Fixation of Cells & Nuclei 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 how to perform fixation on single cell and nuclei suspensions for use with the GEM-X Flex Gene Expression workflow. This protocol also provides guidance on storage of fixed cells and post-storage processing.

Prior to fixation, samples can also be labeled using a specific protein binding molecule, such as an antibody conjugated to a Feature Barcode oligonucleotide.

Consult Demonstrated Protocol Cell Surface and Intracellular Protein Labeling for GEM-X Flex Gene Expression (CG000781) for guidance.

Additional Guidance

This protocol was demonstrated using primary cells (including peripheral blood mononuclear cells - PBMCs), dissociated tumor cells (DTCs), cell lines (including Jurkat, K562, 293T), and nuclei suspensions from several tissue types. See tested tissue list for more information.

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	PN
For Cell Thaw & Sample Fixation		
10x Genomics	GEM-X Flex Sample Preparation v2 Kit *Kit components are listed on the next page*	1000781
Millipore Sigma	Bovine Serum Albumin In DPBS (10%) *Alternative to Thermo Fisher product*	A1595
Thermo Fisher Scientific	UltraPure Bovine Serum Albumin (BSA, 50 mg/ml) *Alternative to Millipore Sigma product*	AM2616
	Formaldehyde (37% by Weight/ Molecular Biology), Fisher BioReagents	BP531-25
	Nuclease-free Water (not DEPC-Treated)	AM9937
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
	*Corning RPMI 1640 1X with L-Glutamine	10-040-CM
VWR	*Seradigm Premium Grade Fetal Bovine Serum (FBS)	97068-085
**Only needed for cell thawing.*		
For Sample Filtration		
Sysmex	Sterile Single-Pack CellTrics Filters	04-004-2326
Miltenyi Biotec	Pre-Separation Filters (30 µm) *Alternative to Sysmax product*	130-041-407
Choose either Sysmex or Miltenyi Biotec filter.		

Vendor	Item	PN
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 the stock to 1:100 and mix 1:1 with the sample. For example, add 10 µl diluted dye to 10 µl sample.	
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
	Trypan Blue Stain (0.4%)	T10282
	*DAPI solution, 1 mg/mL	62248
*†Choose Countess II/3, Cellaca, or equivalent fluorescent counter. *Choose either AOPI, NucSpot, PI, or DAPI solution. If the sample has no debris, Trypan Blue can be used.*		
For Storage & Post-Storage Processing		
Acros Organics	Glycerol, 99.5%, for molecular biology, DNAse, RNAse and Protease free, *Alternative to Millipore Sigma product*	327255000
Millipore Sigma	Glycerol for molecular biology, ≥99.0%, *Alternative to Acros Organics product*	G5516-100ML
	Albumin, Bovine Serum, 10% Aqueous Solution, Nuclease-Free *Alternative to Thermo Fisher product*	126615
	Protector RNase Inhibitor	3335402001
Thermo Fisher Scientific	UltraPure BSA (50 mg/mL) *Alternative to Millipore Sigma product*	AM2616
Additional Materials		
Eppendorf	DNA LoBind Tubes 2.0 ml	022431048
	ThermoMixer C	5382000023
VWR	Vortex Mixer	10153-838

This list may not include some standard laboratory equipment.

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

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

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

Preparation - Buffers

Buffers for Fixation - Prepare fresh			
Fixation Buffer B *Maintain at room temperature*	**Stock**	**Final**	**Per Sample (µl) +10%**
Nuclease-free Water	-	-	435
Conc. Fix & Perm Buffer B (10x Genomics PN-2001301) *Thaw at room temperature. Vortex, check for precipitation, and centrifuge briefly. Maintain at room temperature.*	10X	1X	55
Formaldehyde*	37%	4%	60
Quenching Buffer B *Maintain at 4°C*	**Stock**	**Final**	**Per Sample (µl) + 10%**
Nuclease-free Water	-	-	962.5
Conc. Quench Buffer B (10x Genomics PN-2001300) *Thaw at room temperature. Vortex and centrifuge briefly. Maintain at 4°C*	8X	1X	137.5
Additional Buffers			
PBS + 0.04% BSA *(maintain at 4°C)*			
Additive C (10x Genomics PN-2001332) *Thaw at room temperature. Vortex and check for precipitation. Maintain at room temperature.*			
Formaldehyde should always be used with adequate ventilation, preferably in a fume hood. Follow appropriate regulations.			

Buffers for Storage of Fixed Samples - Prepare fresh

50% Glycerol Solution
For long-term storage of fixed samples

- Mix an equal volume of nuclease-free water and 99% Glycerol, Molecular Biology Grade.
- Filter through a 0.2 µm filter.
- Store at room temperature in 2-ml DNA LoBind tubes.

All buffer preparations should be fresh.

Tips & Best Practices

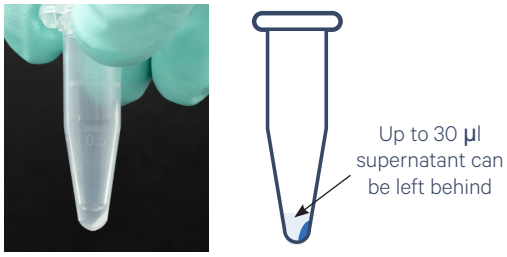
The following recommendations are critical for optimal performance of the GEM-X Flex Gene Expression assay.

Sample Quality

- Use high-quality single cell or nuclei suspensions that can withstand the fixation steps.
- Perform pilot experiments to determine if the sample type is suitable for the fixation.
- Highly viable single cell or nuclei suspensions (>80%) will have the greatest sensitivity and cell recovery. However, the GEM-X Flex Gene Expression assay is robust to samples at much lower viability, with successful results demonstrated even with low viability samples (50% or lower). Low viability samples may have more variable cell calling and lower sensitivity.
- Samples should have minimal debris for best results; debris can have associated RNA that can contribute to noncell background.

Centrifugation & Pellet Resuspension

- Use a swinging-bucket rotor for higher cell/nuclei recovery.
- Pre-fixation centrifugation speed and time may need optimization depending upon sample type.
- When working with samples with low cell numbers (i.e. <300,000 cells), complete removal of the supernatant is not required. Up to 30 µl supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.



- After each buffer addition step, gently mix cells/nuclei 5x, or until the pellet is completely resuspended, without introducing bubbles.

Pre-fixation Cell Counting

- Counting fresh single cell/nuclei suspensions prior to fixation can be skipped if viability information is not needed and if the amount of cells/nuclei do not overly exceed the upper limit recommendations going into fixation (see page 5). If cell/nuclei number exceeds this recommendation, additional Fixation Buffer will be needed.

Fixation Conditions

- Fixation temperature and time depend upon the subsequent use of the fixed sample.

Fixed Sample Use	Fixation Time & Temperature
Fixed sample to be processed immediately	1 h at room temperature (20°C)
Fixed sample to be stored subsequently	16-24 h at 4°~C

DO NOT mix samples with different fixation times in one experiment.

- If planning to store the fixed samples, it is strongly recommended to perform a 16-24 h fixation at 4°C and store the fixed samples at -80°C for best results.

Fixed Cell Counting

- Counting fixed cells can be skipped if planning to run samples in singleplex format and pre-fixation counts are <500,000.
- Counting fixed samples in Quenching Buffer B before hybridization is recommended if samples will be multiplexed and the amounts of cells/nuclei added into each hybridization need to be normalized. Counting is also encouraged if unsure of low input sample amount.
- It is recommended that the sample be stained with a fluorescent dye such as PI Staining Solution and counted using an automated fluorescent cell counter or hemocytometer.
- See Appendix for details on fixed cell counting.

Fixed Sample Storage

- Fixed cell or nuclei suspensions can be stored at 4°C for up to 1 week or at -80°C for up to 12 months after resuspending in appropriate reagents.

- Sample storage and post-storage guidelines are provided in the Appendix.

Cell/Nuclei Number Recommendation

- For sample fixation, the recommended minimum number is 25,000 cells or nuclei to ensure there are enough cells/nuclei for the downstream workflow.
- The recommended maximum number for fixation is 10 x 10^6 per 0.5 ml Fixation Buffer B.
- It may be possible to use less than the recommended minimum numbers. The lower cell numbers during fixation will impact the cell input number during hybridization and increase the likelihood of cell/cell pellet loss prior to workflow completion.
- Some cell loss is expected during the fixation steps depending up on the sample type, cell type, and user experience.

Recommended Cell/Nuclei Input For Fixation
25,000–10 x 10^6 cells
Recommended Cell/Nuclei Input Per Hybridization
25,000–500,000 *(if sufficient cells/nuclei are available, it is recommended to default to 300,000 fixed cells/nuclei)* *≤100,000 if using leukocytes and splenocytes*

Recommended Cell/Nuclei Input For Fixation

Important Considerations

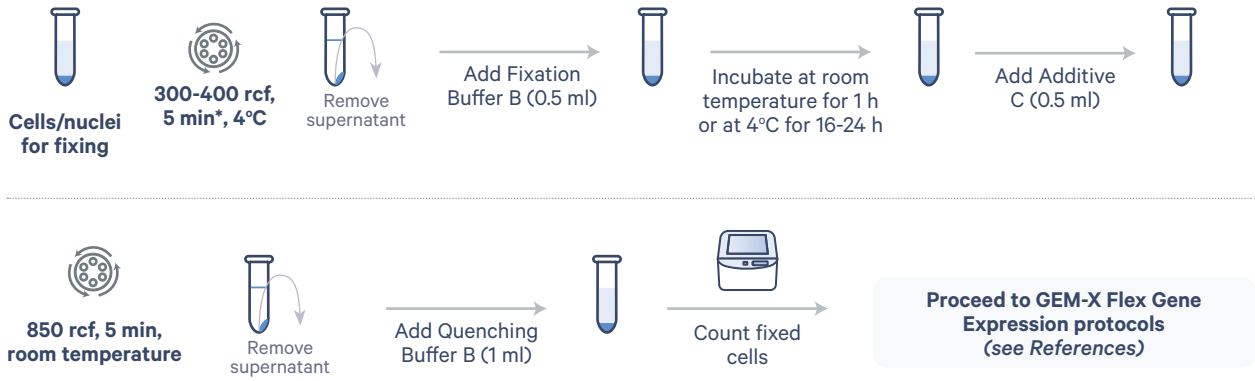
It may be possible to use ≤25,000 cells/nuclei, but it may lead to:

- Loss of pellet
- Not enough cells for storage
- Difficulty in pooling samples in equal number when multiplexing
- Not enough cells left after washing to target maximum cell load (20,000 cells/Probe Barcode)
- Difficulty in counting samples; may require concentrating the sample
- Observance of an atypical library trace and increase in wasted data due to excess supernatant left behind during post hybridization washes

Mitigation Strategies when using Lower Cell Input

- Follow better sample preparation practices including use of a swinging bucket rotor
- During probe hybridization, up to 15 µl supernatant can be left behind to avoid losing the pellet
- During post-hybridization wash, up to 30 µl supernatant can be left behind to avoid losing the pellet
- Follow pooled wash workflow during post-hybridization wash

Fix Sample



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

Fixation Protocol

GEM-X Flex Sample Preparation v2 Kit, 48 rxns (PN-1000781) was used for sample fixation. This protocol is compatible with both cell and nuclei suspensions.

This protocol has been demonstrated using 25,000-10x10^6 cells or nuclei. If cell/nuclei number exceeds this recommendation, additional Fixation Buffer will be needed.

Optional

Label cells with TotalSeq antibody. Refer to Demonstrated Protocol Cell Surface and Intracellular Protein Labeling for GEM-X Flex Gene Expression (CG000781) for details. Optional cell surface and intracellular protein labeling must be completed prior to sample fixation.

Thaw Cells

If using fresh cells or nuclei suspensions, proceed directly to Sample Fixation.

a. Pre-warm **10 ml** complete growth media (RPMI + 10% FBS) for cell thawing at **37°C**.

b. Remove cryovials containing cryopreserved cells from storage, thaw in the water bath at **37°C** for **1–2 min**. Remove from the water bath when a tiny ice crystal remains.

c. Add **1 ml** pre-warmed complete growth media (RPMI + 10% FBS) to the thawed cell vial.

d. Pipette mix the cells and transfer to a 15-ml conical tube containing **9 ml** pre-warmed complete growth media (RPMI + 10% FBS).

e. Centrifuge at **300-400 rcf** for **5 min** (PBMCs/cell lines) or **150 rcf** for **10 min** (dissociated tumor cells) at **room temperature**.

Use of a swinging-bucket rotor is recommended for higher cell recovery. Centrifugation speed and time may need optimization depending upon the sample type.

f. Remove the supernatant without disturbing the pellet.

Up to 30 µl supernatant may be left behind if working with < 300,000 cells. Transfer pipettes may be used to remove supernatant after centrifugation to minimize disturbance to the pellet.

g. Resuspend the pellet in **1 ml** chilled PBS + 0.04% BSA. Gently pipette mix and transfer to a 1.5-ml microcentrifuge tube.

h. Determine and record cell concentration and viability of the sample using an automated cell counter or hemocytometer. If cell debris and large clumps are present, pass the sample through a 40, 70, or 120 µm Cell Strainer.

i. Proceed to Sample Fixation.

Sample Fixation

a. Centrifuge sample at **300-400 rcf** for **5 min** (PBMCs/cell lines) or **150 rcf** for **10 min** (dissociated tumor cells) at **4°C**.

b. Remove the supernatant without disturbing the pellet.

Up to 30 µl supernatant may be left behind if working with < 300,000 cells.

c. Add **0.5 ml** room temperature Fixation Buffer B to the sample pellet and pipette mix 5x.

d. Incubate for **1 h** at **room temperature (20°C)** or for **16-24 h** at **4°C** . If planning to store fixed samples, a **16-24 h** fixation at **4°C** is recommended.

DO NOT agitate or mix the sample during incubation.

If working with splenocytes,, fix for 16-24 h at 4°C.

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. Fixation time and temperature should be consistent across all samples in an experiment.

e. Add **0.5 ml** room temperature Additive C to the sample in Fixation Buffer B and pipette mix 5x.

f. Centrifuge at **850 rcf** for **5 min** at **room temperature**.

g. Remove the supernatant without disturbing the pellet.

Up to 30 µl supernatant may be left behind if working with < 300,000 cells.

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

**If cell numbers are expected to be <100,000, resuspend sample pellet in 200 µl Quenching Buffer B. After counting, add an additional 800 µl Quenching Buffer B before proceeding to GEM-X Flex Gene Expression protocols or storage.*

i. Determine cell concentration of the fixed sample using an automated cell counter or hemocytometer. See Appendix for Fixed Cell/ Nuclei Counting.

For accurate cell counting, it is strongly recommended that the cell/nuclei suspension be stained with a fluorescent dye such as AO/PI Staining Solution and counted using an automated fluorescent cell counter.

j. 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 4°C for up to 1 week or at -80°C for up to 12 months, depending upon the reagents used for storage. See Appendix for guidance on storage and post-storage processing.

Appendix

Fixed Sample Storage Guidance

Fixed samples can be stored for short or long-term.

Short-term Storage at 4°C

a. Thaw Enhancer (10x Genomics PN-2000482) for **10 min** at **65°C**. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use.

DO NOT keep the thawed reagent on ice, or the solution will precipitate. 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.

b. Add **0.1 volume** pre-warmed Enhancer to fixed sample in Quenching Buffer B.
For example, add 100 µl Enhancer to 1,000 µl fixed sample in Quenching Buffer B. Pipette mix. Alternatively, to conserve the Enhancer volume, centrifuge cells at 850 rcf, remove 500 µl Quenching Buffer B, and add 50 µl Enhancer to the sample.

c. Store sample at **4°C** for up to **1 week**.

Long-term Storage at −80°C

a. Thaw Enhancer (10x Genomics PN-2000482) for **10 min** at **65°C**. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use.

DO NOT keep the thawed reagent on ice, or the solution will precipitate. 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.

b. Add **0.1 volume** pre-warmed Enhancer to fixed sample in Quenching Buffer B.
For example, add 100 µl Enhancer to 1,000 µl fixed sample in Quenching Buffer B. Pipette mix. Alternatively, to conserve the Enhancer volume, centrifuge cells at 850 rcf, remove 500 µl Quenching Buffer B, and add 50 µl Enhancer to the sample.

c. Add 50% glycerol for a final concentration of 10%. For example: add 275 µl 50% glycerol to 1,100 µl fixed sample in Quenching Buffer B and Enhancer. Pipette mix.

d. Store at **−80°C** for up to **12 months**.

If planning to store the fixed samples, it is strongly recommended to perform a 16-24 h fixation at 4°C during the fixation step and store the fixed samples at −80°C for best results.

Post-Storage Processing

Samples may undergo a color change during storage (e.g. black, light gray, or green), however this will not impact assay performance.

If samples were stored at −80°C, thaw at room temperature until no ice is present.

a. Centrifuge sample at **850 rcf** for **5 min** at **room temperature**.

b. Remove the supernatant without disturbing the pellet.

Up to 30 µl supernatant may be left behind if working with < 300,000 cells.

c. Resuspend cell pellet in **1 ml** Quenching Buffer B and keep on ice.

d. Determine cell concentration of the fixed sample using an automated cell counter or hemocytometer. See Fixed Cell/Nuclei Counting.

e. Proceed **immediately** to the appropriate GEM-X Flex Gene Expression protocols (see References).

Fixed Sample Shipping Guidance

a. Fixed samples resuspended in Quenching Buffer B supplemented with Enhancer can be shipped with a cold pack. See Short-term Storage for details.

b. Fixed samples resuspended in Quenching Buffer B supplemented with Enhancer and Glycerol can be shipped on dry ice. See Long-term Storage for details.

Fixed Cell/Nuclei Counting

- Accurate sample counting is critical for achieving desired cell recovery. Table 1 shows the combination of counters and dyes tested for counting cells/nuclei post-hybridization and post-hybridization wash.
- It is strongly recommended that the fixed sample be stained with a fluorescent dye such as PI staining solution and counted using an automated fluorescent cell counter or hemocytometer.
- The use of fluorescent dye during cell counting enables accurate quantification even in the presence of sub-cellular debris.
- Automated fluorescent cell counters are strongly recommended when counting fixed cells.
- Ensure that the counter laser/filter setup is compatible with the fluorescent dye used.
- Ensure cells are well-focused under brightfield before switching to the fluorescent channel for counting.
- Increase exposure time to help adjust signal to noise during counting.

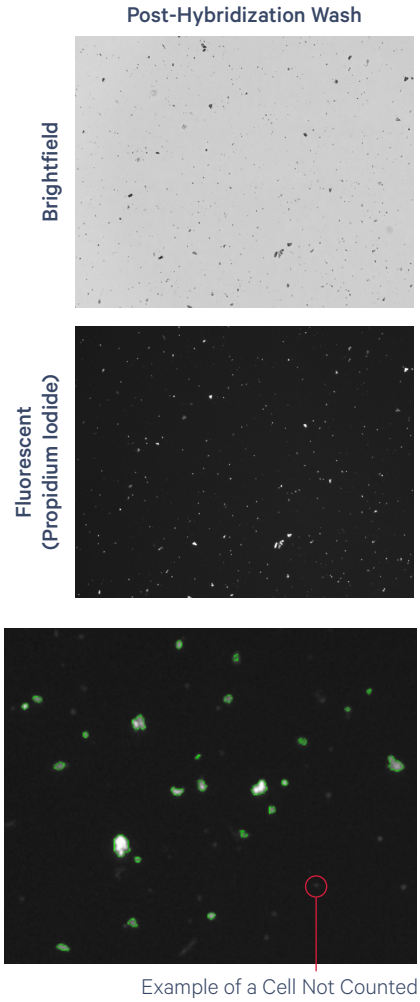
Counter Type	Fluorescent Dye	Counting Comparison
Cellaca *Range: 1 x 10^5–1 x 10^7cells/ml* *Automated exclusion of debris from cell count*	• Propidium Iodide • NucSpot 470 • DAPI	Comparable counting results at both counting steps for all three dyes
Countess II FL/Countess 3 FL *Range: 1 x 10^4–1 x 10^7cells/ml (optimal 1 x 10^5–4 x 10^6)* *Manual debris exclusion from cell count post-image capture, using gates on the instrument program*	• Propidium Iodide • NucSpot 470 • DAPI	Comparable counting results at both counting steps for the three dyes
Cellometer K2 *Range: 1 x 10^5–1 x 10^7cells/ml* *Debris exclusion from cell count by adjusting instrument program settings before image capture*	• Propidium Iodide • NucSpot 470	Comparable counting results at both counting steps for the two dyes Propidium Iodide stained nuclei require longer exposure compared to NucSpot 470 but can still be relatively dimmer

- Perform visual inspection to confirm that the counting number is accurate. For example, after obtaining the counting number, switch between the brightfield and fluorescent channels to make sure the counts include minimal debris and the most cells.

Counting using PI Staining Solution

This protocol provides instructions for counting samples using 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.

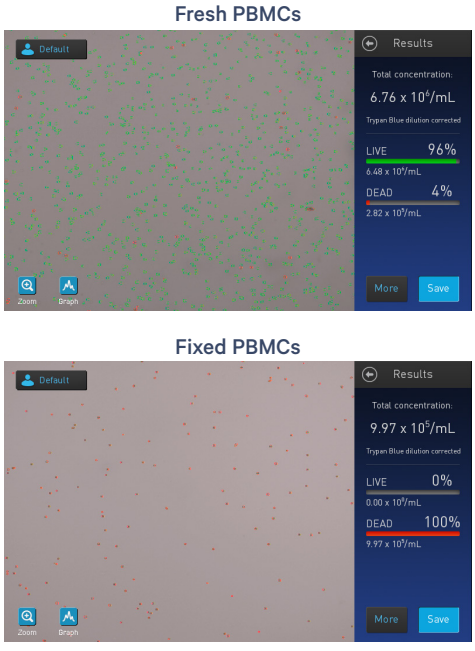
Brightfield & Fluorescent Images from Automated Cell Counter - Samples Stained with Propidium Iodide



- Add **25 µl** 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. Ensure that this dilution factor is accounted for during counting. For example, because a 1:1 dilution was performed, the final cell conentration should be multiplied by two.
- Add **25 µl** sample to Mixing Row of plate containing 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.

Samples stained with PI staining solution can also be counted using the Countess II/3 or K2 Automated Cell Counter. Refer to manufacturer's instructions for details.

Brightfield image from Automated Cell Counter – Samples Stained with Trypan Blue



Counting using Trypan Blue (Only for Debris-free Samples)

Debris-free samples (cells or nuclei suspensions) can also be counted using trypan blue. This protocol provides instructions for counting sample using trypan blue and a hemocytometer or Countess II Automated Cell Counter.

- Mix **1 part** 0.4% trypan blue and **1 part** sample.
- Transfer **10 µl** sample to a Countess II Cell Counting Slide chamber or a hemocytometer.
- Insert the slide into the Countess II Cell Counter and determine the cell concentration. Or if using a hemocytometer, count fixed cells by placing the hemocytometer under the microscope.
- The majority of fixed cells or nuclei suspensions will be stained with trypan blue stain and appear nonviable.

References

1. GEM-X Flex Gene Expression Reagent Kits for Singleplexed Samples (CG000786)
2. GEM-X Flex Gene Expression Reagent Kits for Singleplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture (CG000788)
3. GEM-X Flex Gene Expression Reagent Kits for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture (CG000789)
4. GEM-X Flex Gene Expression Reagent Kits User Guide for Multiplexed Samples (CG000787)
5. Blood Fixation and Cell Isolation for GEM-X Flex Gene Expression (CG000785)

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

Document Revision Summary

Document Number	CG000782
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