Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling

Introduction

Chromium Fixed RNA Profiling 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 Chromium Fixed RNA Profiling 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 Protein Labeling for Chromium Fixed RNA Profiling with Feature Barcode technology (CG000529) 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. Optimize this protocol (e.g., centrifugation conditions) based on sample type.

Cells carry potentially hazardous pathogens. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage, and disposal of biological materials.

Specific Reagents & Consumables

Vendor	Item	Part Number
For Cell Thaw & Sample Fixation		
10x Genomics	Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit	1000414
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)	130-041-407
Choose either Sysmex or Miltenyi Biotec filter.		

For Cell Counting		
Nexcelom Biosciences	ViaStain PI Staining Solution	CS1-0109-5mL
	ViaStain AOPI Staining Solution *Alternative to PI Staining Solution.*	CS2-0106-5mL
	†Cellaca MX High-throughput Automated Cell Counter	MX-112-0127
Thermo Fisher Scientific	†Countess II FL Automated Cell Counter *Discontinued*	AMAQAF1000
	Countess II FL Automated Cell Counting Chamber Slides	C10228
	Trypan Blue Stain (0.4%)	T10282
	Ethidium Homodimer-1	E1169
	†Choose either Countess or Cellaca.	
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	126615
	Protector RNase Inhibitor	3335402001
Thermo Fisher Scientific	UltraPure BSA (50 mg/mL)	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.

Preparation – Buffers

Buffers for Fixation - Prepare fresh			
Fixation Buffer Maintain at room temperature	**Stock**	**Final**	**Per Sample (µl)**
Nuclease-free Water	-	-	791.9
Conc. Fix & Perm Buffer* (10x Genomics PN-2000517)	10X	1X	100
Formaldehyde	37%	4%	108.1
Quenching Buffer Maintain at 4°C	**Stock**	**Final**	**Per Sample (µl)**
Nuclease-free Water	-	-	875.0
Conc. Quench Buffer* (10x Genomics PN-2000516)	8X	1X	125.0

Additional Buffers

PBS + 0.04% BSA *(maintain at 4°C)*

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 LoBind tubes.

Additional Buffers

0.5X PBS + 0.02% BSA (*maintain at 4°C*)
For post-storage processing; use RNase-free BSA

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

* *Included in the 10x Genomics Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit, 16 rxns (PN-1000414).*

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

All buffer preparations should be fresh.

Tips & Best Practices

The following recommendations are critical for optimal performance of the Chromium Fixed RNA Profiling assay.

Sample Quality

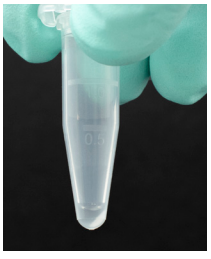
- Use high quality single cell or nuclei suspensions that can withstand the fixation steps.
- Perform pilot experiments to determine if the sample type is suitable for the fixation.
- Highly viable single cell or nuclei suspensions (>80%) will have the greatest sensitivity and cell recovery. However, the Chromium Fixed RNA Profiling 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 non-cell background.

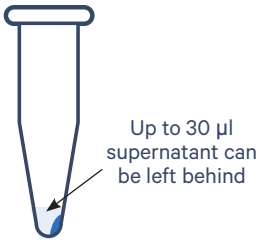
Cell/Nuclei Number

- This fixation protocol requires ≥300,000 cells or ≥500,000 nuclei.

Centrifugation & Pellet Resuspension

- Use a swinging-bucket rotor for higher cell/nuclei recovery.
- Centrifugation speed and time may need optimization depending upon the sample type.
- When working with samples with low cell numbers (i.e. <500,000 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.

Fixation Conditions

- Fixation temperature and time depends 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 incubation at 4°C and store the fixed samples at -80°C for best results.

Fixed Cell Counting

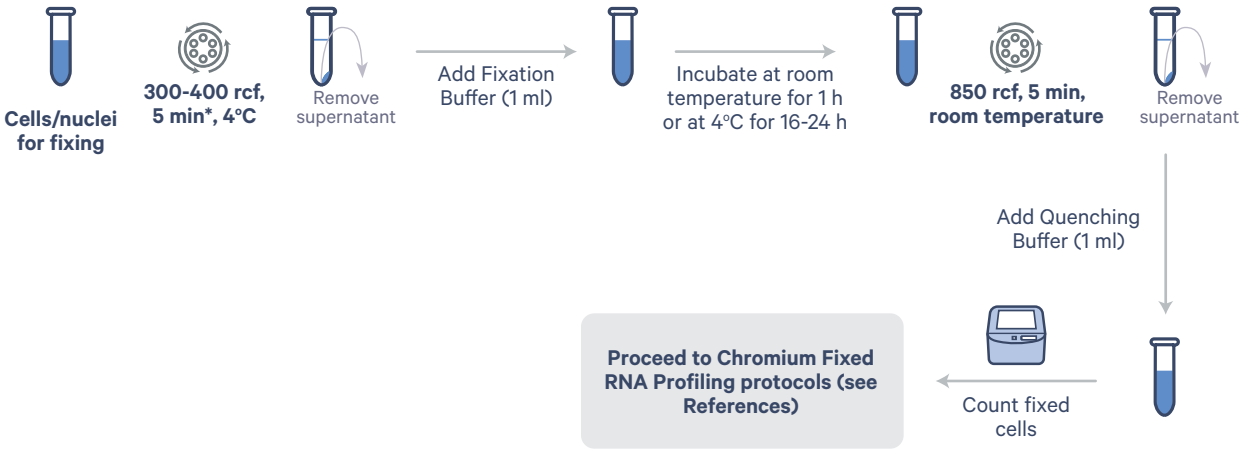
- Accurate sample counting is critical for optimal assay performance.
- It is strongly recommended that the sample be stained with a fluorescent dye such as Ethidium Homodimer-1 or PI Staining Solution and counted using an automated fluorescent cell counter (Countess II Automated Cell Counter or a Cellaca counter).
- 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 6 months after resuspending in appropriate reagents.
- Sample storage and post-storage guidelines are provided in the Appendix.

Protocol Overview : Sample Fixation

Fix sample



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

Fixation Protocol

Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit, 16 rxns (PN-1000414) was used for sample fixation. This protocol is compatible with both cell and nuclei suspensions.

This protocol has been demonstrated using 300,000-10x10^6 cells or 500,000-10x10^6 nuclei. If cell/nuclei number exceeds this recommendation, additional Fixation Buffer will be needed.

Optional

Label cells with TotalSeq-B antibody. Refer to Demonstrated Protocol Cell Surface Protein Labeling for Chromium Fixed RNA Profiling for Singleplexed Samples with Feature Barcode technology (CG000529) for details. Optional cell surface protein labeling must be completed prior to sample fixation.

Thaw Cells

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

a. Pre-warm **10 ml** media (RPMI + 10% FBS) for cell thawing.

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 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 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 **4°C**.

Use of 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.

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 (Countess II/Cellaca MX) or hemocytometer. If cell debris and large clumps are present, pass sample through a 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.

c. Add **1 ml** Fixation Buffer 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.

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

f. Remove the supernatant without disturbing the pellet.

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

h. Determine cell concentration of the fixed sample using an Automated Cell Counter (Countess II/Cellaca MX) 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 Ethidium Homodimer-1 or AO/PI Staining Solution and counted using an automated fluorescent cell counter.

i. Proceed **immediately** to appropriate Chromium Fixed RNA Profiling 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 6 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.

b. Add **0.1 volume** pre-warmed Enhancer to fixed sample in Quenching Buffer. For example, add 100 µl Enhancer to 1,000 µl fixed sample in Quenching Buffer. Pipette mix.

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.

b. Add **0.1 volume** pre-warmed Enhancer to fixed sample in Quenching Buffer. For example, add 100 µl Enhancer to 1,000 µl fixed sample in Quenching Buffer. Pipette mix.

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 and Enhancer. Pipette mix.

d. Store at **−80°C** for up to **6 months**.

If planning to store the fixed samples, it is strongly recommended to perform a 16-24 h incubation at 4°C 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.

c. Resuspend cell pellet in **1 ml** 0.5X PBS + 0.02% BSA* (optionally supplemented with 0.2 U/µl RNase Inhibitor) or Quenching Buffer and keep on ice.

**Use RNase-free BSA at this step. See Specific Reagents & Consumables for details.*

d. Determine cell concentration of the fixed sample using an Automated Cell Counter (Countess II/ Cellaca MX) or hemocytometer. See Fixed Cell/ Nuclei Counting.

e. Proceed **immediately** to appropriate Chromium Fixed RNA Profiling protocols (see References).

Fixed Sample Shipping Guidance

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

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

Fixed Cell/Nuclei Counting

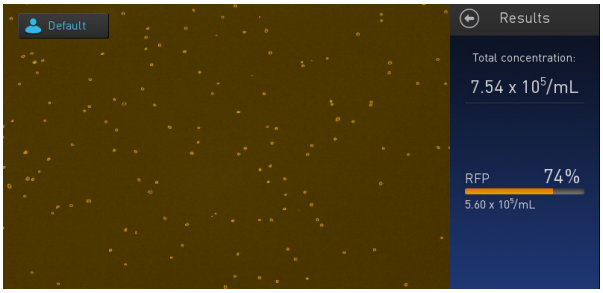
- Accurate sample counting is critical for optimal assay performance.
- It is strongly recommended that the fixed sample be stained with a fluorescent dye such as Ethidium Homodimer-1 or PI staining solution and counted using an automated fluorescent cell counter (Countess II Automated Cell Counter or a Cellaca counter).

Counting using Ethidium Homodimer-1

This protocol provides instructions for counting samples using Ethidium Homodimer-1 and the Countess II FL Automated Cell Counter (with RFP light cube) to enable accurate quantification even in the presence of sub-cellular debris. The optimal cell concentration for the Countess is 1,000-4,000 cells/µl. Refer to manufacturer's instructions for details on operations.

- Vortex Ethidium Homodimer-1, centrifuge briefly, and dilute the concentrated stock as per manufacturer's instructions (~1:100 dilution).
- Aliquot **10 µl** diluted Ethidium Homodimer-1 in a tube.
- Gently mix the sample. Immediately add **10 µl** sample to **10 µl** diluted Ethidium Homodimer-1. Gently pipette mix 10x.

Fixed PBMCs Stained with Ethidium Homodimer-1



- Transfer **10 µl** sample to a Countess II Cell Counting Slide chamber.
- Insert the slide into the Countess II FL Cell Counter. Image the sample using the RFP setting for fluorescent illumination and filtering. Optimize focus and exposure settings.
- Confirm the absence of large clumps using the bright-field mode. Make sure the cell counter is circling RFP positive cells. Note the RFP-positive concentration. Multiply by dilution factor 2 to determine cell concentration.

Samples stained with Ethidium Homodimer-1 can also be counted using Cellaca counter. Refer to manufacturer's instructions for details.

Counting using PI Staining Solution

This protocol provides instructions for counting sample using PI staining solution and the Cellaca Counter to enable accurate quantification even in the presence of sub-cellular 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** 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 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 Countess II FL Automated Cell Counter. Refer to manufacturer's instructions for details.

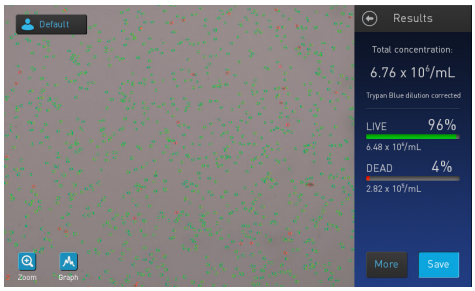
Counting using Trypan Blue (Only for Debris-free Samples)

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.

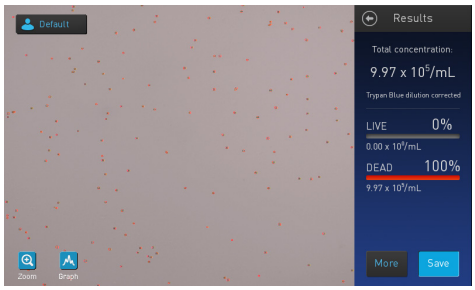
- Mix **1 part** 0.4% trypan blue and **1 part** sample.
- Transfer **10 µl** sample to a Countess II Cell Counting Slide chamber or a hemocytometer.
- Insert the slide into the Countess II Cell Counter and determine the cell concentration. Or if using hemocytometer, count fixed cells by placing hemocytometer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

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

Fresh PBMCs



Fixed PBMCs



References

1. Chromium Fixed RNA Profiling Reagent Kits User Guide for Multiplexed Samples (CG000527)
2. Chromium Fixed RNA Profiling Reagent Kits User Guide for Singleplexed Samples with Feature Barcode technology for Cell Surface Protein (CG000477)

Document Revision Summary

Document Number	CG000478
Title	Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling
Revision	Rev C
Revision Date	February 2023
General Changes	Updated for general minor consistency of language and terms throughout
Specific Changes	Added Quenching Buffer for resuspension of samples during post-storage processing. Removed −20°C from long-term storage. Included table for the preparation of 0.5X PBS + 0.02% BSA.

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