Tissue Fixation & Dissociation for Chromium Fixed RNA Profiling

Introduction

This protocol outlines how to perform tissue fixation followed by dissociation of the fixed tissue for use with Chromium Fixed RNA Profiling workflow. Storage recommendations for the fixed tissue and post-storage processing are listed in the Appendix.

Additional Guidance

This protocol was demonstrated using 25 mg of various tissue types, including human flash frozen liver, spleen, testes, ileum, duodenum, pancreas, kidney, lung, colon, tonsil, lymph, uterus, and also fresh (not frozen) human uterus.

See Appendix for cell yields derived from 25 mg of indicated tissue types. Optimize the protocol based on tissue type, tissue density, and dissociation efficiency.

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 Tissue Mincing & Fixation		
10x Genomics	Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit	1000414
Millipore Sigma	Protector RNase Inhibitor	3335399001
	Albumin, Bovine Serum, 10% Aqueous Solution, Nuclease-Free	126615
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
	Pyrex Petri Dish*	08-748D
	General-Purpose Forceps*	10-270
	Standard Dissecting Scissors*	08-951-20
	Wide-Bore Pipette Tips RT LTS 1000 uL*	FLW 768A/8 (30389218)
	**May be procured from alternative vendors*	
Corning	Phosphate-Buffered Saline, 1X *without Calcium and Magnesium*	21-040-CV
VWR	Tris Buffer, 1M sterile solution, pH 8.0	E199-100ML
Additional Materials		
Blade; Lab Weighing Scale		

This list may not include some standard laboratory equipments.

For Tissue Dissociation		
Millipore Sigma	Liberase TL	05401020001
Miltenyi Biotec	gentleMACS Octo Dissociator with Heaters	130-096-427
	gentleMACS C Tubes	130-093-237
For Sample Filtration		
Sysmex	Sterile Single-Pack CellTrics Filters (70 µm; 30 µm)	04-004-2326
Miltenyi Biotec	MACS SmartStrainers (70 µm)	130-098-462
	MACS SmartStrainers (30 µm)	130-098-458
Choose either Sysmex or Miltenyi Biotec filter.		
For Cell Counting		
Nexcelom Biosciences	ViaStain AOPI Staining Solution	CS2-0106-5mL
	Cellaca MX High-throughput† Automated Cell Counter	MX-112-0127
Thermo Fisher Scientific	Countess II FL Automated Cell Counter†	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
	Protector RNase Inhibitor	3335402001
VWR	Vacuum Filter/Storage System (0.2 µm)	29442-936
Additional Materials		
Eppendorf	DNA LoBind Tubes 2.0 ml	022431048
	ThermoMixer C	5382000023

This list may not include some standard laboratory equipments.

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

Preparation - Buffers

All buffer preparations should be fresh.

Buffers for Fixation - Prepare fresh (1 ml)			
Fixation Buffer Maintain at room temperature	**Stock**	**Final**	**Per 25 mg tissue (µl)**
Nuclease-free Water	-	-	791.9
Conc. Fix & Perm Buffer* (10x Genomics PN 2000517)	10X	1X	100.0
Formaldehyde	37%	4%	108.1
Tissue Resuspension Buffer Maintain at 4°C	**Stock**	**Final**	**Per sample (µl)**
PBS	1X	0.496X	496.0
Tris buffer (pH 8.0; mM)	1000	50	50.0
BSA (RNase free)	10%	0.02%	2.0
RNase Inhibitor (U/µl)	40	0.24	6.0
Nuclease-free Water	-		446.0
Quenching Buffer Maintain at 4°C	**Stock**	**Final**	**Per 25 mg tissue (µl)**
Nuclease-free Water	-	-	875.0
Conc. Quench Buffer* (10x Genomics PN 2000516)	8X	1X	125.0

Dissociation Solution Prepare 2 ml

- Reconstitute 5mg Liberase by adding 1 ml sterile water. Agitate at 2-8 °C until dissolved. Store stock solution in single-use aliquots at -15 to -25°C
- Prepare RPMI + 0.2 mg/ml Liberase *(Add 80 µl Liberase stock solution into 1,920 µl of RPMI, mix, maintain at 4°C)*
- Warm Dissociation Solution for 10 min at 37°C before use.

Additional Buffers

PBS + 0.04% BSA *(maintain at 4°C)*

Buffers for Storage of Fixed Samples - Prepare fresh

50% Glycerol Solution
(needed for long-term storage of fixed samples)

- Mix an equal volume of 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

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

Tips & Best Practices

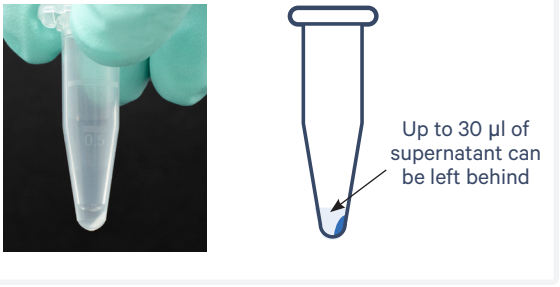
The following recommendations are critical for optimal performance of the Chromium Fixed RNA Profiling assay.

Tissue Quality & Processing

- Perform pilot experiments to determine if the tissue type is suitable for fixation and dissociation for preparing single cell suspension(s).
- Ensure that variation in cell yields from different tissue types is considered when performing the protocol.
- The tissue should be minced only on a glass surface. DO NOT use plastic petri dish for mincing tissue.

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.



Fixed Cell Counting

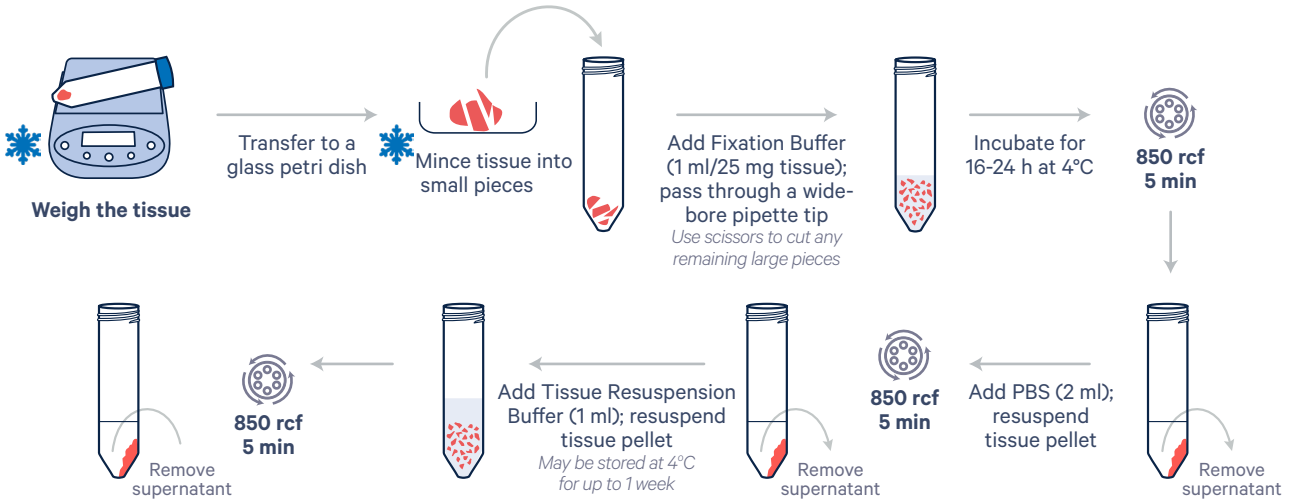
- Samples should have minimal debris for best results; debris can have associated RNA that can contribute to non-cell background.
- Accurate sample counting is critical for optimal assay performance.
- It is strongly recommended that the sample be stained with a fluorescent nucleic acid dye such as Ethidium Homodimer-1 or AO/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

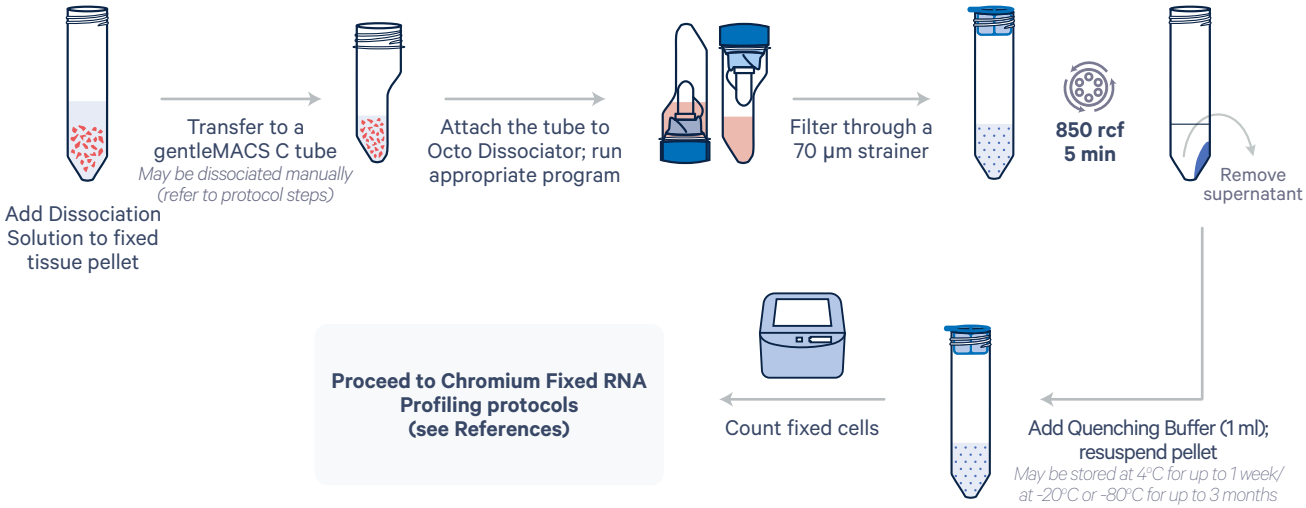
- The tissue pieces post-fixation can be stored at 4°C for up to 1 week.
- Cell suspension derived from fixed dissociated tissues can be stored at 4°C for up to 1 week or at -20°C or -80°C for up to 3 months after resuspending in appropriate reagents.
- Sample storage guidelines are provided in the Appendix.

Protocol Overview : Tissue Fixation & Dissociation

1. Tissue Fixation



2. Fixed Tissue Dissociation



Tissue Fixation & Dissociation Protocol

This protocol was demonstrated using 25 mg fresh or flash frozen tissue. Chromium Next GEM RNA Profiling Sample Fixation Kit, 16 rxns (PN-1000414) was used for tissue fixation.

1. Fix Tissue

If using frozen tissue, maintain the tissue on dry ice before and after weighing. Use a pre-chilled petri dish placed on ice while mincing the tissue.

a. Weigh tissue to determine Fixation Buffer volume. 1 ml Fixation Buffer/25 mg tissue will be used at step 1c.

b. Place the tissue on a pre-chilled glass petri dish maintained on ice and using a blade, mince tissue finely (enables passing through a 1 ml wide-bore pipette tip).

c. Transfer the tissue pieces to a centrifuge tube and add **1 ml** Fixation Buffer/25 mg tissue. Using a wide-bore (1.5 mm) 1-ml pipette, pass the tissue up and down. If larger tissue pieces fail to pass through the pipette, use dissection scissors to cut the tissue pieces further until they pass through the pipette. Maintain on ice.

d. Incubate for **16-24 h** at **4°C**.

DO NOT agitate or mix the sample during incubation.

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 tissue pellet.

g. Add **2 ml** chilled PBS and resuspend the tissue pellet.

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

i. Remove the supernatant without disturbing the tissue pellet.

j. Add **1 ml** Tissue Resuspension Buffer, resuspend the tissue pellet, and maintain on ice.

Fixed tissue pieces can be stored at 4°C for up to 1 week. See Appendix for guidance on short-term storage and post-storage processing of fixed tissue pieces.

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

l. Remove the supernatant without disturbing the fixed tissue pellet.

m. Proceed to tissue dissociation.

2. Dissociate Fixed Tissue

a. Warm Dissociation Solution for **10 min** at **37°C** before use.

b. Add **2 ml** pre-warmed Dissociation Solution to the sample.

Use 2 ml Dissociation Solution for up to 100 mg tissue. Use only 2 ml Dissociation Solution per C tube.

c. Dissociate tissue. Using an Octo Dissociator is highly recommended. Alternatively, tissue may be dissociated manually.

- **Octo Dissociator:**
  Transfer to Miltenyi C tubes. The minimum Dissociation Solution required for using the C tubes is 2 ml. Place the C tube in the Octo Dissociator and run following program:
- Incubate for **20 min** at **37°C**, **50 rpm**.
- Spin for **30 sec** at **37°C**, **2,000 rpm** - clockwise
- Spin for **30 sec** at **37°C**, **2,000 rpm** - counterclockwise
- Detach the C tubes and proceed to step 2d. **OPTIONAL:** Centrifuge the C tubes at **300 rcf** briefly to collect all the cells at the bottom of the tube. Resuspend the pellet in the supernatant and proceed to step 2d.

OR

- **Manual Dissociation:**
  Incubate for **20 min** at **37°C** shaking the tube intermittently. Using a silanized glass pipette, triturate the tissue pieces 15-20X (until solution begins to turn cloudy) to obtain a single cell suspension. Proceed to step 2d.

If tissue is tough to triturate and/or solution is not cloudy after trituration, see Troubleshooting guidance

d. Pass the dissociated tissue through a 70 µm filter to remove debris and undissociated tissue pieces.

e. **OPTIONAL**: Perform an additional wash of the 70 µm filter by adding **2 ml** PBS to the filter. Collect the filtrate in the same tube as step 2d.

TIPS *Use of swinging-bucket rotor is recommended for higher cell recovery.*

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

g. Remove the supernatant without disturbing the pellet.

h. Resuspend pellet in **1 ml** chilled Quenching buffer.

i. **OPTIONAL:** Pass sample through a 30 µm filter.

j. Determine the cell concentration using a Countess II FL Automated Cell Counter or hemocytometer. See Appendix for Fixed Cell Counting.

TIPS *For accurate cell counting, it is strongly recommended that the cell/nuclei suspension be stained with a fluorescent nucleic acid dye such as Ethidium Homodimer-1 or AO/PI Staining Solution and counted using an automated fluorescent cell counter.*

For lower than expected cell yields, see Troubleshooting guidance.

k. 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 -20°C or -80°C for up to 3 months, depending upon the buffers used. See Appendix for guidance on short and long-term storage and post-storage processing of fixed cells.

Appendix

Fixed Sample Storage Guidance

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

Short-term Storage at 4°C (for fixed tissue pieces & cells)

a. Thaw Enhancer 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** of pre-warmed Enhancer to fixed tissue pieces in Tissue Resuspension Buffer or fixed cells in Quenching Buffer. For example, add 100 µl Enhancer to 1,000 µl fixed cells in Quenching Buffer. Pipette mix.

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

Long-term Storage at −20°C or −80°C (for cells only)

a. Thaw Enhancer 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** of 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 of fixed sample in Quenching Buffer and Enhancer. Pipette mix.

d. Store at **−20°C** or **−80°C** for up to **3 months**.

TIPS *Storing fixed cells at −80°C is recommended for best results.*

Fixed Tissue Pieces – 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 −20°C or −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 tissue pellet.

c. Proceed to Dissociate Fixed Tissue protocol (step 2a).

Fixed Cells – 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 −20°C or −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) 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 Appendix for Fixed Cell Counting.

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

Fixed Sample Shipping Guidance

a. Fixed single cells resuspended in Quenching Buffer or fixed tissue pieces resuspended in Tissue Resuspension Buffer and supplemented with Enhancer can be shipped with a cold pack. See Short-term Storage for details.

b. Fixed single cells 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

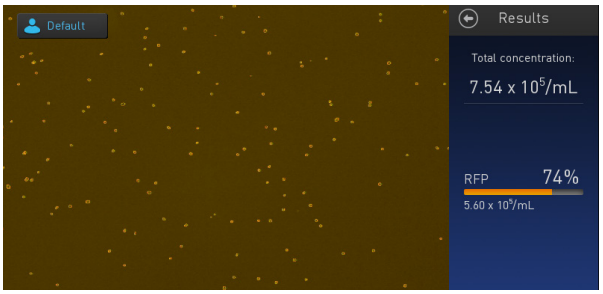
- Accurate sample counting is critical for optimal assay performance.
- It is strongly recommended that the fixed sample be stained with a fluorescent nucleic acid dye such as Ethidium Homodimer-1 or AO/PI staining solution and counted using an automated fluorescent cell counter (Countess II Automated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

Representative Cells Stained with Ethidium Homodimer-1



- 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 AO/PI Staining Solution

This protocol provides instructions for counting sample using AO/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** 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.

Cell Yields from Fixed Dissociated Tissues

Listed below are the cell yields from 25 mg of various flash-frozen tissue types processed as described in this protocol:

Tissue Type	Cell Yields/25 mg tissue
Jejunum	1.37×10^6
Colon*	0.04×10^6
Lung	0.25×10^6
Kidney	2×10^6
Liver	1.8×10^6
Spleen	9.6×10^6
Ileum	0.14×10^6
Testes	2.05×10^6
Duodenum	0.32×10^6
Breast Cancer	0.96×10^6
Melanoma	3.18×10^6
Ovarian Cancer	0.93×10^6
Pancreatic Cancer	1.78×10^6 cells

**Lower than expected yield from 25 mg colon tissue; using >25 mg colon tissue recommended for this assay.*

Troubleshooting

Problem	Solution
Lower than expected cell yield after dissociation	• Increase the tissue dissociation time • If using an Octo Dissociator, after transferring the dissociated cells from the C tube, perform an additional PBS rinse of the C tube and pass the rinse through the 70 µm strainer in step 2e to collect additional cells • If manually dissociating tough tissue, use the back of a 1-ml syringe plunger to push any undissociated tissue pieces through the 70 µm strainer

References

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

Document Revision Summary

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