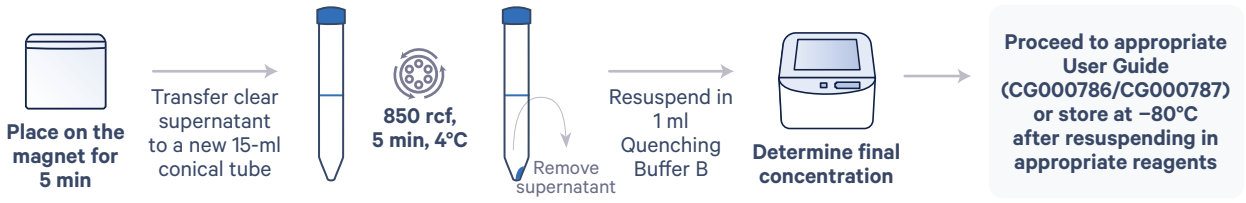
Second Separation



Third Separation

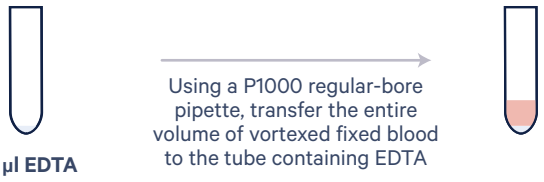


Residual RapidSpheres Removal

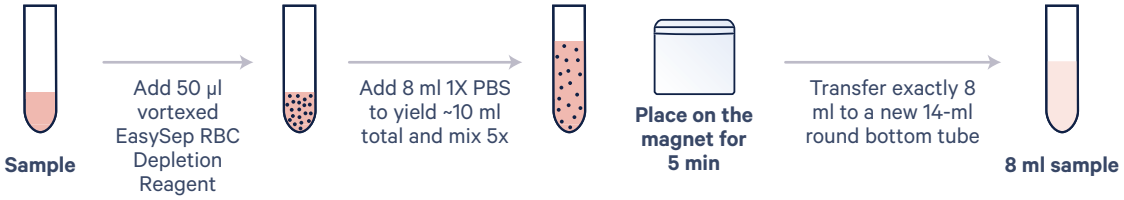


3. Cell Isolation
Option B. Leukocyte Isolation

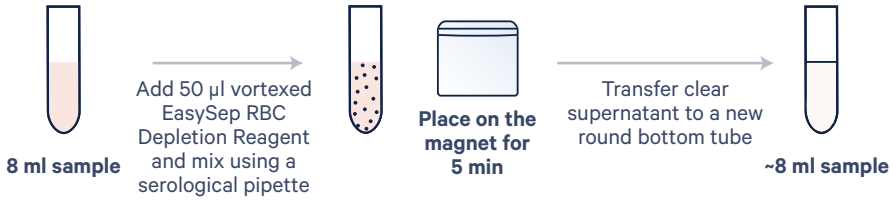
All separation steps were performed in 14-ml round bottom tubes



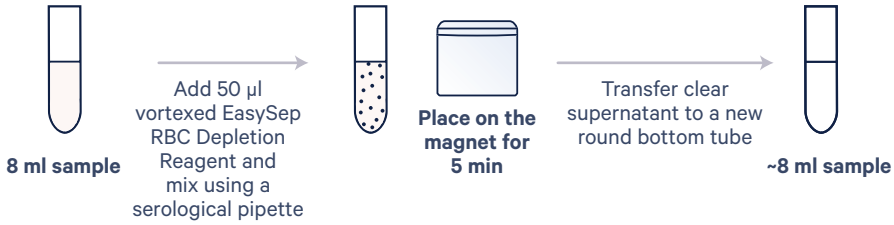
First Separation



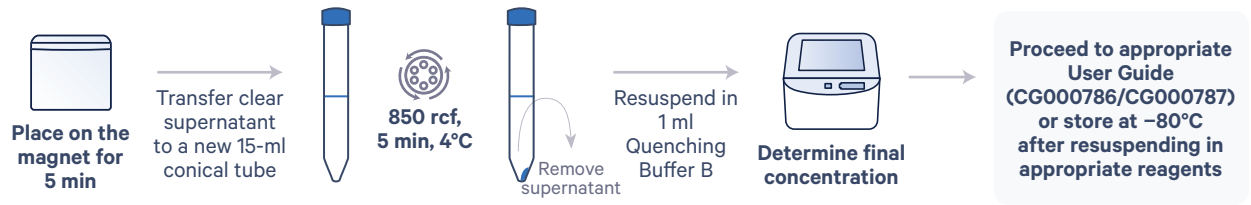
Second Separation



Third Separation



Residual EasySep RBC Depletion Reagent Removal



Blood Collection

Use vacutainer tubes containing K2 EDTA.

DO NOT use any other anticoagulant vacutainer to avoid loss of monocytes.

Protocol

1. Blood Fixation

Prepare

- Prepare Fixation Buffer and maintain at room temperature.

Buffers for Fixation - Prepare fresh				
Fixation Buffer Maintain at room temperature	**Stock**	**Final***	**For 1 Sample (µl)**	**For 4 Samples + 10% (µl)**
Formaldehyde	37%	4%	540.5	2378.2
1X PBS	-	-	3459.5	15221.8

** Final concentration in buffer + sample mix. Use formaldehyde with adequate ventilation, preferably in a fume hood. Follow appropriate regulations.*

Steps

a. Add **4 ml** Fixation Buffer to a 50-ml tube.

b. Invert the vacutainer tubes 3x.

c. Using a P1000 regular-bore pipette, add **1 ml** blood to the tube containing 4 ml Fixation Buffer.

Pipette up and down 2x to remove residual blood sample from the tip.

d. Swirl 50-ml tube to mix. DO NOT invert.

e. **Immediately** place at appropriate temperature and incubate. The fixation temperature and time will depend on the protocol option selected (see table below).

Protocol Option	Fixation Temperature	Fixation Time
Option A. PBMC Isolation	4°C	Overnight (20–24 h) to 1 week
Option B. Leukocyte Isolation	Room temperature	Overnight (20–24 h)

f. Proceed **immediately** to Post-Fixation Processing or store at -80°C after resuspending in 50% glycerol for a final concentration of 10%.

Fixed blood can be stored at –80°C for up to 12 months. See Appendix for guidance on storage and post-storage processing of fixed blood.

2. Post-Fixation Processing

Prepare

- Pre-cool centrifuge.
- Place 65 ml 1X PBS per sample on ice.

Steps

a. Pour or use a serological pipette to add ~**20 ml** cold 1X PBS to the sample. Cap the tube and vortex at high speed for ~20 sec to resuspend the pellet. Ensure the pellet is fully resuspended before moving on to the next step.

The sample might become foamy after vortexing. This is normal and does not affect performance.

b. Add additional 1X PBS to the sample to fill the 50-ml tube by using either a serological pipette or pouring directly.

c. Centrifuge at **850 rcf** for **5 min** at **4°C**.

d. Remove supernatant by quickly pouring out into an appropriate waste container. A serological pipette can also be used for removal.

The 50-ml tube can be left upside down for a few seconds to remove all the supernatant without dislodging the pellet.

e. Add **1.5 ml** cold 1X PBS to the pellet. Cap the tube and resuspend by vortexing at high speed for 20 sec.

The sample might become foamy after vortexing. This is normal and does not affect performance.

f. Proceed **immediately** to either PBMC Isolation (Option A) or Leukocyte Isolation (Option B).

3. Cell Isolation from Fixed Blood

Option A: PBMC Isolation

Prepare

- Pre-cool centrifuge.
- Obtain 0.5 M EDTA.
- Remove EasySep Direct Human PBMC Isolation Cocktail and EasySep Direct RapidSpheres (part of the STEMCELL Technologies EasySep Direct Human PBMC Isolation Kit reagents) from 4ºC storage and keep at room temperature when ready to use.
- Obtain an EasySep Magnet for steps e-h.
- Prepare Quenching Buffer B and maintain at 4ºC/ice.

Buffers for Isolation - Prepare fresh				
Quenching Buffer B Maintain at 4ºC	**Stock**	**Final**	**Per Sample (µl)**	**For 4 Samples + 10% (µl)**
Nuclease-free Water	-	-	875.0	3850.0
Conc. Quench Buffer B (10x Genomics PN-2001300)	8X	1X	125.0	550.0

Steps

a. Add **24 µl** 0.5 M EDTA to a 14-ml Round-Bottom Polypropylene Test Tube.

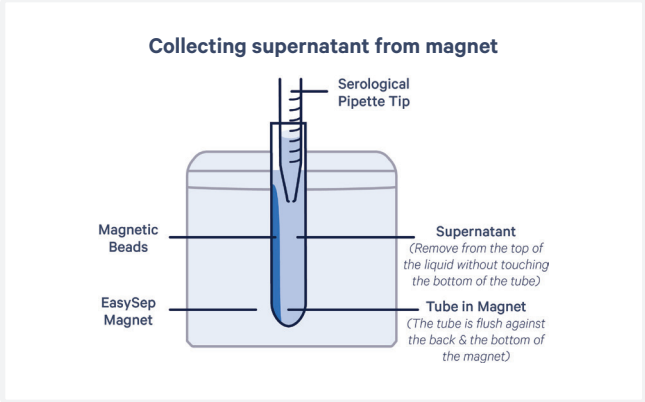
b. Using a P1000 regular-bore pipette, transfer the entire volume of vortexed fixed blood to the tube containing EDTA.

c. Add **150 µl** Isolation Cocktail to the sample. Using a P1000, pipette mix 5x.

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

e. First Separation:

- Vortex RapidSpheres for 30 sec. Add **50 µl** RapidSpheres to the sample.
- Using a serological pipette, add **8 ml** 1X PBS to yield ~10 ml total and mix 5x without introducing bubbles.
- Immediately place the sample tube on an EasySep Magnet and incubate for **5 min**.
- With the tube still on the magnet, transfer exactly **8 ml** from the top of the liquid to a new 14-ml Round-Bottom Polypropylene Test Tube. Supernatant might not be clear at this step.



f. Second Separation:

- Vortex RapidSpheres for 30 sec. Add **50 µl** RapidSpheres to the sample. Using a serological pipette, mix 5x without introducing bubbles.
- Immediately place the tube on an EasySep Magnet and incubate for **5 min**.
- With the tube still on the magnet, transfer all of the clear supernatant to a new 14-ml Round-Bottom Polypropylene Test Tube.

g. Third Separation: Repeat step f one more time for a third separation.

h. Residual RapidSphere Removal:

- Immediately transfer the tube to the EasySep Magnet and incubate for **5 min**.
- With the tube still on the magnet, transfer clear supernatant containing purified cells to a new 15-ml conical tube.

i. Centrifuge sample at **850 rcf** for **5 min** at **4ºC**.

j. Remove the supernatant.

k. Add **1 ml** Quenching Buffer B to the pellet and resuspend using a P1000. Keep the sample on ice. Pellet color could vary from white to pink.

l. Determine the cell concentration using an appropriate automated cell counter. See Appendix for counting guidance.

m. Proceed **immediately** to the appropriate GEM-X Flex Gene Expression protocols (see References) or store the sample after resuspending in appropriate reagents.

Samples can be stored at –80ºC for up to 12 months. See Appendix for guidance on storage and post-storage processing of fixed cells.

Option B: Leukocyte Isolation

Prepare

- Pre-cool centrifuge.
- Obtain 0.5 M EDTA.
- Remove EasySep RBC Depletion Reagent (part of EasySep RBC Depletion Reagent Kit) from 4ºC storage and keep at room temperature when ready to use.
- Obtain an EasySep Magnet for steps c-f.
- Prepare Quenching Buffer B and maintain at 4ºC/ice.

Buffers for Isolation - Prepare fresh				
Quenching Buffer B Maintain at 4°C	**Stock**	**Final**	**Per Sample (µl)**	**For 4 Samples + 10% (µl)**
Nuclease-free Water	-	-	875.0	3850.0
Conc. Quench Buffer B. (10x Genomics PN-2001300)	8X	1X	125.0	550.0

Steps

a. Add **24 µl** 0.5 M EDTA to a 14-ml Round-Bottom Polypropylene Test Tube.

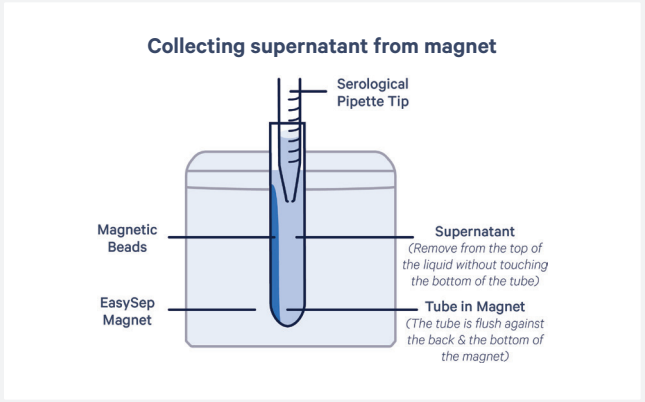
b. Using a P1000 regular-bore pipette, transfer the entire volume of vortexed fixed blood to the tube containing EDTA.

c. First Separation:

- Vortex EasySep RBC Depletion Reagent for 30 sec. Add **50 µl** EasySep RBC Depletion Reagent to the sample.
- Using a serological pipette, add **8 ml** 1X PBS to yield ~10 ml total and mix 5x without introducing bubbles.
- Immediately place the sample tube on an EasySep Magnet and incubate for **5 min**.
- With the tube still on the magnet, transfer exactly **8 ml** from the top of the liquid to a new 14-ml Round-Bottom Polypropylene Test Tube. Supernatant might not be clear at this step.

d. Second Separation:

- Vortex EasySep RBC Depletion Reagent for 30 sec. Add **50 µl** EasySep RBC Depletion Reagent to the sample. Using a serological pipette, mix 5x without introducing bubbles.



- Immediately place the tube on an EasySep Magnet and incubate for **5 min**.
- With the tube still on the magnet, transfer all of the clear supernatant to a new 14-ml Round-Bottom Polypropylene Test Tube.

e. Third Separation: Repeat step d one more time for a third separation.

f. Residual EasySep RBC Depletion Reagent Removal:

- Immediately transfer the tube to the EasySep Magnet and incubate for **5 min**.
- With the tube still on the magnet, transfer clear supernatant containing purified cells to a new 15-ml conical tube.

g. Centrifuge sample at **850 rcf** for **5 min** at **4ºC**.

h. Remove the supernatant.

i. Add **1 ml** Quenching Buffer B to the pellet and resuspend using a P1000. Keep the sample on ice. Pellet color could vary from white to pink.

j. Determine the cell concentration using an appropriate automated cell counter. See Appendix for counting guidance.

Load ≤100k leukocytes per hyb reaction for optimal performance. Higher cell loads can lead to slightly decreased data quality.

k. Proceed **immediately** to the appropriate GEM-X Flex Gene Expression protocols (see References) or store the sample after resuspending in appropriate reagents.

Samples can be stored at –80ºC for up to 12 months. See Appendix for guidance on storage and post-storage processing of fixed cells.

Data Highlights

The representative Data Highlights show key results derived from PBMCs and leukocytes isolated from fixed blood. Data below demonstrates that PBMCs isolated from the blood fixed for up to 7 days at 4ºC, with or without fixed blood storage at −80ºC prior to processing, retain the single cell information (Fig. 1). Similar results were observed in leukocytes isolated from blood fixed overnight (20-24 h) at room temperature or for 7 days at 4ºC, with or without fixed blood storage at −80ºC prior to processing (Fig. 2).

Methods Overview

Blood collected in EDTA vacutainers was mixed with the Fixation Buffer and incubated for the appropriate temperature and time as listed in this protocol. Fixed blood was either stored at −80ºC until processing or directly used for post-fixation processing. Cells (PBMCs and leukocytes) were then isolated. Fixed cells were then hybridized with probe sets, each set containing a unique Probe Barcode to enable sample multiplexing and read-level demux. After overnight hybridization, samples were pooled, washed, and partitioned in the Chromium X instrument, where the probes were ligated along with the addition of a 10x GEM Barcode. This was followed by library construction, sequencing, and data analysis.

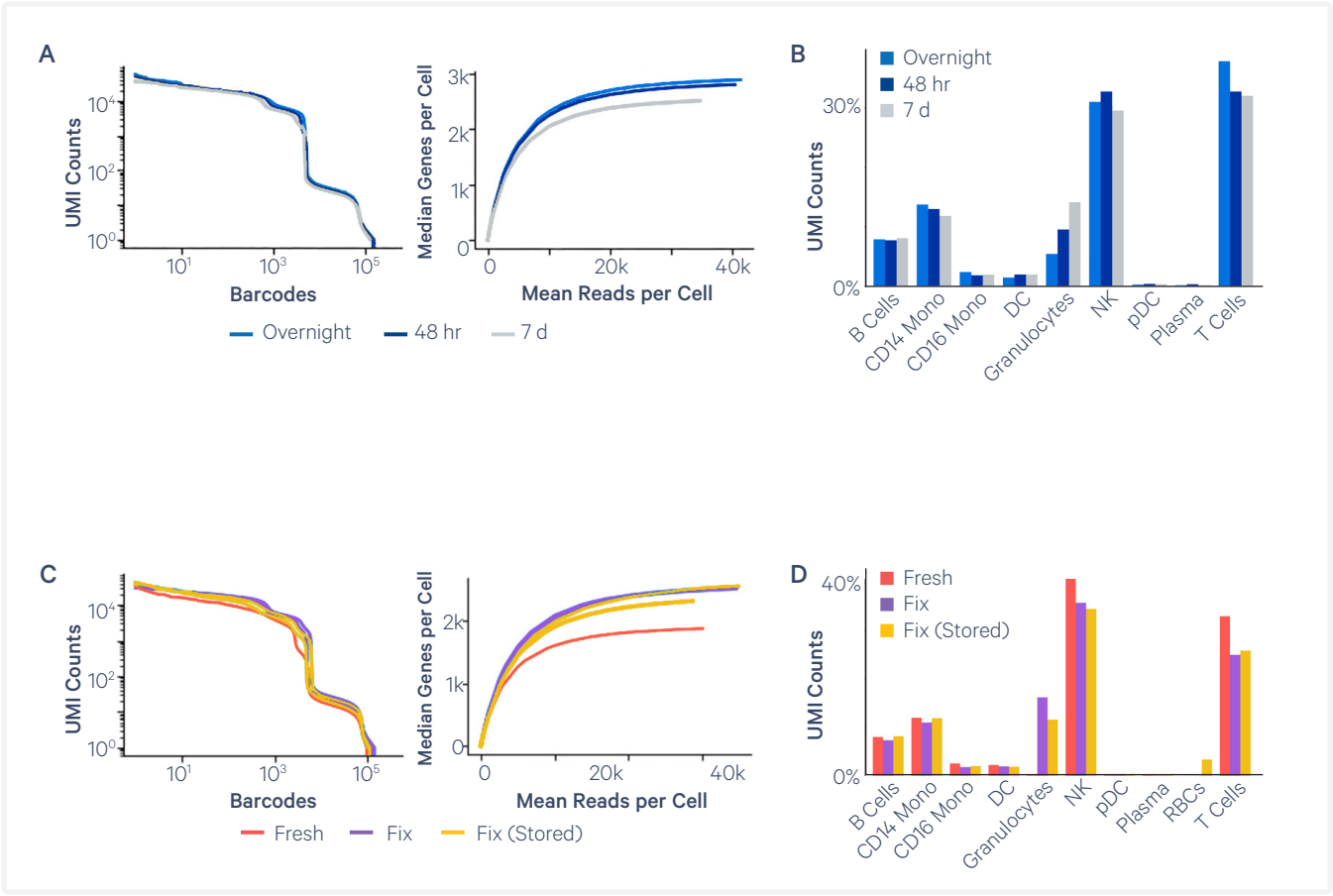


Figure 1. PBMCs isolated from blood fixed overnight, 2 days, or 7 days. Barcode rank plot and assay complexity or sensitivity plots (A) and frequency of cell population (B) across conditions. Barcode rank plot and assay complexity/sensitivity comparison of PBMCs from fresh blood versus PBMCs isolated from fixed blood that was either directly used for post-fixation processing or stored at −80ºC until processing (C). Representative cell population frequency of PBMCs from fresh blood vs. PBMCs from fixed blood that was either directly used or first stored and then used (D).

Data Highlights *(contd)*

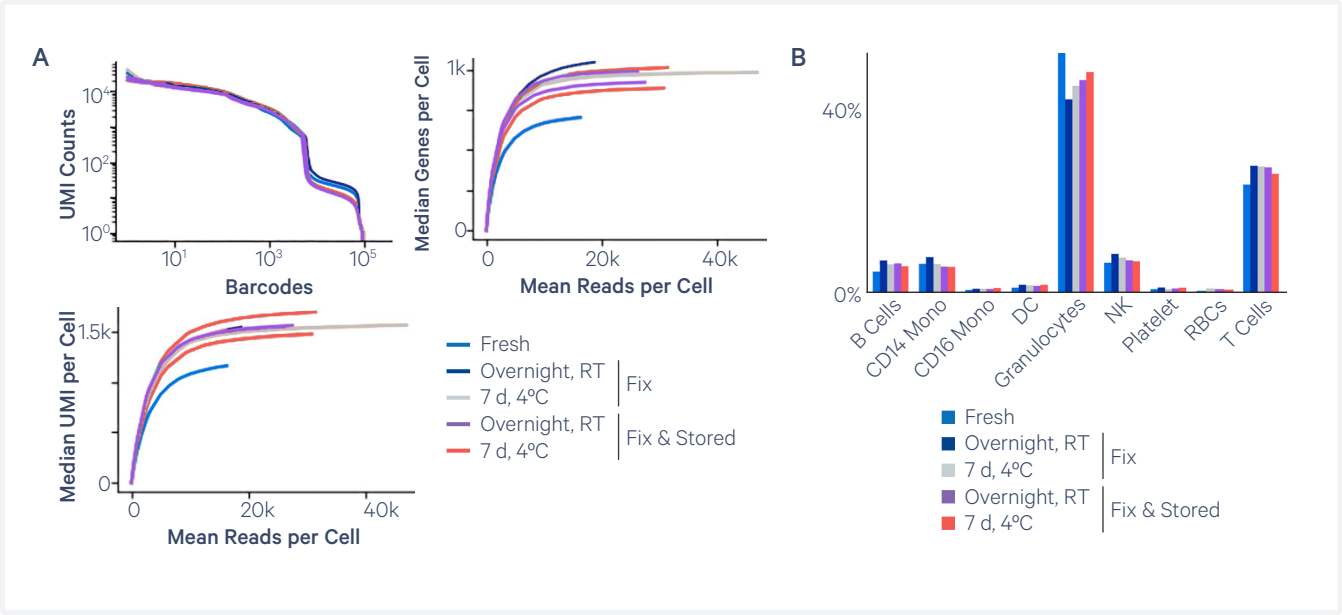


Figure 2. Leukocytes isolated from fresh blood or blood fixed overnight (room temperature) or 7 days (4°C). Fixed blood was either directly used for post-fixation processing and cell isolation or stored at −80°C first. Barcode rank plot and assay complexity/sensitivity plots (A) and frequency of cell populations (B) across conditions.

Appendix

Storage of Fixed Blood

- For storage, add 1.25 ml 50% glycerol to the fixed blood sample for a final concentration of 10% and vortex briefly at high speed to mix.

Post-Storage Processing of Fixed Blood

- When ready to use samples stored at −80°C from this step, thaw at room temperature until no ice remains.
- Proceed to Post-Fixation Processing.

Storage of Cells Isolated from Fixed Blood

- Thaw Enhancer (10x Genomics PN-2000482) for 10 min at 65°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. Once thawed, Enhancer can be kept at 42°C for up to 10 min. If not used within 10 min and precipitation is observed, reheat at 65°C to ensure enhancer is fully dissolved again before use.
- Add 0.1 volume Enhancer (10x Genomics PN 2000482) to the sample in Quenching Buffer B. For example, add 100 µl Enhancer to 1,000 µl of sample in Quenching Buffer B.
- Add 50% glycerol (freshly prepared) for a final concentration of 10%. For example, add 275 µl 50% glycerol to a 1,100 µl sample in Quenching Buffer B and Enhancer.

Post-Storage Processing of Fixed Cells

- When ready to use samples stored at −80°C from this step, thaw at room temperature until no ice remains.
- Centrifuge sample at 850 rcf for 5 min at room temperature. Remove the supernatant without disturbing the pellet.
- Resuspend cell pellet in 1 ml Quenching Buffer B or 0.5X PBS + 0.02% BSA supplemented with 0.2 U/µl RNase Inhibitor and maintain on ice. Use RNase-free BSA at this step. See Specific Reagents & Consumables for details.

0.5X PBS + 0.02% BSA Maintain at 4°C	**Stock**	**Final**	**For 1 sample (µl)**	**For 4 samples + 10% (µl)**
Nuclease-free Water	-	-	493.0	2169.2
1X PBS	1X	0.5X	500.0	2200.0
RNase-free BSA	10.0	0.02	2.0	8.8
RNase Inhibitor	40.0	0.2	5.0	22.0

Fixed Cell Counting

- Accurate sample counting is critical for achieving desired cell recovery.
- The fixed sample 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.

- 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

Compatible User Guides:

1. GEM-X Flex Gene Expression Reagent Kits For Singleplexed Samples (CG000786)
2. GEM-X Flex Gene Expression Reagent Kits For Multiplexed Samples (CG000787)

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

Document Revision Summary

Document Number	CG000785
Title	Blood Fixation and Cell Isolation for GEM-X Flex Gene Expression
Revision	Rev A
Revision Date	October 2024

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