Plate-based Sample Preparation for GEM-X Flex Gene Expression Multiplex Workflow

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. GEM-X Flex Gene Expression Multiplex workflow provides an efficient and cost-effective way to further increase the experiment size and cell number by enabling up to 16 samples to be run within a single GEM reaction.

The purpose of document is to provide sample preparation protocols in a 96-well V-bottom deep-well plate to enable high throughput handling of up to 96 samples for use with the GEM-X Flex Gene Expression multiplex workflow.

This document outlines the following protocols:

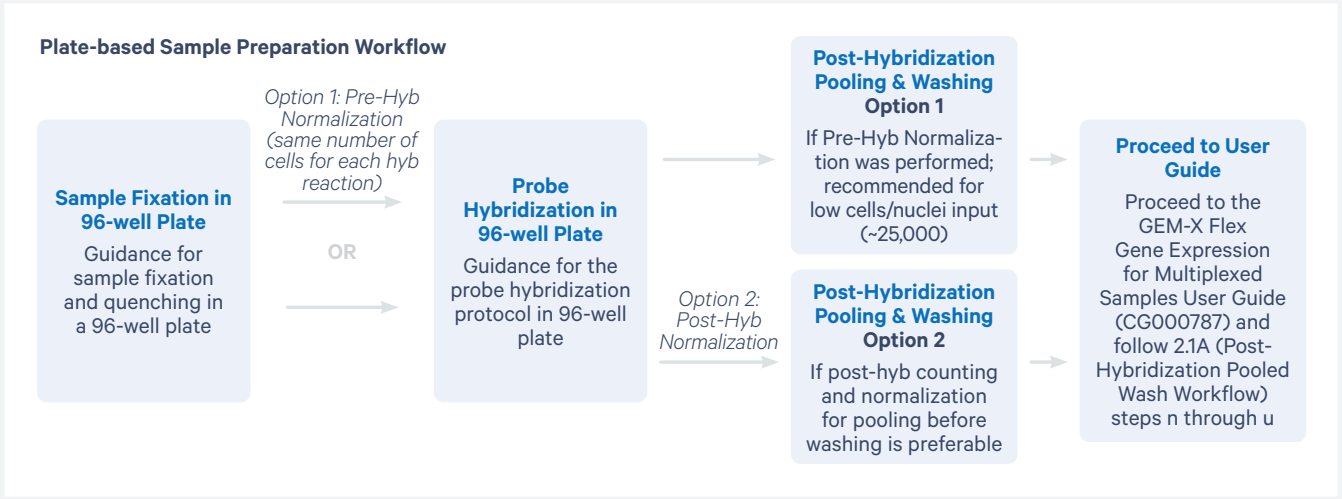
- Fixation of samples (fresh single cell and nuclei suspensions)
- Probe hybridization of fixed and quenched samples
- Post-hybridization pooling & washing of hybridized samples

Additional Guidance

This protocol is compatible with fresh cell/nuclei suspension as well as samples that are already fixed using GEM-X Flex sample preparation Demonstrated Protocols provided on the 10X Genomics support website. See Workflow Overview to choose the appropriate starting point based on the sample.

This document should be used with the GEM-X Flex Reagent Kits for Multiplexed Samples (CG000787) User Guide. Preread the User Guide for additional tips and best practices on multiplexing. The protocols provided here are for the 16-plex pooled wash workflow and can be adapted for any lower plex workflow. After the completion of post-hybridization washing steps described in this document, proceed to the GEM-X Flex Reagent Kits for Multiplexed Samples (CG000787) User Guide.

Workflow Overview



Choose the appropriate starting point based on the sample type:

- If using fresh cell or nuclei suspensions, start with Sample Fixation in 96-well Plate
- If using fixed samples (fixed cells, fixed nuclei, PBMCs and leukocytes derived from fixed blood, cells derived from fixed and dissociated tissue, and nuclei derived from FFPE tissue sections), start with Probe Hybridization in 96-well Plate.

Contents

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.

GEM-X Flex Reagent Kits

GEM-X Flex Sample Preparation v2 Kit PN-1000781

GEM-X Flex Sample Preparation v2 Kit
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 96 samples including storage of the fixed samples.

GEM-X Flex Human Transcriptome Probe, 16 samples, Kit PN-1000785

GEM-X Flex Human Transcriptome Probe Kit
16 samples, PN-1000785
Shipped on dry ice
Store at -20°C

	#	PN
Human WTA Probes BC001	1	2001259
Human WTA Probes BC002	1	2001260
Human WTA Probes BC003	1	2001261
Human WTA Probes BC004	1	2001262

GEM-X Flex Human Transcriptome Probe, 64 samples, Kit PN-1000787

GEM-X Flex Human Transcriptome Probe Kit
64 samples, Module 1 PN-1000787
Shipped on dry ice
Store at -20°C

	#	PN
Human WTA Probes BC001	1	2001259
Human WTA Probes BC002	1	2001260
Human WTA Probes BC003	1	2001261
Human WTA Probes BC004	1	2001262
Human WTA Probes BC005	1	2001263
Human WTA Probes BC006	1	2001264
Human WTA Probes BC007	1	2001265
Human WTA Probes BC008	1	2001266

GEM-X Flex Human Transcriptome Probe Kit
64 samples, Module 2 PN-1000787
Shipped on dry ice
Store at -20°C

	#	PN
Human WTA Probes BC009	1	2001267
Human WTA Probes BC010	1	2001268
Human WTA Probes BC011	1	2001269
Human WTA Probes BC012	1	2001270
Human WTA Probes BC013	1	2001271
Human WTA Probes BC014	1	2001272
Human WTA Probes BC015	1	2001273
Human WTA Probes BC016	1	2001274

GEM-X Flex Mouse Transcriptome Probe, 16 samples, Kit PN-1000786

GEM-X Flex Mouse Transcriptome Probe Kit
16 samples, PN-1000786
Shipped on dry ice
Store at -20°C

	#	PN
Mouse WTA Probes BC001	1	2001275
Mouse WTA Probes BC002	1	2001276
Mouse WTA Probes BC003	1	2001277
Mouse WTA Probes BC004	1	2001278

GEM-X Flex Mouse Transcriptome Probe, 64 samples, Kit PN-1000788

GEM-X Flex Mouse Transcriptome Probe Kit
64 samples, Module 1 PN-1000788
Shipped on dry ice
Store at -20°C

	#	PN
Mouse WTA Probes BC001	1	2001275
Mouse WTA Probes BC002	1	2001276
Mouse WTA Probes BC003	1	2001277
Mouse WTA Probes BC004	1	2001278
Mouse WTA Probes BC005	1	2001279
Mouse WTA Probes BC006	1	2001280
Mouse WTA Probes BC007	1	2001281
Mouse WTA Probes BC008	1	2001282

GEM-X Flex Mouse Transcriptome Probe Kit
64 samples, Module 2 PN-1000788
Shipped on dry ice
Store at -20°C

	#	PN
Mouse WTA Probes BC009	1	2001283
Mouse WTA Probes BC010	1	2001284
Mouse WTA Probes BC011	1	2001285
Mouse WTA Probes BC012	1	2001286
Mouse WTA Probes BC013	1	2001287
Mouse WTA Probes BC014	1	2001288
Mouse WTA Probes BC015	1	2001289
Mouse WTA Probes BC016	1	2001290

GEM-X Flex Hybridization & Wash Kit, 24 rxns, PN-1000789

GEM-X Flex Hybridization & Wash Kit
24 rxns, PN-1000789
Shipped on dry ice
Store at -20°C

	#	PN
Hyb Buffer B	1	2001312
Conc. Post-Hyb Buffer B	1	2001308
Enhancer	1	2000482

Additional Kits, Reagents & Equipment

Vendor	Item	PN
For Sample Fixation		
Thermo Fisher Scientific	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 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.		
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
96-well Deep Well Plates		
Axygen	96-well Clear V-Bottom 500 µL Polypropylene Deep Well Plate	P-96-450V-C-S
Corning	96-well Expanded Volume Polypropylene Not Treated Microplate, Standard Height, V-Bottom, Sterile	3344

- *Choose either Axygen or Corning plate based on availability & preference*
- *All the deep-well plates are not individually packaged and do not have lids. To prevent potential RNase/sample cross contamination in such case, promptly clean up the working bench. The microplate lids can also be purchased separately from Azenta Life Sciences (Microplate 96 Lid (4ti-0282).*
- *Alternative standard well plates may be used but the total reaction volumes may need to be modified. See Tips & Best Practices for more information.*
- *For overnight hybridization in a thermal cycler, samples can also be transferred to 96-well PCR plates, for example Eppendorf twin.tec PCR Plates 96 EP0030129504*

Vendor	Item	PN
Sealing Films		
Bio-Rad	Microseal 'C' PCR Plate Sealing Film, adhesive	MSC1001
	Microseal 'B' PCR Plate Sealing Film, adhesive	MSB1001
Applied Biosystems	MicroAmp Optical Adhesive Film	4311971
Choose Bio-Rad or Applied Biosystems films based on availability & preference..		
Temperature-controlled Instruments for Incubation		
Eppendorf	Thermo Mixer w/ heated lid	5308000003
	ThermoMixer C Accessory, Smartblock Thermoblock	5363000039
Analytik Jena	Biometra TAdvanced 96 SG/S	846-x-070-241/ 846-x-070-251 *(x = 2 for 230 V; 4 for 115 V; 5 for 100 V, 50-60 Hz)*
Eppendorf	Mastercycler X50s/X50a	6311000010/ 6313000018
Bio-Rad	PTC Tempo Deepwell Thermal Cycler	12015392
	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (discontinued)	1851197
-	Oven *Use any oven with consistent heating*	-
• *Choose either thermomixer, thermal cycler, or oven based on availability & preference.*		
• *If using thermal cyclers, choose Analytik Jena, Bio-Rad, or Eppendorf based on availability & preference.*		
• *For select instruments, ramp rates should be adjusted for all steps as described below: Analytik Jena Biometra TAdvanced 96 SG/S: 2°C/sec for both heating and cooling, Eppendorf Mastercycler X50s/X50a: 3°C/sec heating and 2°C/sec cooling*		
For Sealing		
Bio-Rad	Film Sealing Roller for PCR Plates	MSR0001
Applied Biosystems	MicroAmp Adhesive Film Applicator	4333183
Additional Materials		
Eppendorf	DNA LoBind Tubes 2.0 ml	022431048
	DNA LoBind Tubes 1.5 ml	022431021
	DNA LoBind Tubes 5.0 ml	0030108310
Corning	Corning Centrifuge Tubes with CentriStar Cap (15 ml), sterile	430790
	Self-Standing Polypropylene Centrifuge Tubes (50 ml), sterile	430921
VWR	Vortex Mixer	10153-838

This list may not include some standard laboratory equipment.

Tips & Best Practices

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

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 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.

96-well Plates

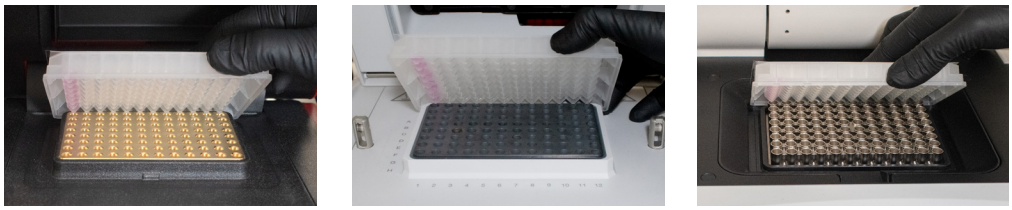
96-well Plate	Compatible with	Notes
Recommended deep-well (≥350 µl) plates		
Axygen/Corning V-bottom deep-well	Thermal Cycler, Thermomixer, and Oven	-
Alternative deep-well (≥350 µl) plates		
Flat- or round-bottom deep-well	Thermomixer and Oven*	• Minimal testing has been done with this plate type • No expected change in data quality • Cell loss might be greater than the V-bottom plates • Not recommended for low cell input (10,000-25,000)
Alternative standard-well (≤350 µl) plates		
V-bottom standard-well	Thermomixer, and Oven*	• Minimal testing has been done with this plate type • No expected change in data quality • During fixation, scale down the Fixation Buffer B and Additive C volumes keeping the ratio 1:2. To avoid overflow during centrifugation, ensure that the total volume added to each well is at least 50 µl less than the plate's maximum working volume. • For storage at −80°C, scale down the volume of reagents. See Appendix.

96-well Plate	Compatible with	Notes
Flat- or round-bottom standard-well	Thermomixer and Oven*	• Minimal testing has been done with this plate type • No expected change in data quality • Cell loss might be greater than the V-bottom plates • During fixation, scale down the Fixation Buffer B and Additive C volumes keeping the ratio 1:2. To avoid overflow during centrifugation, ensure that the total volume added to each well is at least 50 µl less than the plate's maximum working volume. • Not recommended for low cell input (10,000-25,000) • For storage at −80°C, scale down the volume of reagents. See Appendix.

**If a thermal cycler is the only available option, transfer samples to a standard 96-well PCR plate before incubation.*

- Heat the thermal cycler, oven, or thermomixer to 42°C before using. In case of thermal cycler ensure that the lid temp is also set to 42°C.
- When using a thermal cycler, ensure that the plate wells are in alignment with the thermal block.

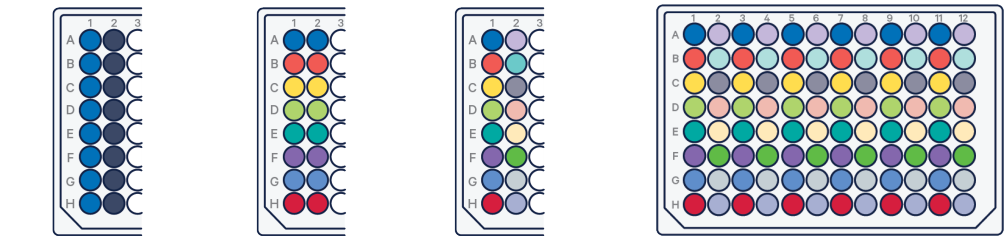
Placement of the Plate in the Thermal Cycler



Sample Setup for Multiplexing in 96-well Plate

- For a 16-plex reaction in a 96-well plate, set up samples in columns instead of rows to facilitate multichannel pipetting.
- Below are a few examples of how to set up a 16-plex multiplexing workflow in the plate with varying number of samples:

Examples of a 16-plex Multiplexing Workflow



One 16-plex *(with sub-pooling)* 2 samples, each split into 8 wells

One 16-plex *(with sub-pooling)* 8 samples, each split into 2 wells

One 16-plex *(no sub-pooling)* 16 samples into 16 wells

Six 16-plexes *(no sub-pooling)* 96 samples, 6 pools of 16-plex

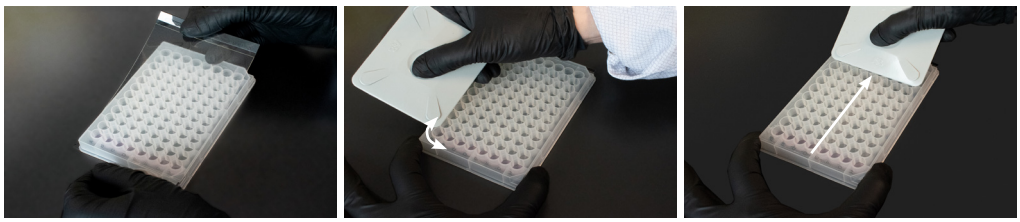
Different colors represent different samples.

Sub-pooling refers to pooling one sample over multiple Probe Barcodes to enable capture of more cells from that sample.

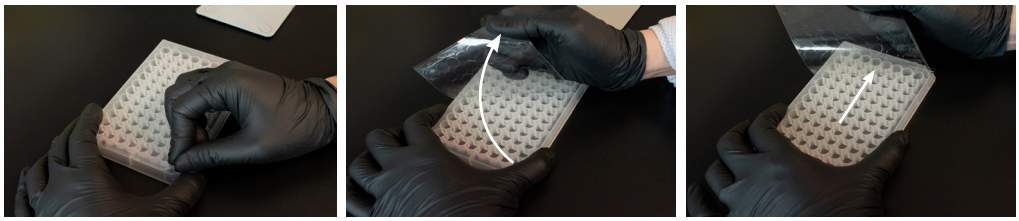
Plate Sealing & Removal

- Use the recommended seals for sealing the plate.
- To apply the seal, place the plate flat on a clean work surface. Peel the backing from the adhesive seal. Align the seal with the plate and apply while firmly holding the plate with one hand. Press on the seal around the edge of each well to ensure uniform adhesion. Use a roller or an applicator to firmly press the seal along rows and columns so each well is sealed.
- To remove the seal, place the plate flat on a clean work surface. Hold the plate down firmly with one hand. Carefully pull the seal using the side tabs while continually holding the plate. Move slowly to ensure that no liquid splashes out of the well.

Sealing the Plate



Removing the Seal



Practice Sample Handling in 96-well Plates

- If needed, practice sample handling in 96-well plates by using nonexperimental samples.
- Follow Fixation protocol steps using 4% formaldehyde for fixation and 1X PBS for wash steps instead of 10x Genomics reagents.

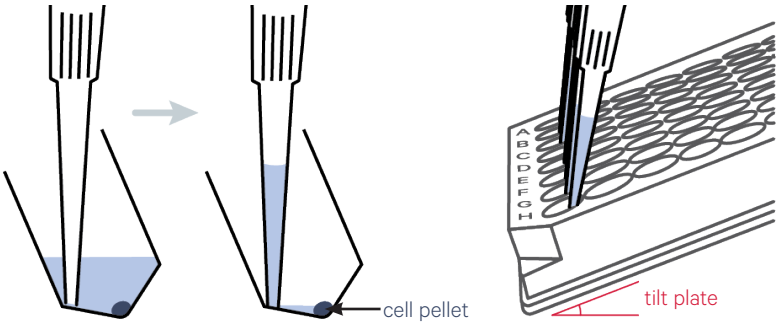
Pipettes

- Use well-calibrated multichannel pipettes for the workflow.

Centrifugation & Supernatant Removal

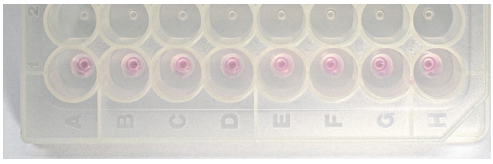
- Use a swinging-bucket rotor for higher cell/nuclei recovery.
- Centrifugation speed and time may need optimization depending upon sample type. Optimize sample-specific centrifugation conditions before moving to plates. The conditions optimized in tubes should apply to plates as well.
- Tilt the plate when removing the supernatant. Rest pipette tips on the bottom seam of the well to aspirate supernatant (see below) without disturbing the pellet.

Pipette Technique for Supernatant Removal from Plates

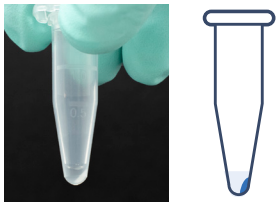


- When working with samples with low cell numbers (i.e. <300,000 cells), complete removal of the supernatant is not required.
- During fixation and probe hybridization, up to **15 µl** supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.
- During Post-Hybridization Pooling and Washing, up to **30 µl** supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.
- If uncertain about the volume remaining in the wells/tubes, add 15 or 30 µl water/PBS to an empty well/tube and use it for a visual estimation.
- After each buffer addition step, gently mix cells/nuclei 5x, or until the pellet is completely resuspended, without introducing bubbles.

Supernatant Removal



During fixation & hybridization, up to 15 µl supernatant may be left behind



During post-hybridization washes, up to 30 µl supernatant may be left behind

Fixation Considerations

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 number of cells/nuclei do not overly exceed the upper limit recommendations going into fixation.

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 recommended to perform a 16-24 h fixation at 4°C and store the fixed samples at -80°C for best results.
- If working with splenocytes, fix for 16-24 h at 4°C.
  If working with leukocytes or human bone marrow mononuclear cells (BMMCs), fix for 16-24 h at room temperature (20°C).

Fixed Cell Counting

- 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 for Fixation & Probe Hybridization

Recommendations

Recommended Cell/Nuclei Input
for Fixation
25,000–2 x 10^6
It may be possible to use ≤25,000 cells/nuclei with limited in-house testing showing successful results from as few as 10,000 cells/nuclei.
Nuclei have been identified as a challenging sample type and can have a higher chance of clumping and post-fixation sample loss.
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 in case of leukocytes isolated from fixed blood (CG000785), splenocytes, human BMMCs (CG000782), and cells from fixed & dissociated spleens and pancreas (CG000783), as higher cell loads may lead to a slight decrease in data quality.

Recommended Cell/Nuclei Input
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 • Use a V-bottom plate • Follow centrifugation & supernatant removal guidance provided in this document

- 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.

Cell Number Normalization

- During a multiplex Flex experiment, it is recommended to have an equal representation of each sample in the final pool. This can be achieved either through normalization of cell numbers prior to hybridization, or after hybridization during pooling samples.

Pre-Hybridization Normalization	Post-Hybridization Normalization
• Recommended for samples with low cells/nuclei input (~25,000) • Use the same number of cells/nuclei in each well for hybridization • Skip sample counting before pooling the samples and pool the entire volume of each sample • See the Pre-Hybridization Normalization section under Tips & Best Practices in the GEM-X Flex Gene Expression Reagent Kit for Multiplex samples User Guide (CG000787) for more information.	• Use different numbers of cells/nuclei during probe hybridization • Count after hybridization and use an equal number for pooling

- Based on the Pre- vs Post-Hybridization Normalization, choose one of the following options for Post-Hybridization Pooling & Washing:
  - Option 1: Post-hybridization Pooling & Washing protocol if Pre-Hyb Normalization was performed. Post-hybridization counting can be skipped.
  - Option 2: Post-hybridization Pooling & Washing protocol if Pre-Hyb Normalization was not performed and Post-hybridization counting is preferred.

Additional Tips & Best Practices

See the following topics in the GEM-X Flex Multiplexing User Guide (CG000787) for additional tips and best practices:

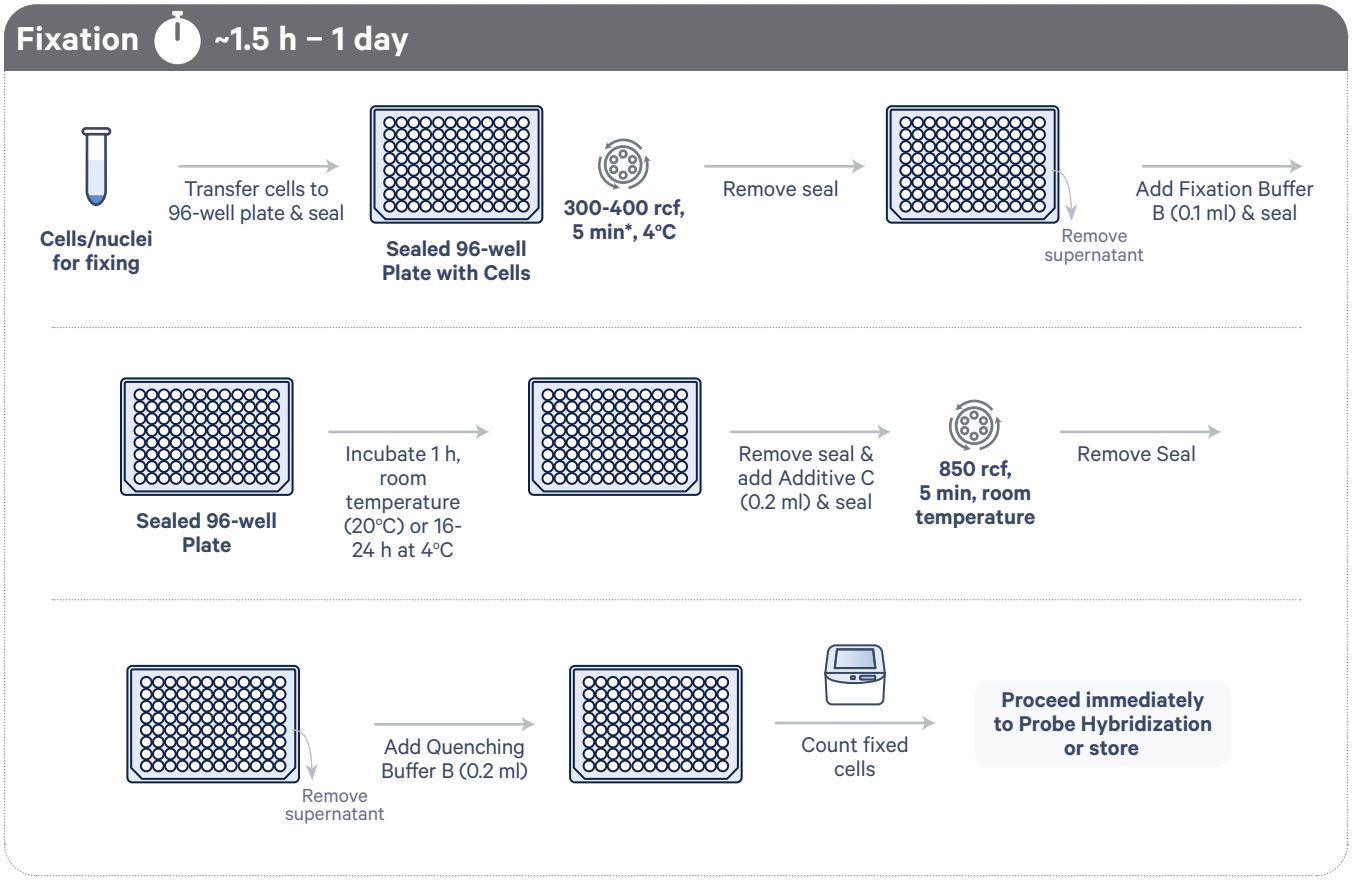
- Post-Hybridization Pooling & Washing Guidance: For more information on Pooled Wash workflow
- Multiplexing Experiment Design for guidance on sample sub-pooling and on pooling samples with different RNA content

1. Sample Fixation in 96-well Plate

1.0 Overview

GEM-X Flex Sample Preparation v2 Kit (PN-1000781) is used for sample fixation. This protocol is compatible with fresh cell and nuclei suspensions. If starting with samples other than fresh cells/nuclei suspensions (e.g. fresh/frozen tissues or fresh blood) or samples that are already fixed (e.g. FFPE samples), see the support website for sample-specific Demonstrated Protocols for sample preparation prior to Probe Hybridization in 96-well plate.

The steps described here provide guidance for sample fixation and quenching in a 96-well plate. If bulk fixation and quenching is preferred, follow Demonstrated Protocol CG000782 and after counting, proceed to Probe Hybridization in 96-well plate. If using cryopreserved cells or nuclei suspensions, thaw samples before proceeding to Sample Fixation. See Appendix for Thawing Protocol.



1.1 Preparation – Buffers

Buffers for Fixation – Prepare fresh

Fixation Buffer B *Maintain at room temperature*	**Stock**	**Final**	**1X* + 10% (µl)**	**8X* + 10% (µl)**	**16X* + 10% (µl)**
Nuclease-free Water	-	-	87.1	696.8	1,393.6
Conc. Fix & Perm Buffer B (10x Genomics PN-2001301)	10X	1X	11.0	88.0	176.0
Thaw at room temperature. Vortex, check for precipitation, & centrifuge briefly. Maintain at room temperature.					
Formaldehyde**	37%	4%	11.9	95.2	190.4
Total	**-**	**-**	**110.0**	**880.0**	**1760.0**
**X represents the number of sample wells*					
***Formaldehyde should always be used with adequate ventilation, preferably in a fume hood. Follow appropriate regulations.*					

Quenching Buffer B*** *Maintain at 4°C*	**Stock**	**Final**	**1X* + ~7% (µl)**	**8X* + ~7% (µl)**	**16X* + ~7% (µl)**
Nuclease-free Water	-	-	187.6	1,500.8	3,001.6
Conc. Quench Buffer B (10x Genomics PN-2001300)	8X	1X	26.8	214.4	428.8
Thaw at room temperature. Vortex and centrifuge briefly. Maintain at 4°C.					
Total	**-**	**-**	**214.4**	**1715.2**	**3,430.4**
**X represents the number of sample wells*					
****If planning to store the sample, 0.2 ml Quenching Buffer B will be required per sample well during Post-Storage Processing.*					

Additional Buffers
Additive C (10x Genomics PN-2001332) *Thaw at room temperature. Vortex and check for precipitation. Maintain at room temperature. If precipitate is observed, heat at 42°C for 10 min.*

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.

1.2 Fixation Protocol Steps

a. Transfer 25,000–2 x 10^6 cells into each well of a 96-well plate.

b. Seal the plate with a plate seal.

c. Centrifuge sample at **300–400 rcf** for **5 min** (PBMCs/cell lines) at **4°C**.

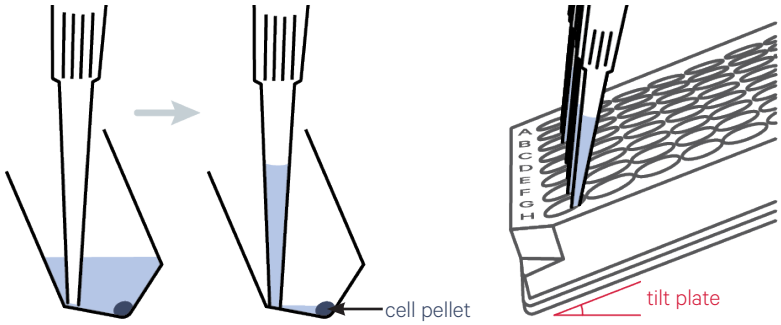
d. Carefully remove the seal while steadily holding the plate.

e. Remove the supernatant without disturbing the pellet.

TIPS *Tilt the plate when removing the supernatant. Rest pipette tips on the bottom seam of the well wall to aspirate supernatant as shown below without disturbing the pellet. Up to 15 µl supernatant can be left behind.*

Proceed immediately to the next step to prevent sample loss due to drying.

Pipette Technique



f. Add **0.1 ml room temperature** Fixation Buffer B to the sample pellet and pipette mix 5x.

g. Seal the plate with a plate seal.

h. 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. If working with leukocytes or human BMMCs, fix for 16-24 h at room temperature (20°C).

To minimize variability for room temperature fixations, incubation at controlled 20°C temperature (e.g. with a thermomixer with a plate adaptor and no shaking or oven or thermal cycler) is recommended. Fixation time and temperature should be consistent across all samples in an experiment.

i. Carefully remove the seal while steadily holding the plate.

j. Add **0.2 ml room temperature** Additive C to the sample in Fixation Buffer B and pipette mix 5x.
At this stage, if necessary, samples may be maintained at room temperature for up to an hour. This short stopping point can be used to facilitate the efficient processing of a large number of samples.

k. Seal the plate with a plate seal.

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

m. Carefully remove the seal while steadily holding the plate.

n. Remove the supernatant without disturbing the pellet.

TIPS *Tilt the plate when removing the supernatant. Rest pipette tips on the bottom seam of the well wall to aspirate supernatant without disturbing the pellet. Up to 15 µl supernatant may be left behind.*

Proceed immediately to the next step to prevent sample loss due to drying.

o. Add **0.2 ml** chilled Quenching Buffer B to the sample pellet and pipette mix 5x and keep on ice.

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

TIPS *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.*

q. Proceed **immediately** to Probe Hybridization or store the sample after resuspending in appropriate reagents.

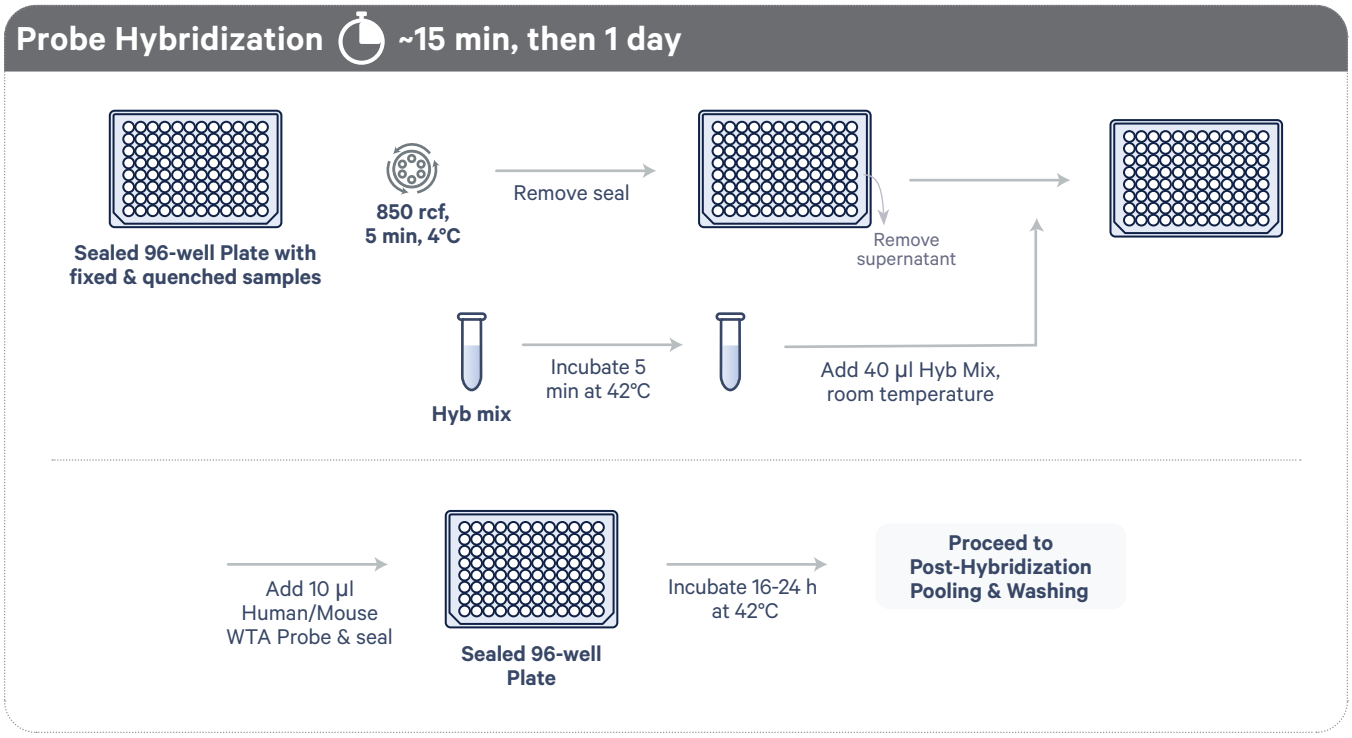
STOP *Samples can be stored at 4°C for up to 1 week or at -80°C for up to 12 months, depending upon the reagents used for storage. See Appendix for guidance on storage and post-storage processing.*

2. Probe Hybridization in 96-well Plate

2.0 Overview

Before starting this protocol:

- Ensure that the samples have been appropriately fixed and quenched.
  If starting with samples that were fixed in tubes, for example fixed cells and nuclei (CG000782), cells derived from fixed and dissociated tissue (CG000783), nuclei derived from FFPE tissue sections (CG000784), and PBMCs and leukocytes derived from fixed blood (CG000785), transfer samples suspended in Quenching Buffer B to an appropriate 96-well plate before starting.
- Determine the number of cells that will be used in each hybridization reaction. Recommended sample input for hybridization is 25,000–500,000 fixed cells/nuclei. If sufficient cells are available, it is recommended to default to 300,000 fixed cells/nuclei for setting up hybridization. DO NOT exceed 500,000 cells in one hybridization reaction.
  In case of leukocytes isolated from fixed blood, splenocytes, human BMMCs, and cells from fixed & dissociated spleen and pancreas tissues, it is recommended to use ≤100,000 cells as higher cell loads may lead to a slight decrease in data quality.



2.1 Preparation - Buffers

Buffers for Hybridization - Prepare fresh

Prepare Hyb Mix at the start of Probe Hybridization. Pipette mix 10x.

Hyb Mix *Maintain at room temperature*	**PN**	**1X* +20% (µl)**	**8X* + 20% (µl)**	**16X* +20% (µl)**
Hyb Buffer B *Thaw at 42°C. Add warm to the mix and if appears milky keep it back on 42°C.*	2001312	42.0	336.0	672.0
Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.*	2000482	6.0	48.0	96.0
Total	**-**	**48.0**	**384.0**	**768.0**

**X represents the number of sample wells*

2.2 Probe Hybridization Protocol Steps

a. Prepare Hyb Mix at **room temperature**. Pipette mix 10x. DO NOT keep at 4°C.

b. Incubate Hyb Mix at **42°C** for **5 min** before use.

c. Seal the plate containing fixed and quenched samples with a plate seal.

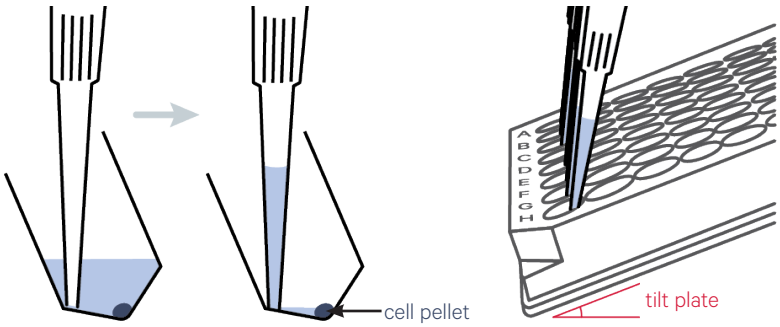
d. Centrifuge fixed cells/nuclei resuspended in Quenching Buffer B at **850 rcf** for **5 min** at **4°C**.
If performing pre-hyb normalization, ensure that the same number of cells are present in each of the 96-well plate. The extra sample can be transferred to a new 96-well plate or tube for storage.

e. Carefully remove the seal while steadily holding the plate.

f. Remove the supernatant.

TIPS *Tilt the plate when removing the supernatant. Rest pipette tips on the bottom seam of the well wall to aspirate supernatant without disturbing the pellet. Up to 15 µl supernatant may be left behind. Proceed immediately to the next step to prevent sample loss due to drying.*

Pipette Technique



g. Resuspend cell pellet with **40 µl** Hyb Mix. Keep samples at room temperature. DO NOT place samples on ice.

h. Add **10 µl** Human/Mouse WTA Probe to the 40 µl mixture of Hyb Mix and fixed sample and gently pipette mix 10x.

i. **Firmly** seal the plate with a **new** micro film seal with a film sealing adaptor or roller.

j. Incubate sample for **16-24 h** at **42°C**.
Incubation can be performed in a thermomixer with heated lid and no shaking, an oven, or a thermal cycler. If using a thermal cycler, ensure that the lid temperature is also set at 42°C.
Incubation for less than 16 h is not recommended. Incubation time should be consistent across all samples in an experiment.

k. After incubation, proceed to Post-Hybridization Pooling & Washing.

3. Post-Hybridization Pooling & Washing

3.0 Overview

This section provides guidance on Pooled Wash workflow for Post-Hybridization Pooling & Washing. Two options are provided for executing the protocol.

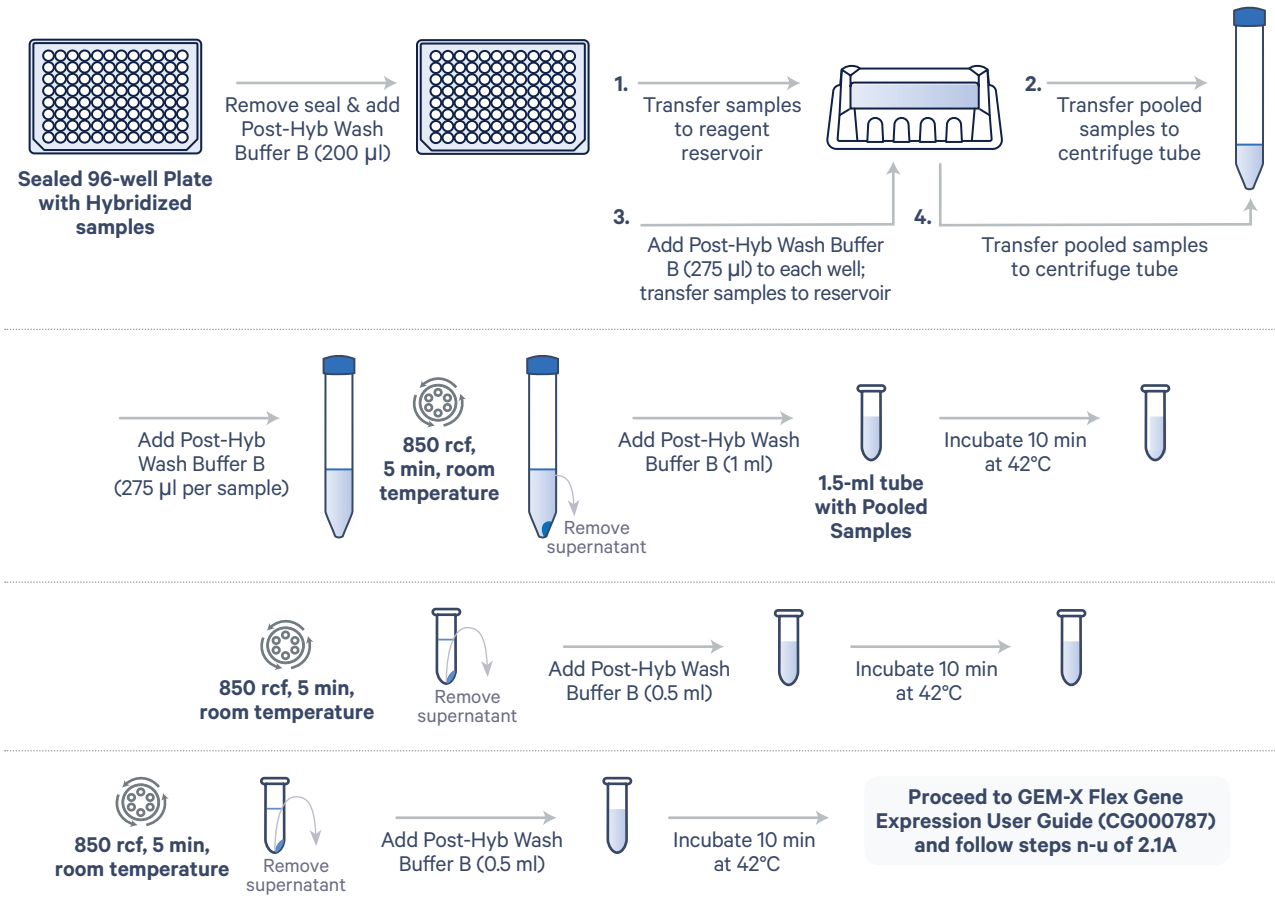
Choose Option 1 if Pre-hyb normalization was performed during probe hybridization. In this case, the entire volume of sample can be pooled without counting.

Choose Option 2 if Pre-hyb normalization was not performed during probe hybridization. In this case, the samples are counted before pooling.

Preread and have available GEM-X Flex Gene Expression Reagents Kit for Multiplexed Samples User Guide (CG000787) before starting these protocols.

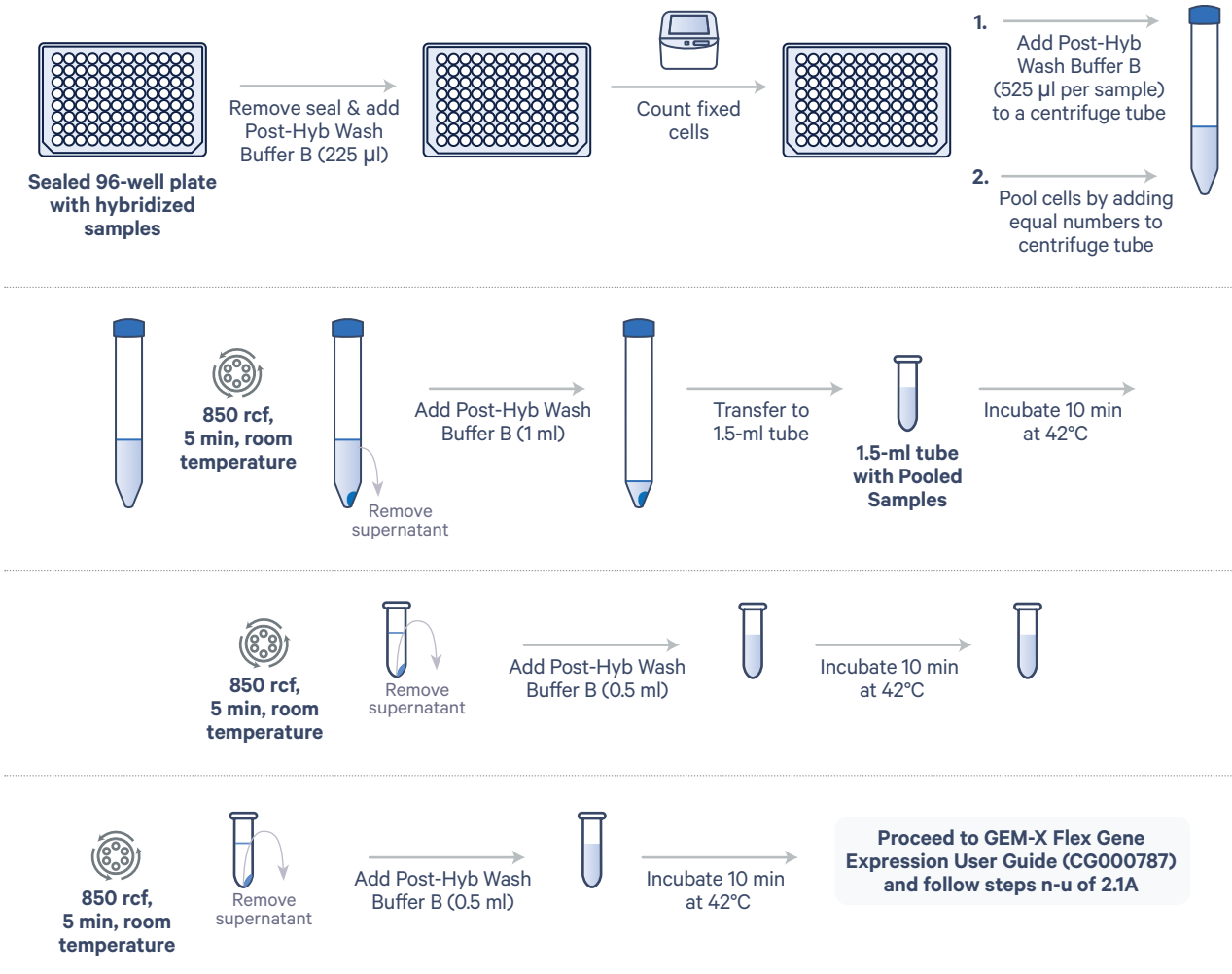
Option 1: Post-Hybridization Pooling & Washing

Post-hybridization pooling & washing protocol if pre-hyb normalization was performed



Option 2: Post-Hybridization Pooling & Washing

Post-hybridization pooling & washing protocol if pre-hyb normalization was not performed & post-hyb counting is preferred.



3.1 Preparation - Buffers

Buffers for Post-Hybridization Pooling & Washing - Prepare fresh

Prepare Post-Hyb Buffer B at the start of this step Pipette mix 10x.

Post-Hyb Buffer B *Maintain at room temperature*	**PN**	**Pooling 16 samples (ml)** *10% Overage included)*
Nuclease-free Water	-	13.86
Conc. Post-Hyb Buffer B.	2001308	0.77
Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.*	2000482	0.77
Total	**-**	**15.4**

3.2A Protocol Steps: Option 1

Post-hybridization pooling & washing protocol if pre-hyb normalization was performed.

a. Prepare Post-Hyb Wash Buffer B. Vortex briefly and keep at room temperature. DO NOT keep at 4°C.

b. Carefully remove the seal while steadily holding the plate.

c. Add **200 µl** Post-Hyb Wash Buffer B to each well.

d. Using a multichannel pipette, pool samples by transferring from the wells to a reagent reservoir.

e. Pipette mix and using a P1000 pipette, transfer pooled samples to a 5-ml (for pooling 4 samples) or 15-ml (for pooling 8 or 16 samples) centrifuge tube.

f. Add **275 µl** Post-Hyb Wash Buffer B to each well. Pipette carefully to avoid buffer overflow. Pipette mix and transfer samples into the same reservoir.

g. Pipette mix and transfer pooled samples to the same centrifuge tube.

h. Add **275 µl** Post-Hyb Wash Buffer B **per sample well** into the centrifuge tube. For example, for pooling samples from 16 wells, add 4.4 ml buffer (275 µl x 16). *This will lead to adding a total of 750 µl post-hyb wash buffer B to each 50 µl sample.*

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

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

k. Add **1 ml** Post-Hyb Wash Buffer B to the tube. Pipette mix and transfer to a 1.5-ml tube.

l. Incubate at **42°C** for **10 min** in a thermomixer or a heat block.

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

n. Remove the supernatant without disturbing the pellet. Up to 30 µl supernatant may be left behind if working with <300,000 cells.

o. Resuspend cell pellet in **0.5 ml** Post-Hyb Wash Buffer B. Pipette mix 5x.

p. Incubate at **42°C** for **10 min** in a thermomixer or a heat block.

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

r. Remove the supernatant without disturbing the pellet. Up to 30 µl supernatant may be left behind if working with <300,000 cells.

s. Resuspend cell pellet in **0.5 ml** Post-Hyb Wash Buffer B. Pipette mix 5x.

t. Incubate at **42°C** for **10 min** in a thermomixer or a heat block.

u. Proceed to 2.1A (Post-Hybridization Pooled Wash Workflow) of GEM-X Flex Gene Expression Reagents Kit for Multiplexes Samples User Guide (CG000787) and follow steps **n** (Prepare Post-Hyb Resuspension Buffer B) through **u**.

3.2B Protocol Steps: Option 2

Post-hybridization pooling & washing protocol if pre-hyb normalization was not performed and post-hyb counting is preferred.

a. Prepare Post-Hyb Wash Buffer B. Vortex briefly and keep at room temperature. DO NOT keep at 4°C.

b. Carefully remove the seal while steadily holding the plate.

c. Add **225 µl** Post-Hyb Wash Buffer B to each well and pipette mix 5x.

d. Determine cell concentration using an automated cell counter or a hemocytometer. Calculate the total cell number present in each well. *The sample can be diluted 1:2 to 1:4 with PBS before counting depending on the cell input into hybridization.*

e. Add **525 µl** Post-Hyb Wash Buffer B **per sample well** into a 5-ml (for 4 pooling samples) or 15-ml (for pooling 8 or 16 samples) centrifuge tube. For examples, for pooling samples from 16 wells, add 8.4 ml (525 µl * 16) buffer.

f. Pool an equal number of cells from different hybridization reactions and add to the tube containing Post-Hyb Wash Buffer B. *See the User Guide (CG000787) for Post-Hybridization Pooling Calculation.*

Optional: Nuclei samples or other sample types prone to clumping can be filtered with a 30 µm filter (Sysmex CellTrics or Miltenyi Biotec PreSeparation Filters) during pooling by adding the filter on top of the tube containing Post-Hyb Wash Buffer B.

g. Mix by inverting 5x.

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

i. Remove the supernatant without disturbing the pellet. Up to 30 µl supernatant may be left behind if working with <300,000 cells.

j. Add **1 ml** Post-Hyb Wash Buffer B to the tube. Pipette mix and transfer to a 1.5-ml tube.

k. Incubate at **42°C** for **10 min** in a thermomixer or a heat block.

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

m. Remove the supernatant without disturbing the pellet. Up to 30 µl supernatant may be left behind if working with <300,000 cells.

n. Resuspend cell pellet in **0.5 ml** Post-Hyb Wash Buffer B. Pipette mix 5x.

o. Incubate at **42°C** for **10 min** in a thermomixer or a heat block.

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

q. Remove the supernatant without disturbing the pellet. Up to 30 µl supernatant may be left behind if working with <300,000 cells.

r. Resuspend cell pellet in **0.5 ml** Post-Hyb Wash Buffer B. Pipette mix 5x.

s. Incubate at **42°C** for **10 min** in a thermomixer or a heat block.

t. Proceed to 2.1A (Post-Hybridization Pooled Wash Workflow) of GEM-X Flex Gene Expression Reagents Kit for Multiplexes Samples User Guide (CG000787) and follow steps **n** (Prepare Post-Hyb Resuspension Buffer B) through **u**.

Appendix

Fixed Sample Storage Guidance

Fixed samples can be stored in the plate for short or long-term. The volumes recommended below are for 96-well V-bottom deep-well (~350 µl) plates. If using standard-well plates (≤350 µl), the volumes can be scaled down while keeping the ratios same (see below). To avoid overflow during centrifugation, ensure that the total volume added to each well is at least 50 µl less than the plate's maximum working volume.

Short-term Storage in 96-well Plates 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 prewarmed Enhancer to fixed sample in Quenching Buffer B.
For example, add 20 µl Enhancer to 200 µl fixed sample in Quenching Buffer B. Pipette mix.
Example volumes if using standard-well plates with a 250 µl working volume: Add 15 µl Enhancer to 150 µl fixed sample in Quenching Buffer B.

c. **Firmly** seal the plate with a **new** micro film seal with a film sealing adaptor or roller. After proper sealing with a film, the plate can also be covered with a PCR plate lid.

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

Long-term Storage 96-well Plates 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 prewarmed Enhancer to fixed sample in Quenching Buffer B.
For example, add 20 µl Enhancer to 200 µl fixed sample in Quenching Buffer B. Pipette mix.
Example volumes if using standard-well plates with a 250 µl working volume: Add 15 µl Enhancer to 150 µl fixed sample in Quenching Buffer B.

c. Add 50% glycerol for a final concentration of 10%. For example: add 55 µl 50% glycerol to 220 µl fixed sample in Quenching Buffer B and Enhancer. Pipette mix.
Example volumes if using standard-well plates with a 250 µl working volume: Add 41.3 µl 50% glycerol.

d. **Firmly** seal the plate with a **new** micro film seal with a film sealing adaptor or roller. After proper sealing with a film, the plate can also be covered with a PCR plate lid.

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

TIPS *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**. Carefully remove the seal while steadily holding the plate

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 **0.2 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 Step 2 Probe Hybridization in 96-well Plate.

Fixed Sample Shipping Guidance

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

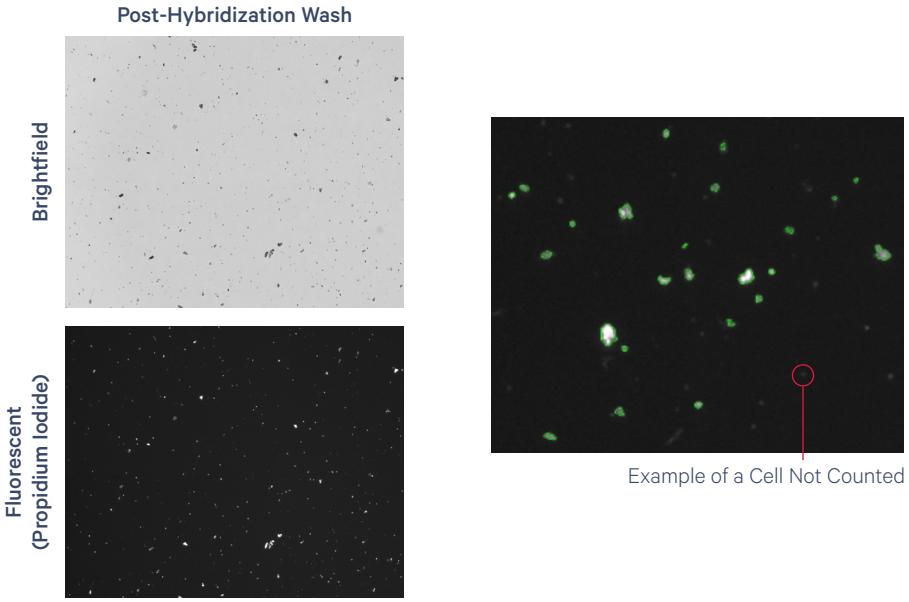
Fixed Cell/Nuclei Counting

- 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.

- 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

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



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

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. See 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. Ensure that this dilution factor is accounted for during counting. For example, because a 1:1 dilution was performed, the final cell concentration 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. See 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. See manufacturer's instructions for details.

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

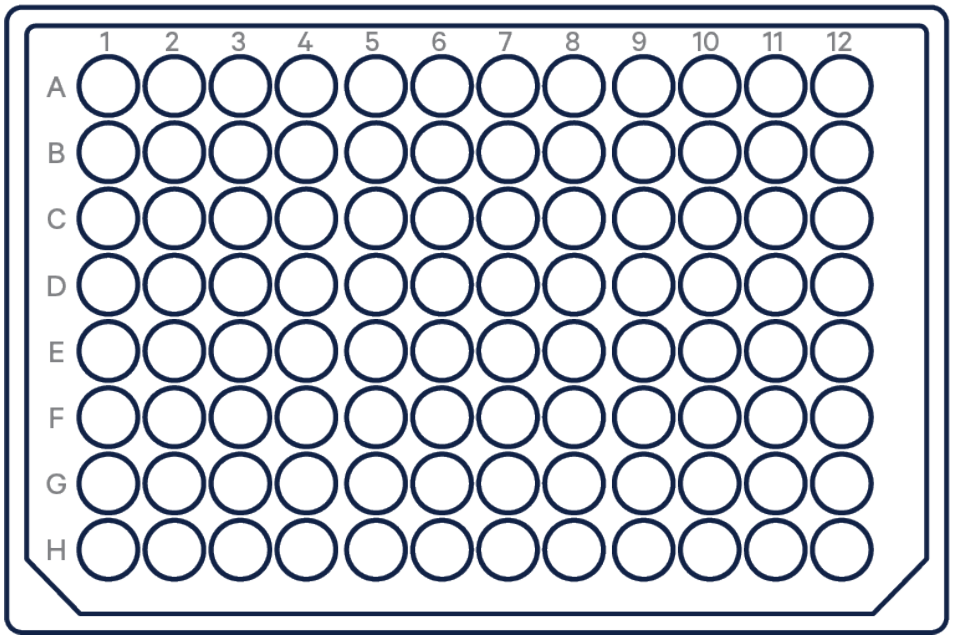
Debris-free samples (cells or nuclei suspensions) can also be counted using trypan blue. This protocol provides instructions for counting sample using trypan blue and a hemocytometer or Countess II Automated Cell Counter.

- 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 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.

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

Visual Layout of a 96-well V-bottom Deep-well Plate

Use this plate layout to mark the position of samples and probes.



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