User Guide | CG000789 | Rev B

GEM-X

Flex Gene Expression Reagent Kits

for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture

For use with:

GEM-X Flex Sample Preparation v2 Kit *PN-1000781*

GEM-X Flex GEM & Library Kit, *4 rxns PN-1000782*

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

GEM-X Flex Supplemental Wash Kit, *64 rxns PN-1000828*

GEM-X Flex Human Transcriptome Probe Kit
16 samples PN-1000785 | 64 samples PN-1000787

GEM-X Flex Mouse Transcriptome Probe Kit
16 samples PN-1000786 | 64 samples PN-1000788

GEM-X Flex Gel Bead Kit, *4 rxns PN-1000790*

Flex Feature Barcode Kit, *64 rxns PN-1000628*

GEM-X Flex Gene Expression Chip Kit, *4 chips PN-1000791*

Dual Index Kit TS Set A, *96 rxns PN-1000251* & Dual Index Kit TN Set A, *96 rxns PN-1000250*

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

Notices

Document Number

CG000789 | Rev B

Legal Notices

Instrument & Licensed Software Updates Warranties

Updates to existing Instruments and Licensed Software may be required to enable customers to use new or existing products. In the event of an Instrument failure resulting from an update, such failed Instrument will be replaced or repaired in accordance with the 10x Limited Warranty, Assurance Plan or service agreement, only if such Instrument is covered by any of the foregoing at the time of such failure. Instruments not covered under a current 10x Limited Warranty, Assurance Plan or service agreement will not be replaced or repaired.

Support

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6230 Stoneridge Mall Road
Pleasanton, CA

Document Revision Summary

Document Number

CG000789 | Rev B

Title

GEM-X Flex Gene Expression Reagent Kits for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture

Revision

Rev A to Rev B

Revision Date

March 27, 2025

Description of Changes

- Updated for general minor consistency of language, format, and terms throughout.
- Removed 48 rxns from GEM-X Flex Sample Preparation v2 Kit in GEM-X Flex Reagent Kits on page 8
- Updated Fixed RNA Feature Barcode Multiplexing Kit name to Flex Feature Barcode Kit in GEM-X Flex Reagent Kits on page 11
- Added leukocytes and bone marrow mononuclear cells to Probe Hybridization on page 51
- Added note on adding one more PCR cycle in case of intracellular only workflow to Sample Index PCR on page 97

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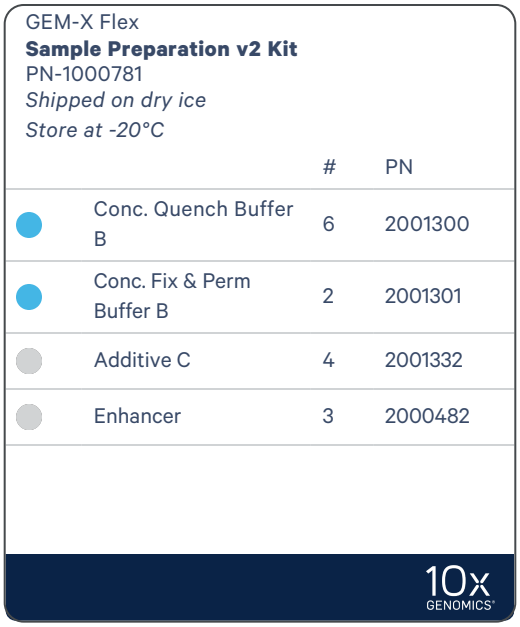
Introduction

GEM-X Flex Reagent Kits

Refer to SDS for handling and disposal information

GEM-X Flex Sample Preparation v2 Kit PN-1000781*



*This kit provides sufficient reagents to process:

- 48 samples when using the Demonstrated Protocol Fixation of Cells and Nuclei for GEM-X Flex Gene Expression (CG000782)
- 48 cell surface protein labeled samples that follow the 1- or 2-wash workflow of the Demonstrated Protocol Cell Surface & Intracellular Protein Labeling for GEM-X Flex Gene Expression (CG000781)
- 24 intracellular protein labeled or cell surface protein labeled samples that follow the no wash workflow of the Demonstrated Protocol Cell Surface & Intracellular Protein Labeling for GEM-X Flex Gene Expression (CG000781)

GEM-X Flex Reagent Bundle for Multiplexing - Human

Reagent Kit Bundle	Part Number (Kit)	Components	Component Part Number	Quantity
GEM-X Flex Gene Expression Human 4-plex, 16 samples	1000793	GEM-X Flex GEM & Library Kit, 4 rxns	1000782	1
		GEM-X Flex Gel Bead Kit, 4 rxns	1000790	1
		GEM-X Flex Hybridization & Wash Kit, 24 rxns	1000789	1
		GEM-X Flex Human Transcriptome Probe Kit, 16 samples	1000785	1
GEM-X Flex Gene Expression Human n-plex, 64 samples	1000829	GEM-X Flex GEM & Library Kit, 4 rxns	1000782	4
		GEM-X Flex Gel Bead Kit, 4 rxns	1000790	4
		GEM-X Flex Hybridization & Wash Kit, 24 rxns	1000789	3
		GEM-X Flex Human Transcriptome Probe Kit, 64 samples	1000787	1
		GEM-X Flex Supplemental Wash Kit, 64 rxns	1000828	1
GEM-X Flex Gene Expression Human 16-plex, 64 samples	1000794	GEM-X Flex GEM & Library Kit, 4 rxns	1000782	1
		GEM-X Flex Gel Bead Kit, 4 rxns	1000790	1
		GEM-X Flex Hybridization & Wash Kit, 24 rxns	1000789	3
		GEM-X Flex Human Transcriptome Probe Kit, 64 samples	1000787	1
GEM-X Flex Gene Expression Human 16-plex, 256 samples	1000795	GEM-X Flex GEM & Library Kit, 4 rxns	1000782	4
		GEM-X Flex Gel Bead Kit, 4 rxns	1000790	4
		GEM-X Flex Hybridization & Wash Kit, 24 rxns	1000789	11
		GEM-X Flex Human Transcriptome Probe Kit, 64 samples	1000787	4

GEM-X Flex Reagent Kits for Multiplexing - Mouse

Reagent Kit Bundle	Part Number (Kit)	Components	Component Part Number	Quantity
GEM-X Flex Gene Expression Mouse 4-plex, 16 samples	1000797	GEM-X Flex GEM & Library Kit, 4 rxns	1000782	1
		GEM-X Flex Gel Bead Kit, 4 rxns	1000790	1
		GEM-X Flex Hybridization & Wash Kit, 24 rxns	1000789	1
		GEM-X Flex Mouse Transcriptome Probe Kit, 16 samples	1000786	1
GEM-X Flex Gene Expression Mouse n-plex, 64 samples	1000831	GEM-X Flex GEM & Library Kit, 4 rxns	1000782	4
		GEM-X Flex Gel Bead Kit, 4 rxns	1000790	4
		GEM-X Flex Hybridization & Wash Kit, 24 rxns	1000789	3
		GEM-X Flex Mouse Transcriptome Probe Kit, 64 samples	1000788	1
		GEM-X Flex Supplemental Wash Kit, 64 rxns	1000828	1
GEM-X Flex Gene Expression Mouse 16-plex, 64 samples	1000798	GEM-X Flex GEM & Library Kit, 4 rxns	1000782	1
		GEM-X Flex Gel Bead Kit, 4 rxns	1000790	1
		GEM-X Flex Hybridization & Wash Kit, 24 rxns	1000789	3
		GEM-X Flex Mouse Transcriptome Probe Kit, 64 samples	1000788	1
GEM-X Flex Gene Expression Mouse 16-plex, 256 samples	1000799	GEM-X Flex GEM & Library Kit, 4 rxns	1000782	4
		GEM-X Flex Gel Bead Kit, 4 rxns	1000790	4
		GEM-X Flex Hybridization & Wash Kit, 24 rxns	1000789	11
		GEM-X Flex Mouse Transcriptome Probe Kit, 64 samples	1000788	4

GEM-X Flex Reagent Kits

GEM-X Flex GEM & Library Kit, 4 rxns, PN-1000782

GEM-X Flex
GEM & Library Kit
4 rxns, PN-1000782
Shipped on dry ice
Store at -20°C

		#	PN
○	Reducing Agent B	1	2000087
○	Amp Mix C	1	2001311
●	Pre-Amp Primers B*	1	2000529
●	GEM Enzyme Mix B	1	2001302
●	GEM Reagent Mix	1	2000491

10x GENOMICS

*This tube is only used in the Optional: Gene Expression Library Cycle Number Determination Using qPCR on page 116 in this User Guide.

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

10x GENOMICS

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

10x GENOMICS

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

10x GENOMICS

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

10x GENOMICS

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

10x GENOMICS

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

10x GENOMICS

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

10x GENOMICS

GEM-X Flex Gel Bead Kit, 4 rxns, PN-1000790

GEM-X Flex
Gel Bead Kit
4 rxns, PN-1000790
Shipped on dry ice
Store at -80°C

	#	PN
Single Cell TL v1 Gel Beads (4 rxns)	1	2000538

10x GENOMICS

GEM-X Flex Supplemental Wash Kit, 64 rxns, PN-1000828*

GEM-X Flex
Supplemental Wash Kit
64 rxns, PN-1000828
Shipped on dry ice
Store at -20°C

		#	PN
●	Conc. Post-Hyb Buffer B	4	2001308
●	Enhancer	3	2000482

10x GENOMICS

*Included with GEM-X Flex Gene Expression Human/Mouse n-plex, 64 samples (1000829/1000831) kit to provide additional reagents required for washing a larger number of pools that are possible with this kit.

*Purchase separately if planning to follow the Individual Wash protocol during the Post-Hybridization Wash step (Step 2.1B) as additional Conc. Post-Hyb Buffer B and Enhancer is required for washing samples before pooling.

Flex Feature Barcode Kit, 64 rxns, PN-1000628*

Fixed RNA Feature Barcode Multiplexing Kit, PN-1000628 has been renamed as Flex Feature Barcode Kit, PN-1000628 without any change in function.

Flex Feature Barcode Kit
64 rxns, Module 1 PN-1000628
Shipped on dry ice
Store at -20°C

		#	PN
●	Antibody Multiplexing Barcode AB001	1	2000917
●	Antibody Multiplexing Barcode AB002	1	2000918
●	Antibody Multiplexing Barcode AB003	1	2000919
●	Antibody Multiplexing Barcode AB004	1	2000920
●	Antibody Multiplexing Barcode AB005	1	2000921
●	Antibody Multiplexing Barcode AB006	1	2000922
●	Antibody Multiplexing Barcode AB007	1	2000923
●	Antibody Multiplexing Barcode AB008	1	2000924
●	Pre-Amp Primers C	1	2000953
○	Amp Mix	1	2000047

10x GENOMICS

Flex Feature Barcode Kit
64 rxns, Module 2 PN-1000628
Shipped on dry ice
Store at -20°C

		#	PN
●	Antibody Multiplexing Barcode AB009	1	2000925
●	Antibody Multiplexing Barcode AB010	1	2000926
●	Antibody Multiplexing Barcode AB011	1	2000927
●	Antibody Multiplexing Barcode AB012	1	2000928
●	Antibody Multiplexing Barcode AB013	1	2000929
●	Antibody Multiplexing Barcode AB014	1	2000930
●	Antibody Multiplexing Barcode AB015	1	2000931
●	Antibody Multiplexing Barcode AB016	1	2000932

10x GENOMICS

*Flex Feature Barcode Kit, 64 rxns, PN-1000628 will support 64 reactions using this User Guide.

GEM-X Flex Gene Expression Chip Kit, 4 chips PN-1000791

Partitioning Oil B
Shipped at ambient temperature
Store at ambient temperature

	#	PN
Partitioning Oil B	4	2001213

Recovery Agent
Shipped at ambient temperature
Store at ambient temperature

	#	PN
Recovery Agent	4	220016

GEM-X
FX Chip & Gaskets
Shipped at ambient temperature
Store at ambient temperature

	#	PN
GEM-X FX Chip	4	2001257
X/iX Chip Gasket, 2-pack	2	3000656

10x GENOMICS

Dual Index Kit TS Set A, 96 rxns PN-1000251

Dual Index Kit TS Set A
Shipped on dry ice
Store at -20°C

	#	PN
Dual Index Plate TS Set A	1	3000511

Dual Index Kit TN Set A, 96 rxns PN-1000250

Dual Index Kit TN Set A
Shipped on dry ice
Store at -20°C

	#	PN
Dual Index Plate TN Set A	1	3000510

10x Genomics Accessories

Product	Part Number (Kit)	Part Number (Item)
10x Vortex Adapter	120251	330002
10x Magnetic Separator B*	1000709 (Chromium X/iX Accessory Kit)/ 1000707 (GEM-X Transition Kit)	2001212
Chromium X Series Chip Holder		3000598
**10x Magnetic Separator (PN-230003) & Magnetic Separator B (PN-2001212) can be used interchangeably.*		

Third-Party Items

Successful execution of GEM-X Flex Gene Expression workflow requires third-party reagents, kits, and equipment in addition to those provided by 10x Genomics. All third-party reagents and consumables should be obtained prior to starting this library construction workflow.

Refer to the GEM-X Flex Gene Expression - Protocol Planner (CG000780) for a detailed list of the following third-party items:

- Additional reagents, kits, and equipment
- Recommended pipette tips
- Recommended thermal cyclers

10x Genomics has tested all items listed in the Protocol Planner. These items perform optimally with the assay. Substituting materials may adversely affect assay performance.

Protocol Steps & Timing

Steps	Timing	Stop & Store
Sample Labeling & Fixation	Variable*	STOP 4°C ≤1 week/-80°C ≤12 months
	*Refer to the appropriate Demonstrated Protocols for details.	
Step 1: Probe Hybridization (page 48)		
1.1 Probe Hybridization (page 51)	16-24 h	
Step 2: Post-Hybridization Washing & Pooling (page 54)		
2.1 Post-Hybridization Pool & Wash (page 56)	90 min	STOP -80°C ≤12 months
Step 3: GEM Generation and Barcoding (page 70)		
3.1 Prepare GEM Master Mix + Sample Dilution (page 73)	30 min	
3.2 Load GEM-X Chip (page 79)	10 min	
3.3 Run the Chromium X/iX (page 81)	6 min	
3.4 Transfer GEMs (page 82)	5 min	
3.5 GEM Incubation (page 83)	125 min	STOP 4°C ≤1 week (GEMs) -80°C ≤12 months (washed undiluted sample from step 2.1)
Step 4: GEM Recovery and Pre-Amplification (page 84)		
4.1 Post-GEM Incubation – Recovery (page 86)	10 min	
4.2 Pre-Amplification PCR (page 87)	55 min	STOP 4°C ≤72 h/-20°C ≤1 week
4.3 DNA Cleanup – SPRIselect (page 88)	30 min	STOP 4°C ≤72 h/-20°C ≤4 weeks
Step 5: GEM-X Flex – Gene Expression Library Construction (page 89)		
5.1 Sample Index PCR (page 91)	40 min	STOP 4°C ≤72 h
5.2 Post Sample Index PCR Size Selection – SPRIselect (page 93)	30 min	STOP 4°C ≤72 h/-20°C long term
5.3 Post Library Construction QC (page 94)	60 min	
Step 6: GEM-X Flex – Protein Expression Library Construction (page 95)		
6.1 Sample Index PCR (page 97)	40 min	STOP 4°C ≤72 h
6.2 Post Sample Index PCR Size Selection – SPRIselect (page 99)	30 min	STOP 4°C ≤72 h/-20°C long term
6.3 Post Library Construction QC (page 100)	60 min	

Sample Preparation

This User Guide is compatible with intact, fresh cells that are labeled with appropriate antibody-oligonucleotide conjugates and fixed. See below for the documents needed to prepare samples for this User Guide. Consult GEM-X Flex Gene Expression - Protocol Planner (CG000780) for additional details.

1	**Sample Labeling**	**Cell Surface & Intracellular Protein Labeling** Label cells with appropriate cell surface and/or intracellular antibody-oligonucleotide conjugates* **Demonstrated Protocol CG000781**
2	**Sample Fixation**	**Sample Fixation** Fix labeled cells. **Demonstrated Protocol CG000782**

*Refer to the Demonstrated Protocol Cell Surface & Intracellular Protein Labeling for GEM-X Flex Gene Expression (CG000781) for more information on the compatible antibodies.

Consult the 10x Genomics support website for additional documentation.

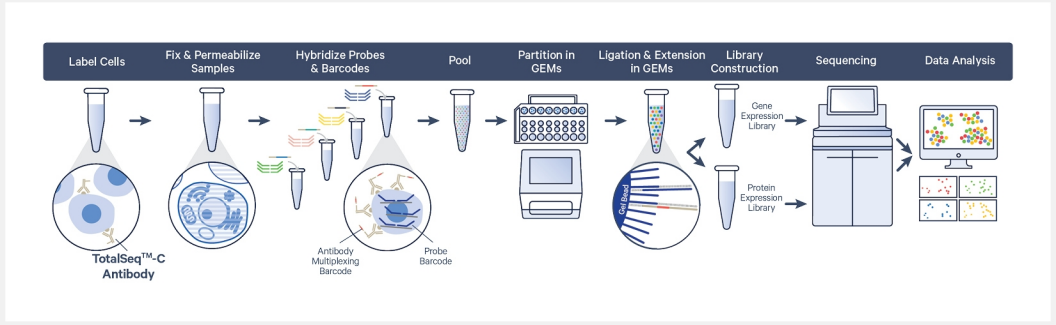
Stepwise Objectives

GEM-X Flex Gene Expression with Feature Barcode technology for protein expression offers comprehensive, scalable solutions to measure gene and protein expression in formaldehyde fixed samples that are labeled with antibody-oligonucleotide conjugates. Protein expression is measured by oligonucleotide conjugated antibodies, which are used to label cells. Gene expression is measured using probe pairs designed to hybridize to mRNA specifically.

For this workflow, labeled and fixed cell suspensions are first hybridized with Probe Barcodes and Antibody Muliplexing Barcodes. Using a microfluidic chip, the hybridized samples are then partitioned into nanoliter-scale Gel Beads-in-emulsion (GEMs). A pool of ~737,000 10x GEM Barcodes (also referred to as 10x Barcodes) is sampled separately to index the contents of each partition.

Inside the GEMs, probes are ligated and the 10x GEM Barcode is added, and all ligated probes within a GEM share a common 10x GEM Barcode. Barcoded and ligated probes are then pre-amplified in bulk, after which gene expression libraries are generated and sequenced.
Similarly, Antibody Multiplexing Barcodes are attached to the Feature Barcode oligonucleotide and the 10x GEM Barcode is added inside the GEMs (barcode oligo capture). The ligated products are then pre-amplified in bulk, after which protein expression libraries are generated and sequenced.

Workflow Overview

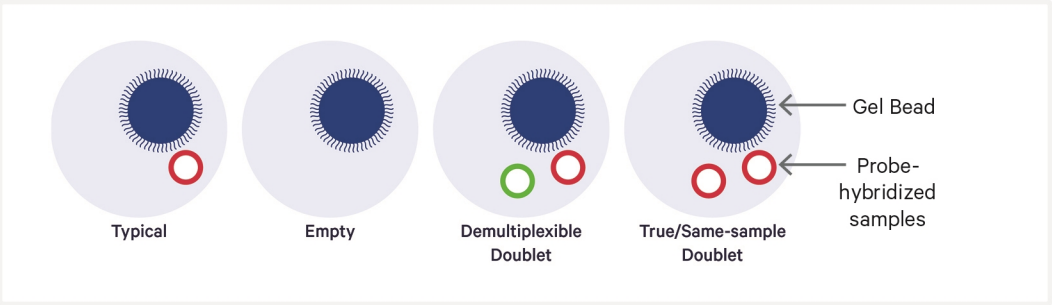


Sample Multiplexing

GEM-X Flex Gene Expression for Multiplexed Samples provides an efficient and cost-effective way to further increase experiment size and cell number by enabling from 4 to 16 samples to be run within a single GEM reaction. Multiplex-compatible GEM-X Flex Human/Mouse Transcriptome Probe kits contain 4 or 16 probe sets, where each probe set includes a Probe Barcode that enables sample multiplexing and downstream demultiplexing. Multiplexing allows for up to 16 unique samples to be barcoded with 16 uniquely barcoded probe sets. Similarly, Fixed RNA Feature Barcode Multiplexing Kit contains 16

unique Antibody Multiplexing Barcodes that allow sample multiplexing and downstream demultiplexing for protein expression.

GEMs Generated During Multiplexing



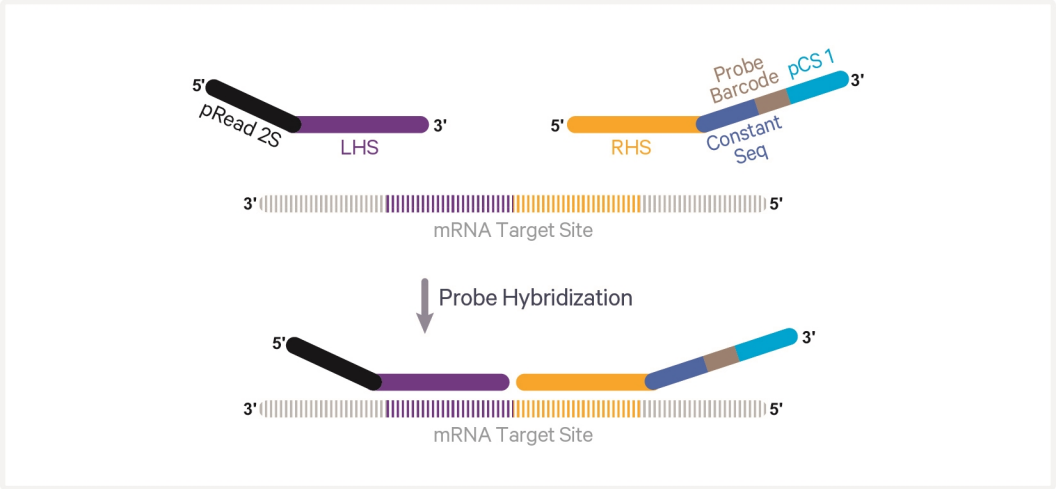
To achieve single cell resolution, cells from any one of the multiplexed samples are delivered at a limiting dilution, such that only 1-10% of GEMs contain a cell hybridized with probes with a given Probe Barcode. The inclusion of the Probe Barcode in each probe pair allows the identification of GEMs containing more than one cell and to demultiplex the data generated from those cells, provided the cells have unique Probe Barcodes.

A high-level overview of each step in this User Guide, including gene and protein expression library construction, is provided in the following sections.

Step 1: Probe Hybridization

The whole transcriptome probe pairs, consisting of a left hand side (LHS) and a right hand side (RHS) for each targeted gene, are added to the fixed sample. Together, probe pairs hybridize to their complementary target RNA in an overnight incubation. Simultaneously, Antibody Multiplexing Barcodes are added to the labeled and fixed sample.

Probe Hybridization



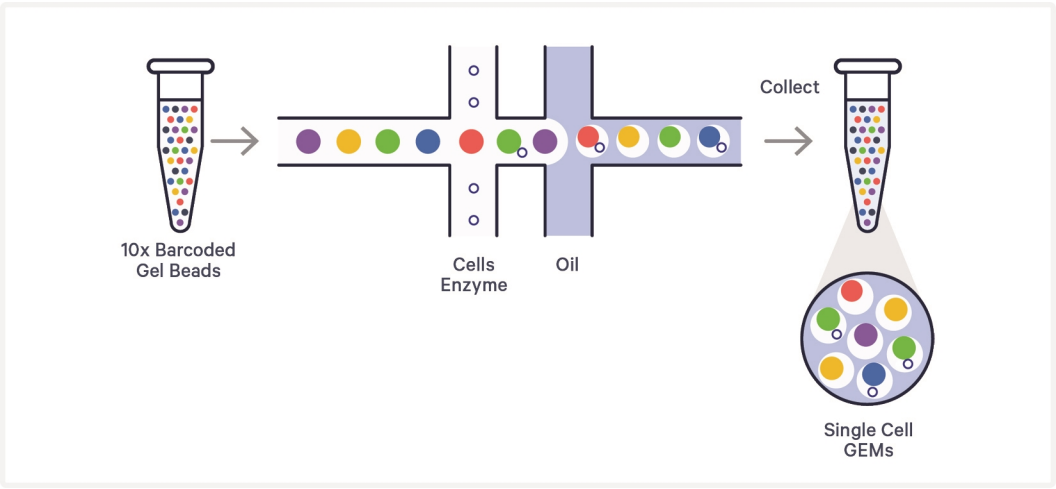
Step 2: Post-Hybridization Washing & Pooling

Samples hybridized with unique Probe Barcodes and Antibody Multiplexing Barcodes can be pooled immediately after hybridization, and washed as a pool (Pooled Wash workflow). Alternatively, samples can be washed individually and pooled after the washing is complete (Individual Wash workflow).

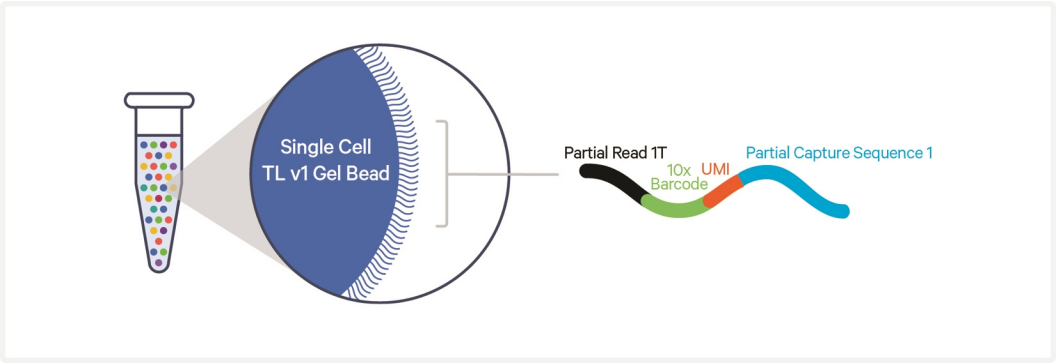
Step 3: GEM Generation & Barcoding

After pooling and washing the samples, GEMs are generated by combining barcoded Gel Beads, a Master Mix containing pooled cells, and Partitioning Oil B onto GEM-X FX Chip. Immediately following GEM generation, the Gel Bead is dissolved, releasing the barcoded Gel Bead primers, and any co-partitioned cell is lysed. Gel Bead primers contain a partial TruSeq Read 1 sequence (partial Read 1T, read 1 sequencing primer), a 16 nt 10x GEM Barcode (or 10x Barcode), a 12 nt unique molecular identifier (UMI), and partial Capture Sequence 1 (sequence complementary to the probe and Antibody Multiplexing Barcode oligonucleotide).

GEM Generation

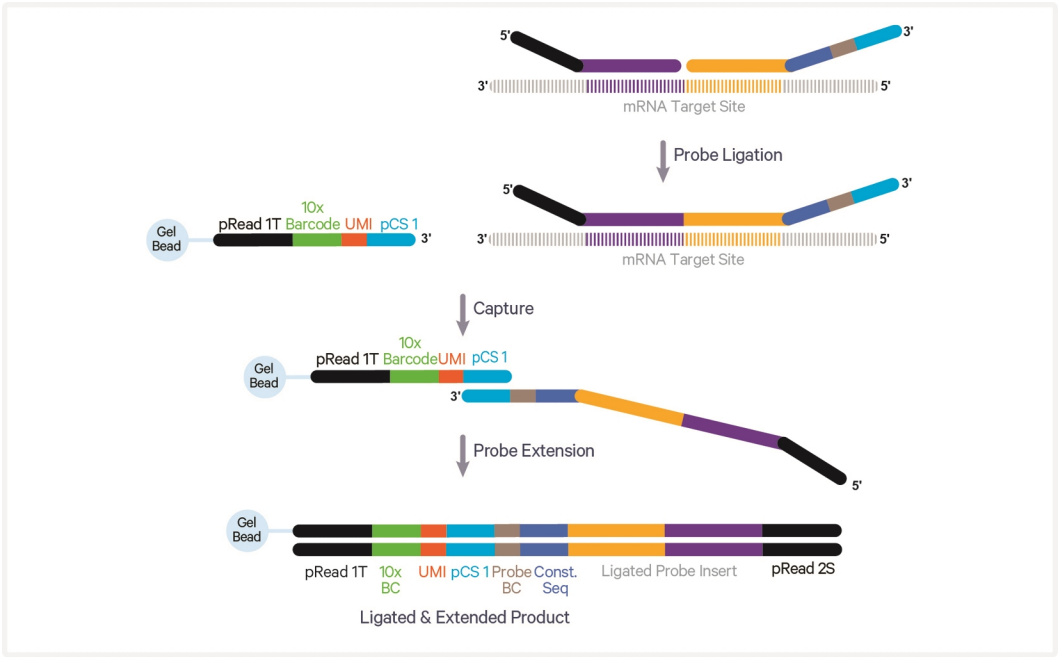


Gel Bead



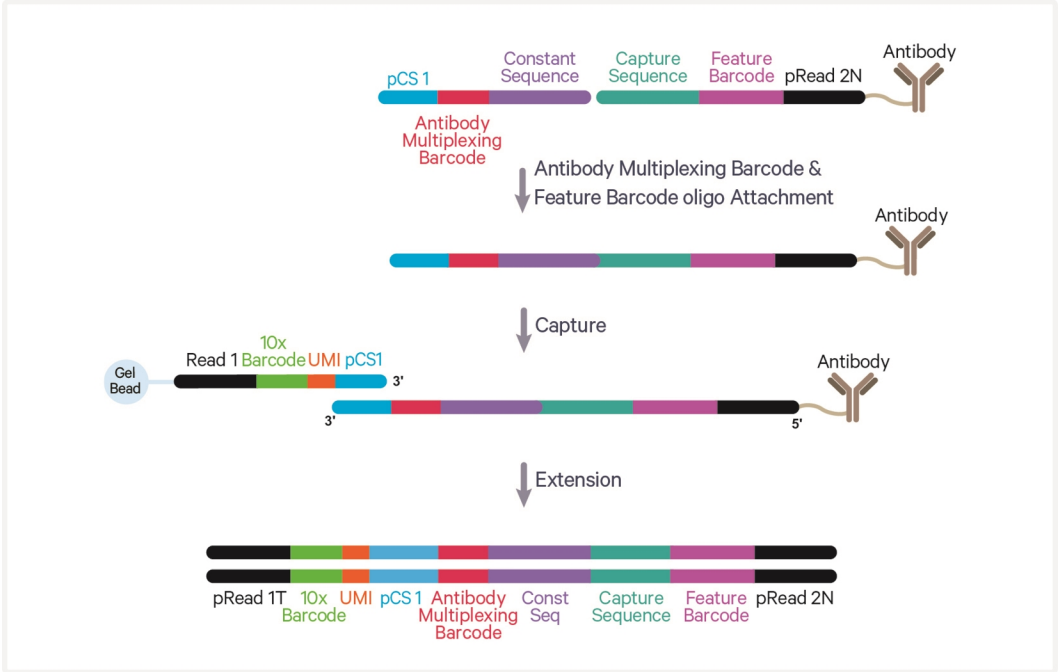
After GEM generation, the partitioned cells, Gel Beads, and Master Mix are placed in a thermal cycler and taken through several steps. First, a ligation step seals the nick between the left hand and right hand probe, while the probes remain hybridized to their target RNA. Second, the Gel Bead primer hybridizes to the capture sequence on the ligated probe pair and is extended by a polymerase to add the UMI, 10x GEM Barcode, and partial Read 1T.

Inside Individual GEMs



Simultaneously, the protein Feature Barcode oligonucleotide is attached to the Antibody Multiplexing Barcode. The Gel Bead primer hybridizes to the capture sequence on the Antibody Multiplexing Barcode that is bound to the protein Feature Barcode (barcode oligo capture) containing (i) a Nextera Read 2 (Read 2N), (ii) a 15 nt Feature Barcode, and (iii) Capture Sequence. Extension by polymerase, produces 10x Barcoded DNA from Antibody Multiplexing Barcode and the protein Feature Barcode.

Inside Individual GEMs - Barcode Oligo Capture

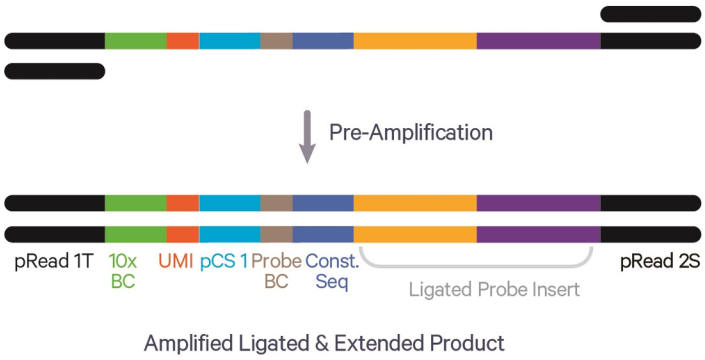


Finally, a heat denaturation step inactivates the enzymes in the GEM reaction.

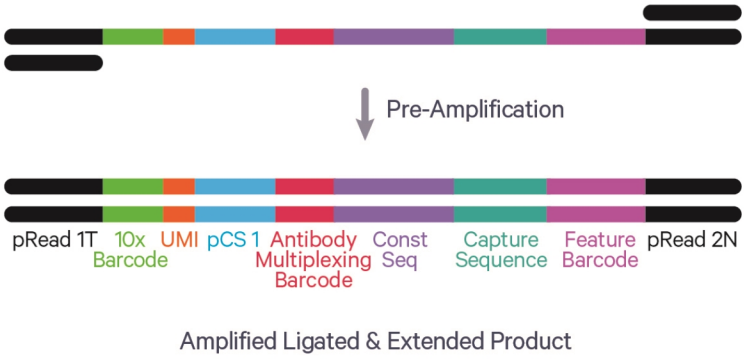
Step 4: GEM Recovery & Pre-Amplification

Once the ligation and barcoding steps are completed, the GEMs are broken by the addition of Recovery Agent, inverting the mixture, and removing the Recovery Agent. A PCR master mix is added directly to the post-GEM aqueous phase to pre-amplify the ligated & extended products, along with barcoded Feature Barcodes identifying proteins. The pre-amplified products are then cleaned up by SPRIselect.

DNA Pre-Amplification - Gene Expression



DNA Pre-Amplification - Feature Barcode

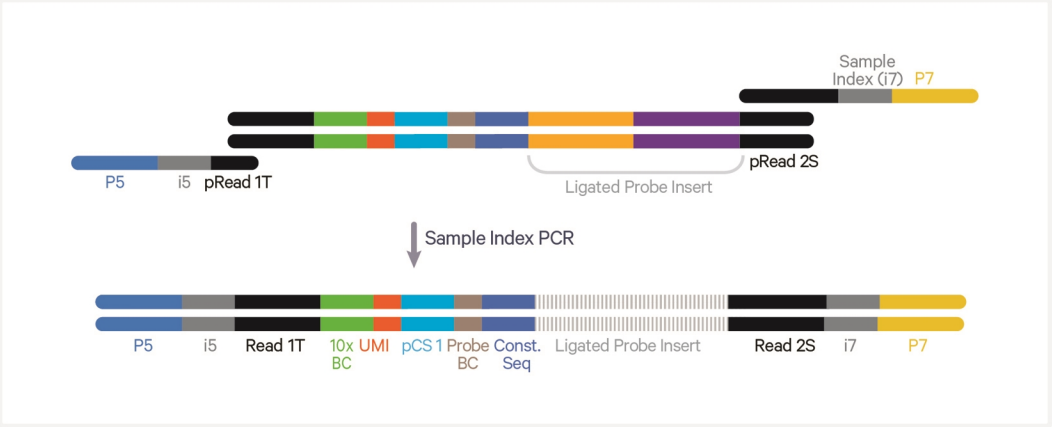


Step 5: GEM-X Flex – Gene Expression Library Construction

The 10x barcoded, ligated probe products undergo indexing via Sample Index PCR. This, in turn, generates final library molecules that are cleaned up by SPRIselect, assessed on a bioanalyzer or a similar instrument, quantified, and then sequenced.

P5, P7, i5 and i7 sample indexes, and Illumina TruSeq Read 1 sequence (Read 1T) and Small RNA Read 2 (Read 2S) sequences are added via Sample Index PCR. The final libraries contain the P5 and P7 priming sites used in Illumina sequencers.

Pooled Amplified DNA Processed in Bulk

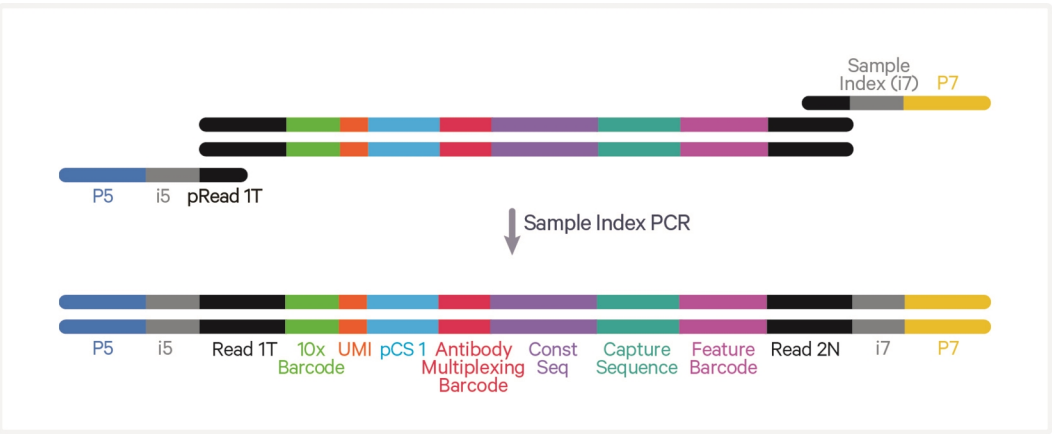


Step 6: GEM-X Flex – Protein Expression Library Construction

The amplified DNA from protein Feature Barcode undergo indexing via Sample Index PCR. This, in turn, generates final library molecules that are cleaned up by SPRIselect, assessed on a bioanalyzer or a similar instrument, quantified, and then sequenced.

P5, P7, i5 and i7 sample indexes, an Illumina TruSeq Read 1 (Read 1T), and a Nextera Read 2 (Read 2N) are added via Sample Index PCR. The final libraries contain the P5 and P7 priming sites used in Illumina sequencers.

Pooled Amplified DNA Processed in Bulk

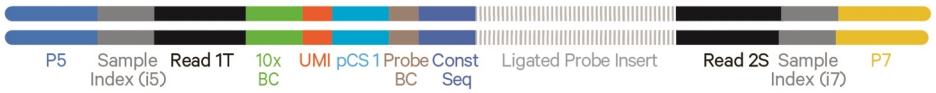


Step 7: Sequencing

A GEM-X Flex – Gene Expression library comprises standard Illumina paired-end constructs which begin and end with P5 and P7. The 16 bp 10x GEM Barcode and 12 bp UMI are encoded in Read 1T. Small RNA Read 2 (Read 2S) sequences the ligated probe insert, constant sequence, and the 8 bp Probe Barcode that identifies the probe set used to hybridize the sample.

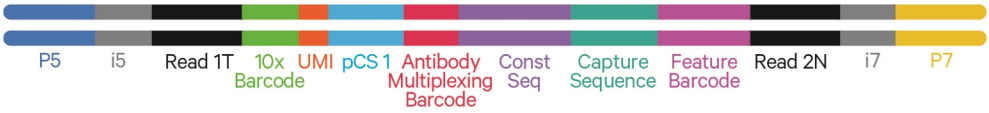
Probe Barcode can be sequenced in both Read 1 and Read 2 sequencing configurations. See GEM-X Flex – Library Sequencing Parameters on page 103 for more details.

GEM-X Flex – Gene Expression Library



A GEM-X Flex – Protein Expression library comprises standard Illumina paired-end constructs which begin and end with P5 and P7. The 16 bp 10x GEM Barcode (10x Barcode) and 12 bp UMI are encoded in Read 1T. Illumina Nextera Read 2 (Read 2N) sequences the Feature Barcode, constant sequence, and Antibody Multiplexing Barcode that identifies the sample.

GEM-X Flex – Protein Expression Library



A single library contains reads derived from up to 16 samples, with cell barcodes composed of a Probe Barcode or an Antibody Multplexing Barcode and a 10x GEM Barcode (10x Barcode).

Antibody Multiplexing Barcode can be sequenced in both Read 1 and Read 2 sequencing configurations. See GEM-X Flex – Library Sequencing Parameters on page 103 for more details.

See Appendix for Oligonucleotide Sequences on page 124

Multiplexing Experiment Design

The following table provides an overview of the multiplexing configurations possible with the different multiplexing kits available for GEM-X Flex Gene Expression assay.

Kit Bundle (PN-Human/ Mouse)	Probe Kit (PN-Human/ Mouse)	# of Samples Pooled	Probe Combination	# of Pools	Cells Recovered
4 Probe Barcodes (BC001-BC004)					
4-plex 16 samples (1000793/ 1000797)	Transcriptome Probe Kit, 16 samples (1000785/ 1000786) - **1 kit**	4 samples	BC001-BC004	4	20,000 cells/Probe Barcode
					80,000 cells/pool
					320,000 cells/kit
16 Probe Barcodes (BC001-BC016)					
n-plex 64 samples (1000829/ 1000831)	Transcriptome Probe Kit, 64 samples (1000787/ 1000788) - **1 kit**	4 samples*	BC001-BC004* BC005-BC008* BC009-BC012* BC013-BC016*	16	20,000 cells/Probe Barcode
					80,000 cells/pool
					1.28 x 10^6 cells/kit
16-plex 64 samples (1000794/ 1000798)	Transcriptome Probe Kit, 64 samples (1000787/ 1000788) - **1 kit**	16 samples	BC001-BC016	4	20,000 cells/Probe Barcode
					320,000 cells/pool
					1.28 x 10^6 cells/kit
16-plex 256 samples (1000795/ 1000799)	Transcriptome Probe Kit, 64 samples (1000787/ 1000788) - **4 kits**	16 samples	BC001-BC016	16	20,000 cells/Probe Barcode
					320,000 cells/pool,
					5.12 x 10^6 cells/kit

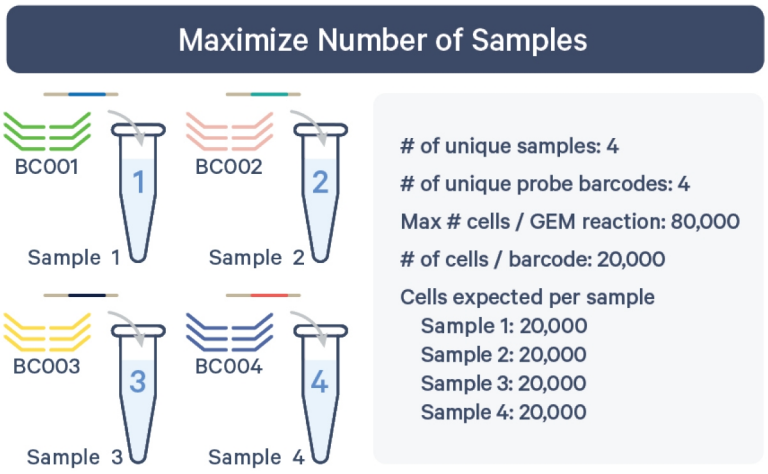
**Other multiplex formats and Probe Barcode combinations also possible*

- Up to 20,000 cells per sample (per Probe Barcode) can be recovered and demultiplexed when using the GEM-X Flex Human/Mouse Transcriptome Probe Kits, 16 & 64 samples.
- GEM-X Flex Gene Expression Human/Mouse 4-plex kits are compatible with and most efficiently used by hybridizing and pooling 4 samples while 16-plex kits are compatible with and most efficiently used by hybridizing and pooling 4 or 16 samples.

  GEM-X Flex Gene Expression Human/Mouse n-plex, 64 samples kit can be used efficiently by pooling an average of 4 samples across 16 GEM reactions. Or alternatively can be used to increase cell load by making 4 pools of 16 samples and running each pool in 4 GEM reactions to capture up to 5.12 x 10^6 cells per kit.

  In addition, other configurations are also compatible. See Appendix for other Alternate Multiplexing Configurations & Pooling Strategies on page 113.
- An example of a 4-plex workflow is demonstrated in the next page.

Example of a 4-plex workflow



Pooling Samples with Different RNA Content

Due to the nature of a multiplexing pool, the sequencing reads for the pool will be distributed to different samples in proportion to their inherent RNA content and all the samples will have the same sequencing saturation. As a result, a sample with cells high in RNA will receive more reads per cell, whereas a sample with cells low in RNA will have proportionally fewer reads per cell, and sequencing saturation will be approximately the same for each sample. Because the distribution of reads across samples is determined by the composition of the pool, it is not possible to add reads to specific samples in the pool.

The recommendation therefore is to

- pool samples when comfortable with sequencing to the same percent saturation for each sample
- keep samples separate if sequencing one closer to saturation than the others is preferred

10x GENOMICS

Tips & Best Practices

Icons

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

GEM-X specific steps

Emulsion-safe Plastics

- Use validated emulsion-safe plastics and other consumables when handling GEMs as some plastics can destabilize GEMs.
- Consult GEM-X Flex Gene Expression - Protocol Planner (CG000780) for a detailed list of plastics and other consumables.

General Reagent Handling

- Fully thaw the reagents at indicated temperatures. Thoroughly mix reagents before use.
- Keep all enzymes and Master Mixes at indicated temperatures during setup and use. Promptly move reagents back to the recommended storage.
- Calculate reagent volumes with indicated % excess of 1 reaction values.
- Cover Partitioning Oil tubes and reservoirs to minimize evaporation.
- Thoroughly mix samples with the beads during bead-based cleanup steps.

Pipette Calibration

- Follow manufacturer's calibration and maintenance schedules.
- Pipette accuracy is particularly important when using SPRIselect reagents.

Probe Hybridization

Sample Input

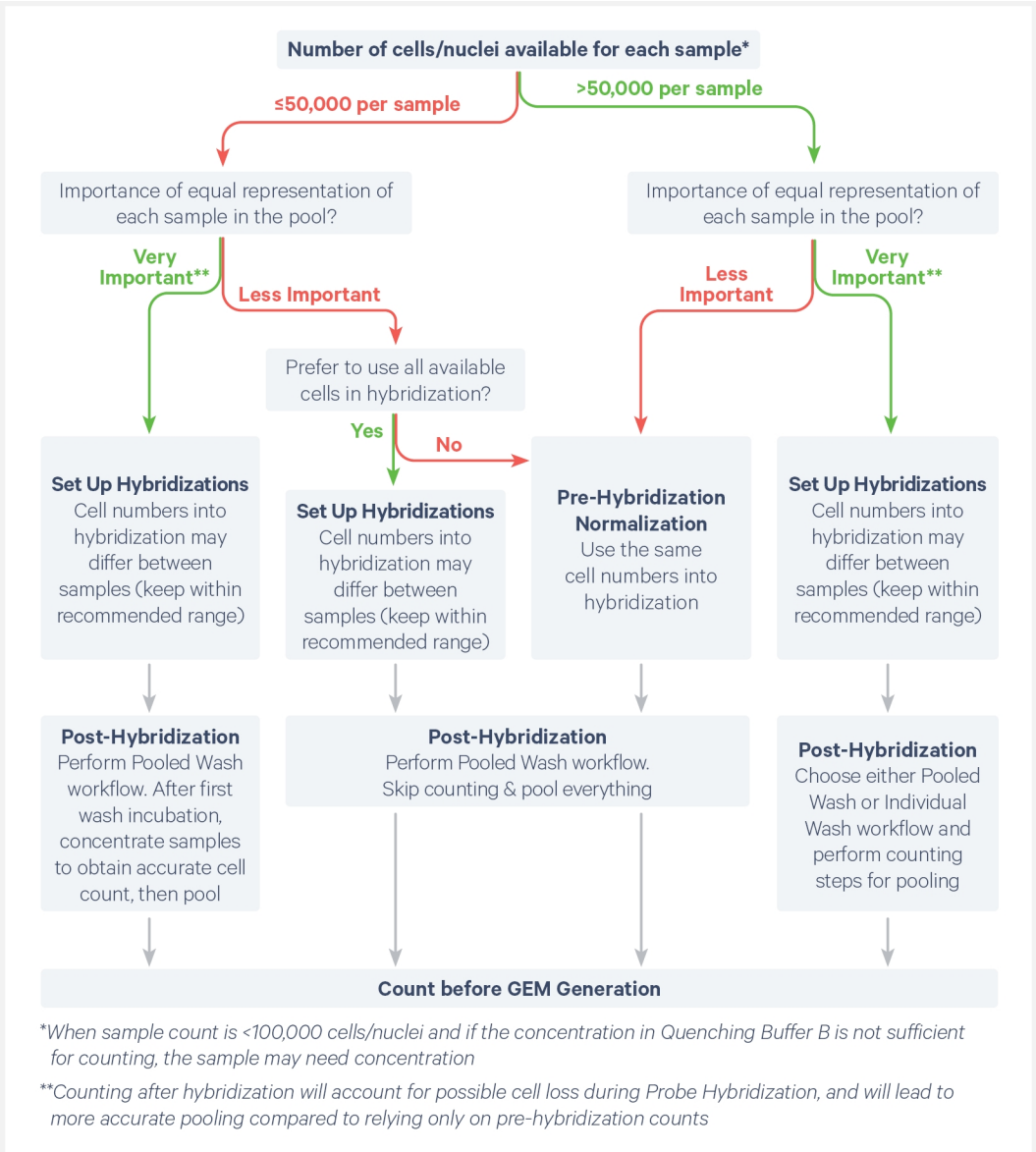
- When working with cells suspension and starting with 25,000 input into fixation, ≤25,000 fixed cells will be moved forward into hybridization.
- Maximum input is **500,000** fixed cells. If sufficient cells are available, it is recommended to default to 300,000 fixed cells for setting up hybridization. However, in case of splenocytes, leukocytes, and bone marrow mononuclear cells, it is recommended to use ≤100,000 cells as higher cell loads may lead to a slight decrease in data quality.
- Using <50,000 cells/barcode may make it difficult to target maximum load.
- It may be possible to use <25,000 cells, 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 cells, may require concentration
  - 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

Pre-Hybridization Normalization

- During multiplexing, if minor differences in cell numbers per sample are acceptable, then it is recommended to start probe hybridization with the same number of cells for all samples that will be pooled together. In this case, the counting before pooling the samples can be skipped and the entire volume of each sample can be pooled (see the flowchart below).

- **Example**: During a 4-plex workflow, with 300,000 to 2 x 10^6 post-fixation count, all the samples are counted and the cell numbers are normalized into probe hybridization (300,000 cells for each sample). In this case, the post-hybridization wash counting can be skipped and all the volume can be pooled together.

Pre-hybridization normalization guidance based on starting cell counts and expected cell recovery during multiplexing.



*When sample count is <100,000 cells/nuclei and if the concentration in Quenching Buffer B is not sufficient for counting, the sample may need concentration

**Counting after hybridization will account for possible cell loss during Probe Hybridization, and will lead to more accurate pooling compared to relying only on pre-hybridization counts

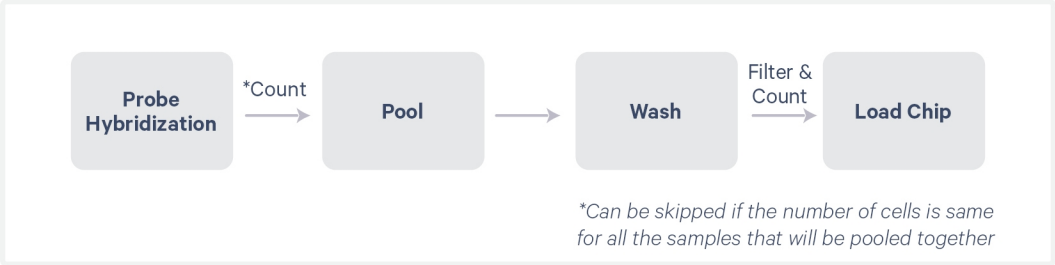
Incubation Time

- Recommended incubation time for probe hybridization is 16-24 h.
- It is recommended to keep the incubation time the same length for all the samples within an experiment.

Post-Hybridization Pooling & Washing Guidance

- Samples hybridized with unique probes can be pooled immediately after hybridization and washed as a pool (**Pooled Wash workflow**). Alternatively, samples can be washed individually and pooled after the washing is complete (**Individual Wash workflow**).
- No difference in data quality is expected between Pooled Wash and Individual Wash workflows.
- The GEM-X Flex Reagent Kits contain sufficient buffer volumes to complete the Pooled Wash workflow. If Individual Wash workflow is chosen, the Supplemental Wash Kit (PN-1000828) is required with the following human/mouse reagent bundles: 4-plex 16 samples (1000793/1000797), 16-plex 64 samples (1000794/1000798), and 16-plex 256 samples (1000795/1000799). The GEM-X Flex Gene Expression n-plex, 64 samples kit bundles contain sufficient reagents for both pooled wash and individual wash workflows.
- After counting, adjust the volume of each sample such that the final washed pool contains an equal number of cells from each sample.

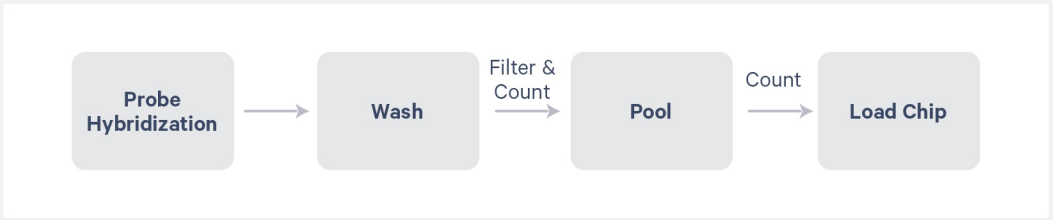
Pooled Wash Workflow



- Ideal for samples with low input cell numbers as samples are pooled together before washing thus minimizing handling multiple tubes compared to the Individual Wash workflow.
- The cell counting is done after probe hybridization to ensure equal representation of each sample in the final pool. This counting step can be skipped if the probe hybridization cell counts were same for all the samples to be pooled together (Pre-Hybridization Normalization).
- If there are ≤50,000 cells and the concentration is under the recommended range of the cell counter, concentrate the sample and count again. See Option 2.1 A: Pooled Wash Workflow on page 57 for details.
- When using fluorescent dyes for counting, fluorescent background on automated counters may be higher due to the buffer composition resulting in inaccurate counting. To reduce the background noise:

- Increase exposure time.
- Further dilute sample 1:2 to 1:4 in PBS before counting. Typically a 1:2 dilution is sufficient to improve the signal to noise.
- The recommended dilution depends on starting cell number, tube size, and the optimal cell concentration and limitation of detection of the counter used. It is not recommended to dilute samples below 100 cells/µl, as it might lead to inaccurate counting results.

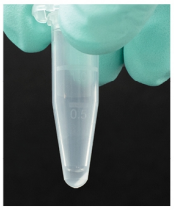
Individual Wash Workflow

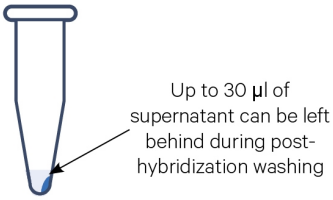


- Ideal for samples with high cell input as samples are washed individually before pooling.
- Cell counting is done immediately after washing to achieve even representation of samples in the pool.
- This workflow allows storage of individual samples after washing, which may be beneficial if a sample needs to be rerun in a singleplex format.

Post-Hybridization Sample Washing & Recovery

- Using a swinging bucket centrifuge can increase cell recovery during washing.
- When performing **post-hybridization washing** with <300,000 cells, complete removal of the supernatant is not required. Up to 30 µl of supernatant may be left behind to optimize cell recovery without significantly impacting assay performance. If uncertain about the volume remaining in the tube, add 30 µl water/PBS to an empty tube of the same size and use it for a visual estimation.





Cell Counts for Chip Loading

- The GEM-X Flex Gene Expression is designed to target 500-20,000 cells per Probe Barcode with a per sample undetected multiplet rate of 0.2% to 8.0%. The solution can target up to 80,000 cells in a 4-plex workflow, up to 160,000 in an 8-plex workflow, and up to 320,000 cells in a 16-plex workflow.
- In a 4-plex workflow, the recommended starting point is to target ~16,000 total cells per GEM reaction (4,000 cells per Probe Barcode), and a multiplet rate of ~1.6%.
- In a 16-plex workflow, the recommended starting point is to target ~64,000 total cells per GEM reaction (4,000 cells per Probe Barcode), and a multiplet rate of ~1.6%.
- The minimum cell input concentration per pool to get maximum cell recovery is 3,343 cells/µl for a 4-plex workflow and 13,371 cells/µl for a 16-plex workflow.
- For each multiplexed sample, assuming 1 unique Probe Barcode is used per sample, the undetected (i.e. with same Probe Barcode) cell multiplet rate for GEM-X FX Chip is approximately 0.4% multiplets per 1,000 cells recovered. Up to 320,000 cells per GEM well can be recovered on GEM-X FX Chip with a low multiplet rate - any GEMs with multiplets derived from dissimilar Probe Barcodes can be demultiplexed.

Undetectable Multiplet Rate (%)	Cells Loaded/ Probe Barcode	Cells Recovered/ Probe Barcode	Cells Equally Distributed On:					
			4 Probe Barcodes		8 Probe Barcodes		16 Probe Barcodes	
			Cells Loaded/ Well	Cells Recovered/ Well	Cells Loaded/ Well	Cells Recovered/ Well	Cells Loaded/ Well	Cells Recovered/ Well
~0.2	725	500	2,900	2,000	5,800	4,000	11,600	8,000
~0.4	1,450	1,000	5,800	4,000	11,600	8,000	23,200	16,000
~0.8	2,900	2,000	11,600	8,000	23,200	16,000	46,400	32,000
~1.6	5,800	4,000	23,200	16,000	46,400	32,000	92,800	64,000
~2.4	8,700	6,000	34,800	24,000	69,600	48,000	139,200	96,000
~3.2	11,600	8,000	46,400	32,000	92,800	64,000	185,600	128,000
~4.0	14,500	10,000	58,000	40,000	116,000	80,000	232,000	160,000
~5.0	18,125	12,500	72,500	50,000	145,000	100,000	290,000	200,000
~6.0	21,750	15,000	87,000	60,000	174,000	120,000	348,000	240,000
~7.0	25,375	17,500	101,500	70,000	203,000	140,000	406,000	280,000
~8.0	29,000	20,000	116,000	80,000	232,000	160,000	464,000	320,000

Sample Filtration

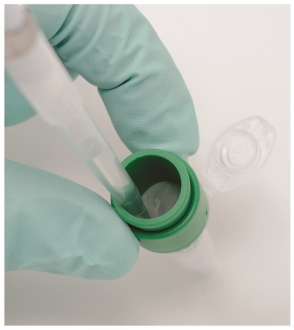
- After post-hybridization wash, pass the sample through a 30 µm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation) into a new 1.5-ml microcentrifuge tube.

DO NOT use 40 µm Flowmi Tip Strainer for filtration.

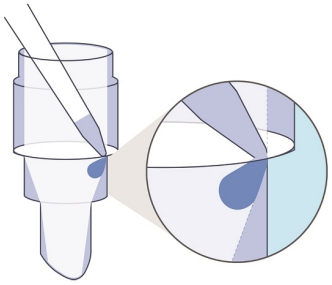
- Hold the pipette tip at an angle and touch the filter membrane where the filter meets the wall. Slowly pipette through the filter. Tap gently or centrifuge briefly if liquid remains at the end of the filter.
- To maximize recovery, residual volume can be pipetted from underneath the filter.

Filtration using 30 µm Filters

Pre-Separation Filters from Miltenyi Biotec



Celltrix Filters from Sysmex



Touch the filter membrane with the tip where the filter meets the wall

Pre-Separation Filters from Miltenyi Biotec

Celltrix Filters from Sysmex

Cell Counting

- Accurate counting is critical for optimal assay performance.
- Fluorescent staining enables accurate counting even in the presence of sub-cellular debris and hence, is strongly recommended.

Combination of counters and dyes tested for counting fixed cells

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 postimage 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 cells are relatively dimmer and require longer exposure compared to NucSpot 470, so NucSpot 470 is preferred.

**Dilute the stock to 1:100 and mix 1:1 with the sample. For example, add 10 µl diluted dye to 10 µl sample.*

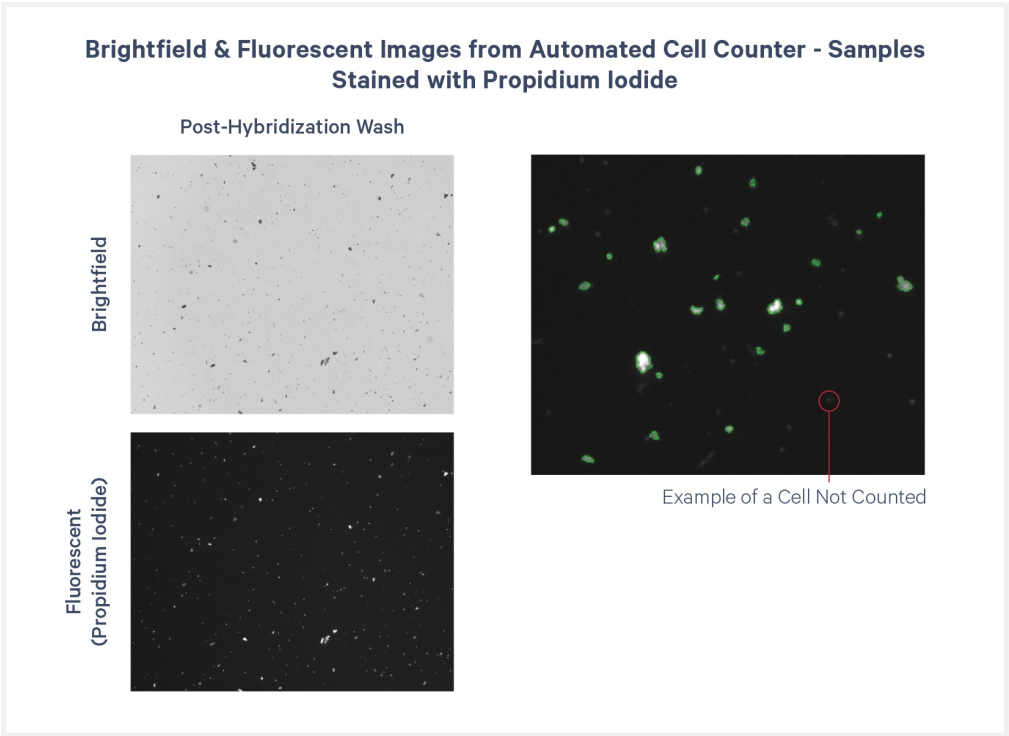
- Focus cells under the brightfield before switching to the fluorescent channel.
- Increase exposure time to help adjust signal to noise during counting.
- Do a final visual inspection to confirm the counts are accurate. After obtaining the counts, switch between brightfield and fluorescent channel to ensure that the counts include minimal to no debris.
- Including debris in the count will result in lower chip loading numbers, which may contribute to lower cell recovery.
- Ensure that the cell counter emission/excitation filter is compatible with the fluorescent dye used.
- If using an automated cell counter, ensure that the cells are being circled correctly. The settings of the automated cell counters may need to be adjusted for optimal cell-detection accuracy.

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. 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 sample 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. See manufacturer's instructions for details.

  Samples stained with PI Staining Solution can also be counted using Countess II FL, Countess 3 FL, and Cellometer K2 cell counters. See manufacturer's instructions for details.



Counting Using Trypan Blue (Only for Debris-Free Samples)

Debris-free samples can also be counted using trypan blue. This protocol provides instructions for counting samples 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 will be stained with trypan blue stain and appear nonviable.

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

GEM-X Chip Handling

- Minimize exposure of reagents, chips, and gaskets to sources of particles and fibers, laboratory wipes, frequently opened flip-cap tubes, clothing that sheds fibers, and dusty surfaces.
- After removing the chip from the sealed bag, use in **≤24 h**.
- Execute steps without pause or delay, unless indicated. When using multiple chips, load, run, and collect the content from one chip before loading the next.
- Fill all unused input wells in rows labeled 1, 2, and 3 on a chip with an appropriate volume of 50% glycerol solution before loading the used wells. DO NOT add glycerol to the wells in the top NO FILL row.
- Avoid contacting the bottom surface of the chip with gloved hands and other surfaces. Frictional charging can lead to inadequate priming of the channels, potentially leading to wetting failures.
- Minimize the distance that a loaded chip is moved to reach the Chromium X/iX.

- Keep the chip horizontal to prevent wetting the gasket with oil or spilling oil, which depletes the input volume and may adversely affect the quality of the resulting emulsion.
- If there is oil on the chip holder, an isopropanol wipe or isopropanol alcohol in a spray bottle with a laboratory wipe can be used to clean the spill.

Chromium X Series Chip Holder

- Chromium X Series Chip Holders encase GEM-X Chips.
- The holder lid flips over to become a stand, holding the chip at 45 degrees for optimal recovery well content removal.
- Squeeze the slider on the back (right side) of the holder toward the middle to unlock the lid and return the holder to a flat position.

GEM-X Chip & Holder Assembly with Gasket

- Close the holder lid. Attach the gasket by holding the tongue (curved end, to the right) and hook the gasket on the left-hand tabs of the holder. Gently pull the gasket toward the right and hook it on the two right-hand tabs.
- DO NOT touch the smooth side of the gasket.
- Open the chip holder.
- Align notch on the chip (upper left corner) and the open holder with the gasket attached.
- Slide the chip to the left until the chip is inserted under the guide on the holder.
- Depress the right-hand side of the chip until the spring-loaded clip engages.
- Keep the assembled unit with the attached gasket until ready for dispensing reagents.

One of the rows of the gasket will not align with the GEM-X chip wells. This is expected and will not impact the assay.

A generic image representative of the GEM-X FX Chip is shown below.

Representative chip image

GEM-X Chip Loading

- Place the assembled chip and holder flat (gasket attached) on the bench with the lid open.
- Dispense at the bottom of the wells without introducing bubbles.
- When dispensing Gel Beads into the chip, wait for the remainder to drain into the bottom of the pipette tips and dispense again to ensure complete transfer.
- See Load GEM-X Chip on page 79 for specific instructions.

Chromium X/iX Firmware

- Firmware version 2.0 or higher is required in the Chromium X/iX to run the GEM-X chips.
- The current firmware version of the instrument can be located using the system menu.
- Consult the Chromium X Series Instrument with Readiness Test User Guide (CG000396) for detailed instructions on updating the firmware.

50% Glycerol Solution for Addition to Unused Chip Wells

- Purchase 50% glycerol solution from Ricca Chemical Company, Glycerin (glycerol), 50% (v/v) Aqueous Solution, PN-3290-32.

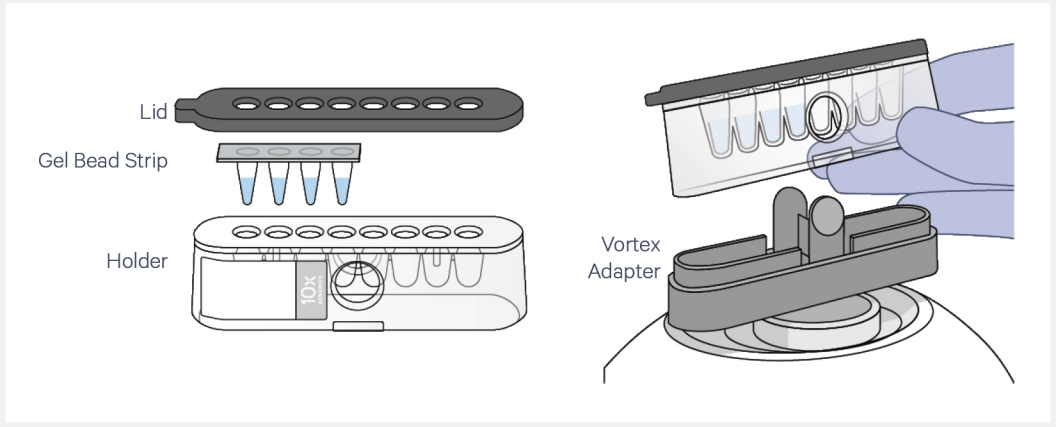
  OR

- Prepare 50% glycerol solution:
  - Mix an equal volume of water and ≥99% Glycerol, Molecular Biology Grade.
  - Filter through a 0.2 µm filter.
  - Store at **−20°C** in 1-ml LoBind tubes. 50% glycerol solution should be equilibrated to room temperature before use.
- Adding glycerol to non-sample chip wells is essential to avoid chip failure.

50% Glycerol Solution for Sample Storage

- Use nuclease-free water and molecular biology grade Glycerol from Millipore Sigma, PN-G5516, to prepare fresh 50% glycerol solution as described previously. DO NOT use 50% glycerol solution from Ricca Chemical Company, Glycerin (glycerol), 50% (v/v) Aqueous Solution, PN-3290-32 as it is not molecular biology grade and has not been tested for sample storage.

Gel Bead Handling

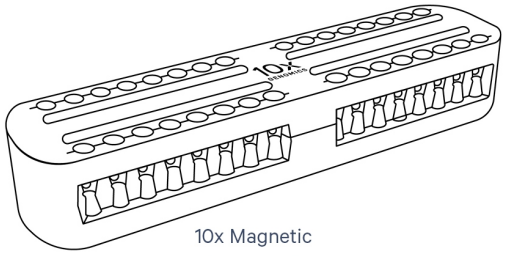


- Use one tube of Gel Beads per sample. DO NOT puncture the foil seals of tubes not used at the time.
- After removing the Gel Bead strip from the packaging, equilibrate the Gel Bead strip to **room temperature** for at least **30 min** before use.

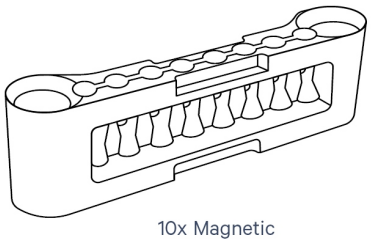
- Store unused Gel Beads at **−80°C** and avoid more than 12 freeze-thaw cycles. DO NOT store Gel Beads at −20°C.
- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**.
- Centrifuge the Gel Bead strip for ~5 sec after removing from the holder. Confirm there are no bubbles at the bottom of tubes and the liquid levels look even. Place Gel Bead strip back in the holder and secure the holder lid.
- If the required volume of beads cannot be recovered, place the pipette tips against the sidewalls and slowly dispense the Gel Beads back into the tubes. DO NOT introduce bubbles into the tubes and verify that the pipette tips contain no leftover Gel Beads. Withdraw the full volume of beads again by pipetting slowly.

10x Magnetic Separator

- Images below are illustrative - actual appearance of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.
- Offers two positions of the magnets (high and low) relative to a tube, depending on its orientation. Flip the magnetic separator over to switch between high (magnet•**High**) or low (magnet•**Low**) positions.
- If using MicroAmp 8-Tube Strips, use the high position (magnet•**High**) only throughout the protocol.



10x Magnetic Separator B

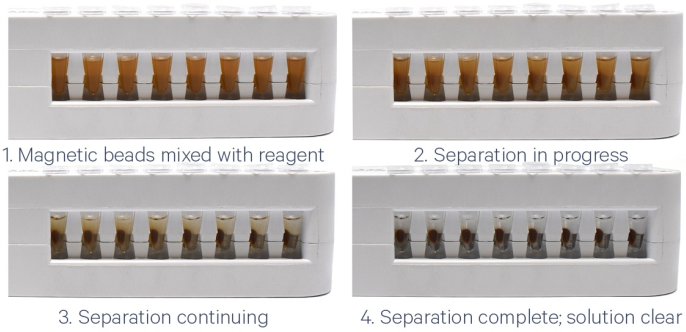


10x Magnetic Separator

Magnetic Bead Cleanup Steps

- During magnetic bead based cleanup steps that specify waiting “until the solution clears”, visually confirm clearing of solution before proceeding to the next step. See panel below for an example.

- The time needed for the solution to clear may vary based on specific step, reagents, volume of reagents etc.
- Images below are representative - actual color of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.
- **Visually Confirm Clearing of Magnetic Bead Solution**



SPRIselect Cleanup & Size Selection

- After aspirating the desired volume of SPRIselect reagent, examine the pipette tips before dispensing to ensure the correct volume is transferred.
- Pipette mix thoroughly as insufficient mixing of sample and SPRIselect reagent will lead to inconsistent results.
- Use fresh preparations of 80% Ethanol.

Tutorial — SPRIselect Reagent: DNA Sample Ratios

SPRI beads selectively bind DNA according to the ratio of SPRIselect reagent (beads).

$$\text{Example Ratio: } \frac{\text{Volume of SPRIselect reagent added to the sample}}{\text{Volume of DNA sample}} = \frac{50\ \mu l}{100\ \mu l} = \mathbf{0.5X}$$

Sample Indices in Sample Index PCR

- Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run.
- Verify and use the specified index plate only. DO NOT use the plates interchangeably.
- Each well in the Dual Index Plate contains a unique i7 and a unique i5 oligonucleotide.

Sample Index PCR Cycles

- Sample index PCR cycle number can be determined using one of the following methods:

  Using qPCR: The cycle numbers are determined by running qPCR assay on the product from Pre-Amplification Cleanup - SPRIselect.

  Using target cell recovery: The cycle numbers are determined using target cell recovery.

- qPCR assay is recommended when working with an unknown tissue type or sample preparation method for the first time. Running qPCR to determine sample index PCR cycles will lead to more reliable library yields, and will reduce the probability of under or over-cycling the final libraries.

10x GENOMICS

Step 1:

Probe Hybridization

1.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Thaw & Keep Warm				
□	Hyb Buffer B	2001312	Thaw at 42°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. DO NOT keep the thawed buffer on ice, or the solution will precipitate. Thawed Hyb Buffer B can be kept at 42°C for up to 1 h.	–20°C
□	Enhancer	2000482	Thaw 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.	–20°C
Place on Ice				
□	Labeled & Fixed Cell Suspension	—	Consult GEM-X Flex Gene Expression - Protocol Planner (CG000780) for details on applicable Demonstrated Protocols.	—
□	Antibody Multiplexing Barcode AB001-AB016	2000917-2000932	Thaw on ice. Vortex and centrifuge briefly.	–20°C
□	Human WTA Probes BC001-BC016 OR Mouse WTA Probes BC001-BC016	2001259-2001274 2001275-2001290	Thaw on ice. Vortex and centrifuge briefly.	–20°C

Probe Hybridization Overview for Multiplexing

To generate GEM-X Flex – Gene Expression & Protein Expression libraries, fixed single cell suspensions are mixed with probes (Probe Barcodes and Antibody Multiplexing Barcodes), and hybridized overnight (16-24 h) at 42°C.

Unique Sample to Unique Probe Barcode

1.1 Probe Hybridization

Before starting this protocol:

- Ensure that the samples have been appropriately labeled and fixed and quenched.
  Consult GEM-X Flex Gene Expression - Protocol Planner (CG000780) for details on the labeling and fixation protocols to use.
- Determine the number of cells for each hybridization reaction.

  When working with cells suspension and starting with 25,000 input into fixation, ≤25,000 fixed cells will be moved forward into hybridization. Maximum input is **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 splenocytes, leukocytes, and bone marrow mononuclear cells, it is recommended to use ≤100,000 cells as higher cell loads may lead to a slight decrease in data quality.

TIPS **Pre-Hybridization Normalization** - In a multiplexing reaction, if the number of cells/nuclei added to each hybridization reaction is the same, the samples can be pooled without counting during Post-Hybridization Wash (step 2.1A/B).

TIPS See Tips & Best Practices for Probe Hybridization on page 33.

a. Set a thermomixer with **heated lid** to 42°C or prepare a thermal cycler with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
42°C	50 µl	Overnight
Step	**Temperature**	**Time**
Pre-equilibrate	42°C	Hold
Probe Hybridization	42°C	16-24 h

b. Prepare Hyb Mix at **room temperature**. Pipette mix 10x.

Hyb Mix *Add reagents in the order listed*		PN	1X* + 20% (µl)	4X* + 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	168.0	672.0
	Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.*	2000482	6.0	24.0	96.0
	Total	-	**48.0**	**192.0**	**768.0**

*X represents the number of samples, for example, 4X = 4 fixed samples

c. Incubate Hyb Mix at **42°C** for **5 min**.

d. Centrifuge fixed sample resuspended in Quenching Buffer B at **850 rcf** for **5 min** at **4°C**.

e. Remove the supernatant.

For <300,000 fixed cells, use a swinging bucket rotor for centrifugation and carefully remove the supernatant without disturbing the pellet. In such cases, complete removal of the supernatant is not required.

TIPS

Up to 15 µl of supernatant may be left behind to optimize cell recovery without significantly impacting assay performance. If uncertain about the volume remaining in the tube, add 15 µl water/PBS to an empty tube of the same size and use it for a visual estimation.

f. Resuspend each pellet in **40 µl** Hyb Mix.

Samples can be transferred to a tube strip if planning to use a thermal cycler for incubation. If using 1.5-ml microcentrifuge tubes, a thermomixer with heated lid will be required for incubation.

When there are ≤50,000 cells, a 1.5-ml tube is preferred (provided that a thermomixer with heated lid is available) to avoid tube transfer and potential cell loss.

Keep sample at **room temperature**. DO NOT place on ice.

g. Add **10 µl** unique Human/Mouse WTA Probes BC001-BC016 (PN-2001259-2001274 or PN-2001275-2001290) to the **40 µl** mixture of Hyb Mix and fixed sample. Record the probes name and part number used for each sample. Only one WTA Probe should be added to each tube containing sample + Hyb mix.

h. Add **2 µl** of any Antibody Multiplexing Barcode (AB001-AB016) to each tube and gently pipette mix 10x with pipette set at 40 µl. Record the Antibody Multiplexing Barcode name and part number used for each sample. *Only one Antibody Multiplexing Barcode should be added to each tube.*

i. Incubate sample for **16-24 h** at **42°C** in a thermal cycler or a thermomixer with heated lid and no shaking.

Incubation for less than 16 h is not recommended. Incubation time should be consistent across all samples in an experiment.

10x GENOMICS

Step 2:

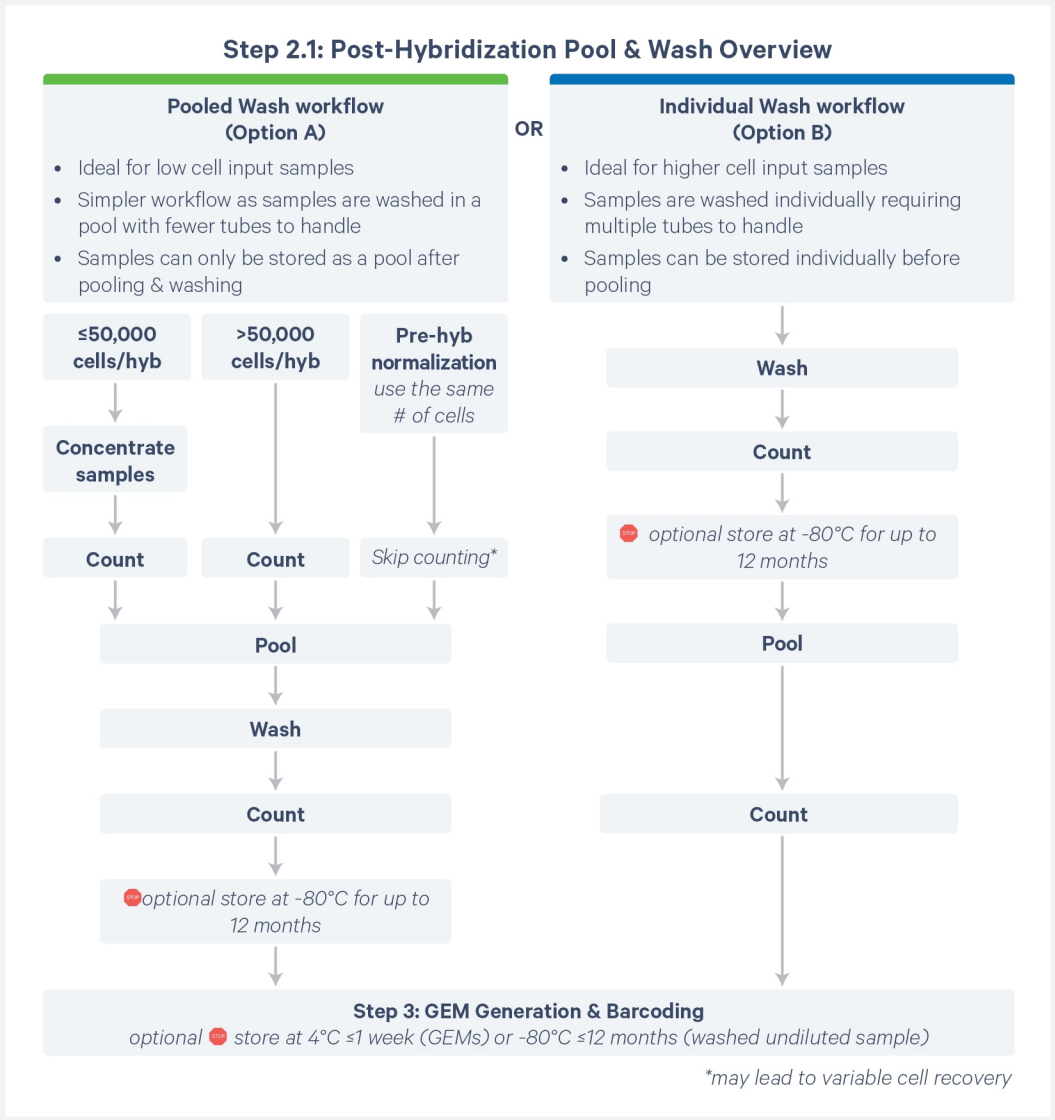
Post-Hybridization Washing & Pooling

2.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Thaw & Keep Warm				
☐	Enhancer	2000482	Thaw 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.	–20°C
Place on Ice				
☐	Conc. Post-Hyb Buffer B	2001308	Thaw at room temperature and keep on ice.	–20°C
Obtain				
☐	Sample Filters Sysmex Sterile Single-pack CellTrics Filters/Miltenyi Biotec Pre-Separation Filters (30 µm)	—	Manufacturer's recommendations.	Ambient
☐	10x Vortex Adapter	330002	See Tips & Best Practices.	Ambient
☐	Glycerol for molecular biology, ≥99% *Prepare fresh 50% glycerol solution for sample storage*	—	See Tips & Best Practices.	—

2.1 Post-Hybridization Pool & Wash

Samples hybridized with unique Probe Barcodes can be pooled immediately after hybridization, and washed as a pool (**Pooled Wash workflow**). Alternatively, samples can be washed individually and pooled after the washing is complete (**Individual Wash workflow**). The following section provides instructions for both **Pooled Wash workflow (Option 2.1A) and Individual Wash workflow (Option 2.1B)**.



Option 2.1 A: Pooled Wash Workflow

During the centrifugation steps, up to 30 µl supernatant may be left behind if working with <300,000 cells. See Post-Hybridization Sample Washing & Recovery on page 36 for more details. If uncertain about the volume remaining in the tube, add 30 µl water/PBS to an empty tube of the same size and use it for a visual estimation.

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

Post-Hyb Wash Buffer B *Add reagents in the order listed*	PN	Pooling 4 samples (ml)*	Pooling 8 samples (ml)*	Pooling 16 samples (ml)*
Nuclease-free Water	-	4.95	7.92	13.86
Conc. Post-Hyb Buffer B	2001308	0.275	0.44	0.77
Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.*	2000482	0.275	0.44	0.77
Total	**-**	**5.5**	**8.8**	**15.40**

**Volumes are in ml and include 10% overage*

The table above provides volumes required when pooling samples into 1 GEM well. For volumes required when pooling samples into >1 GEM wells, see Post-Hyb Wash Buffer B Preparation on page 113.

b. Remove tubes from thermal cycler (8-tube strips) or thermomixer (1.5-ml microcentrifuge tubes) after overnight incubation.

c. Follow either step d or step e depending upon the pre-hybridization cell counts.

d. If the pre-hybridization cell counts were ≤50,000 cells per hybridization: Follow the **Concentrate & Pool workflow** below:

i. Add **750 µl** Post-Hyb Wash Buffer B to the sample in a 1.5-ml microcentrifuge tube and pipette mix 5x.

If the hybridization was performed in 8-tube strips, add 150 µl buffer to the sample, gently pipette mix, and transfer to a 1.5-ml microcentrifuge tube. Wash the tube strips with additional buffer, transfer to the microcentrifuge tube, and add the remaining volume of buffer for a total of 750 µl Post-Hyb Wash Buffer B to the sample.

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

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

iv. **Concentrate** the sample by removing supernatant (~770 µl) leaving ~30 µl behind.

v. Add **150 µl** Post-Hyb Wash Buffer B to the sample and pipette mix 5x.

vi. Determine cell concentration using an automated cell counter or a hemocytometer. Calculate the total cell number present in the tube.

TIPS See Tips & Best Practices for Cell Counting on page 39.

vii. Pool an equal number of cells from different hybridization reactions. See Post-Hybridization Pooling Calculation on page 63 for details. *Optional:* Sample types prone to clumping can be filtered with a 30 µm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation Filters) during pooling.

viii. Proceed to step f.

e. If the pre-hybridization cell counts were >50,000 cells per hybridization:

Follow the instructions below for 8-tube strips or 1.5-ml microcentrifuge tubes.

▲ denotes volumes for 8-tube strips and ■ denotes volumes for 1.5-ml microcentrifuge tubes.

i. Add ▲ **225 µl** or ■ **400 µl** Post-Hyb Wash Buffer B to the sample and pipette mix 5x.

ii. Determine cell concentration using an automated cell counter or a hemocytometer. Calculate the total cell number present in the tube.

TIPS See Tips & Best Practices for Cell Counting on page 39. For 8-tube strips, the sample can be diluted 1:2 to 1:4 with PBS before counting depending on the cell input into hybridization. See the Expected Cell Concentration at Different PBS Dilution on page 113.

Accurate cell counting is critical for optimal assay performance, however, it can be skipped if the probe hybridization cell counts were same for all the samples to be pooled together.

iii. Add ▲ **525 µl** or ■ **350 µl** Post-Hyb Wash Buffer B per sample into a 5-ml (for 4 pooling samples) or 15-ml (for pooling 8 or 16 samples) centrifuge tube.

For examples, for pooling 4 samples, add ▲ 2.1 ml buffer (525 µl * 4) or ■ 1.4 ml buffer (350 µl * 4).

iv. Pool an equal number of cells from different hybridization reactions and add to the tubes containing Post-Hyb Wash Buffer B. See Post-Hybridization Pooling Calculation on page 63 for details. Mix by inverting 5x.

Optional: Sample types prone to clumping can be filtered with a 30 µm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation Filters) during pooling by adding the filter on top of the 5-ml or 15-ml tube containing Post-Hyb Wash Buffer B.

v. Centrifuge each tube containing pooled samples at **850 rcf** for **5 min** at **room temperature**.

vi. Remove the supernatant without disturbing the pellet.

TIPS

Up to 30 µl supernatant may be left behind if working with <300,000 cells. See Post-Hybridization Sample Washing & Recovery on page 36 for more details.

vii. Resuspend cell pellet in **1 ml** Post-Hyb Wash Buffer B and transfer to a 1.5-ml microcentrifuge tube.

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

ix. Proceed to step f.

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

g. Remove the supernatant without disturbing the pellet.

TIPS

Up to 30 µl supernatant may be left behind if working with <300,000 cells. See Post-Hybridization Sample Washing & Recovery on page 36 for more details.

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

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

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

k. Remove the supernatant without disturbing the pellet.

TIPS

Up to 30 µl supernatant may be left behind if working with <300,000 cells. See Post-Hybridization Sample Washing & Recovery on page 36 for more details.

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

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

n. Prepare Post-Hyb Resuspension Buffer B. Pipette mix 10x. Maintain at **4°C.**

Post-Hyb Resuspension Buffer B *Add reagents in the order listed*	PN	1 Pool + 10% (µl)	4 Pools + 10% (µl)
Nuclease-free Water	-	783.75	3135.0
Conc. Post-Hyb Buffer B	2001308	41.25	165.0
Total	**-**	**825.0**	**3300.0**

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

p. Remove the supernatant without disturbing the pellet.

TIPS

Up to 30 µl supernatant may be left behind if working with <300,000 cells. See Post-Hybridization Sample Washing & Recovery on page 36 for more details.

q. Resuspend cell pellet in an appropriate volume of **chilled** Post-Hyb Resuspension Buffer B. Pipette mix 20x. Maintain on ice.

The buffer volume will depend on the targeted cell recovery and the cell stock concentration.

When targeting maximum cell recovery (20,000 cells/Probe Barcode) - resuspend in **250 µl** buffer.

When not targeting maximum cell recovery - follow the steps below to determine the appropriate volume.

- See Cell Suspension Volume Calculator for Multiplexing 4 Samples on page 74 to determine the desired cell stock concentration based on targeted cell recovery.
- Calculate the volume of resuspension buffer using the cells input into probe hybridization, an estimated ~70% cell recovery* during the washing step, and the desired cell stock concentration. See example calculation on the next page.

⚠ ***70% is a conservative estimate for the number of cells recovered during the post-hybridization wash. Cell loss will vary from sample to sample and user to user.**

Example Calculation for a 4-plex Reaction

Cell input into Probe Hybridization:

Sample 1: 300,000, Sample 2: 300,000, Sample 3: 450,000, Sample 4: 500,000

# of cells pooled per sample: 300,000

Total # of cells pooled: 1,200,000

Desired Cell Stock Concentration: 2,000 cells/µl

Volume of Resuspension Buffer

$$= \frac{\text{Total \# of Cells Pooled x Expected Recovery Rate (70\%)}}{\text{Desired Cell Stock Concentration}}$$

$$= \frac{1{,}200{,}000 \times 0.70}{2{,}000 \text{ cells/µl}}$$

=420 µl

r. Pass the sample through a 30 µm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation Filters) into a new 1.5-ml/2-ml microcentrifuge tube and **place on ice**.

DO NOT use 40 µm Flowmi Tip Strainer for filtration.

Filtration is essential for optimal microfluidic performance. Hold the pipette tip at an angle and touch the filter membrane where the filter meets the wall. Slowly pipette through the filter. Tap gently or centrifuge briefly if liquid remains at the end of the filter. To maximize recovery, residual volume can be pipetted from underneath the filter.

TIPS *See Sample Filtration on page 38 for details.*

s. Determine cell concentration of the sample using an automated cell counter or a hemocytometer.

TIPS See Tips & Best Practices for Cell Counting on page 39. As concentrated cell suspensions are required when targeting very high cell loads, a serial dilution may be needed to accurately determine cell concentration.

t. Optional. If the sample concentration is not sufficient to achieve the desired targeted cell recovery, concentrate the sample as follows:

- Centrifuge a known volume of sample at **850 rcf** for **5 min** at **4°C**.
- Carefully remove only a fraction of the supernatant, and pipette thoroughly to resuspend the cell pellet in the remaining volume. The

amount of supernatant removed should be proportional to the desired increase in concentration.

- For example, to increase the concentration of a sample with 25,000 cells as the starting count in probe hybridization and from a starting volume of 400 µl, centrifuge, then remove 350 µl supernatant, and finally resuspend the cell pellet in the remaining 50 µl.
- Recount to confirm final concentration. The concentrated sample may need dilution before counting to avoid loss of sample and going over the limit of the counter.

STOP

u. Store the sample (see Sample Storage below) at **−80°C** for up to **12 months** or proceed **immediately** to the next step. If directly proceeding with next step, the undiluted samples can be placed on ice and then stored later after GEM Incubation. See Prepare GEM Master Mix + Sample Dilution on page 73 for details.

Sample Storage

- Add 0.1 volume Enhancer to sample in Post-Hyb Resuspension Buffer B. For example, add 50 µl Enhancer to 500 µl of sample in Post-Hyb Resuspension Buffer B.
- Add 50% glycerol (freshly prepared) for a final concentration of 10%. For example, add 137.5 µl 50% glycerol to 550 µl sample in Post-Hyb Resuspension Buffer B and Enhancer.
- Store at **−80°C** for up to **12 months**.

Using Stored Samples

- When ready to use samples stored at −80°C from this step, thaw at room temperature until no ice remains and then continue from **2.1n** (Prepare Post-Hyb Resuspension Buffer B) to wash the sample once before proceeding to the step 3.1. Samples may undergo a color change during storage (e.g. black, light gray, or green), however this will not impact assay performance.

Post-Hybridization Pooling Calculation

This section provides calculation for pooling an equal number of cells from each sample. These calculations can be used at steps 2.1d and 2.1e.

- **Count cells and determine Post-Hybridization Cell Concentrations**
- **Calculate Total Cells in Hybridization**

  Total Cells in Hybridization = Post-Hybridization Cell Conc. * Sample Volume
- **Calculate Cells per Sample Added to the Pool**

  Cells per Sample Added to the Pool = Cell count of sample with lowest Total Cells in Hybridization
- **Calculate Sample Volume to be Added for Each Sample**

  $$\text{Sample Volume to be Added for Each Sample} = \frac{\text{Cells per Sample Added to the Pool}}{\text{Post-Hybridization Cell Conc.}}$$

Example Calculation When Pre-hybridization Counts ≥50,000 cells

For pooling an equal number of cells from each of 4 samples

Probe hybridization in 8-tube strips - Four fixed samples hybridized with unique Probe Barcodes, diluted, and counted post-hybridization

Sample Volume = 50 µl Probe hyb rxn + 225 µl Post-Hyb Buffer B - 10 µl for counting

Probe Barcode	Post-Hybridization Cell Conc.	Total Cells in Hybridization	Cells per Sample Added to the Pool	Sample Volume to be Added (µl)
	Cells in 265 µl Post-Hyb Wash Buffer B	Post-Hybridization Cell Conc. * 265	Cell Count of Sample with Lowest Concentration	Cells per Sample Added to the Pool/Post-Hybridization Cell Conc.
BC001	1,200 cells/µl	318,000	238,500	238,500/1,200 = 198.75
BC002	1,600 cells/µl	424,000	238,500	238,500/1,600 = 149.06
BC003	900 cells/µl	238,500	238,500	238,500/900 = 265.0
BC004	1,900 cells/µl	503,500	238,500	238,500/1,900 = 125.52

Probe hybridization in 1.5-ml microcentrifuge tubes - Four fixed samples hybridized with unique Probe Barcodes, diluted, and counted post-hybridization

Sample Volume = 50 µl Probe hyb rxn + 400 µl Post-Hyb Buffer B - 10 µl for counting

Probe Barcode	Post-Hybridization Cell Conc.	Total Cells in Hybridization	Cells per Sample Added to the Pool	Sample Volume to be Added (µl)
	Cells in 440 µl Post-Hyb Wash Buffer	Post-Hybridization Cell Conc. * 440	Cell Count of Sample with Lowest Concentration	Cells per Sample Added to the Pool/Post-Hybridization Cell Conc.
BC001	388 cells/µl	170,720	128,040	128,040/388 = 330
BC002	744 cells/µl	288,672	128,040	128,040/744 = 172.1
BC003	291 cells/µl	128,040	128,040	128,040/291 = 440
BC004	905 cells/µl	398,200	128,040	128,040/905 = 141.5

Option 2.1 B: Individual Wash Workflow

TIPS

If Individual Wash workflow is chosen, the Supplemental Wash Kit (PN-1000828) is required with the following human/mouse reagent bundles: 4-plex 16 samples (1000793/1000797), 16-plex 64 samples (1000794/1000798), and 16-plex 256 samples (1000795/1000799). This workflow will allow an increased flexibility to store samples after washing and later change how samples are pooled before loading the chip.

See Post-Hybridization Pooling & Washing Guidance on page 35 before starting.

During the centrifugation steps, up to 30 µl supernatant may be left behind if working with <300,000 cells. See Post-Hybridization Sample Washing & Recovery on page 36 for more details. If uncertain about the volume remaining in the tube, add 30 µl water/PBS to an empty tube of the same size and use it for a visual estimation.

a. Prepare Post-Hyb Wash Buffer B. Pipette mix 10x and keep at **room temperature**. DO NOT keep at 4°C.

Post-Hyb Wash Buffer B *Add reagents in the order listed*	PN	1X* + 10% (ml)	4X* + 10% (ml)	8X* + 10% (ml)	16X* + 10% (ml)
Nuclease-free Water	-	1.98	7.92	15.84	31.68
Conc. Post-Hyb Buffer B	2001308	0.11	0.44	0.88	1.76
Enhancer	2000482	0.11	0.44	0.88	1.76
Total	**-**	**2.2**	**8.8**	**17.6**	**35.2**

*X represents the number of samples, for example, 4X = 4 samples

b. Remove tubes from thermal cycler (8-tube strips) or thermomixer (1.5-ml microcentrifuge tubes) after overnight incubation.

c. Add **900 µl** Post-Hyb Wash Buffer B to each sample. Pipette mix 5x.

If the hybridization was performed in 8-tube strips, add 175 µl buffer to the sample, gently pipette mix, and transfer to a 1.5-ml microcentrifuge tube. Wash the tube strips with additional buffer, transfer to the microcentrifuge tube, and add the remaining volume of buffer for a total of 900 µl Post-Hyb Wash Buffer B to the sample.

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

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

f. Remove the supernatant without disturbing the pellet.

TIPS

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

See Post-Hybridization Sample Washing & Recovery on page 36 for more details.

g. Resuspend each cell pellet in **0.5 ml room temperature** Post-Hyb Wash Buffer B. Pipette mix 5x.

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

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

j. Remove the supernatant without disturbing the pellet.

TIPS

Up to 30 µl supernatant may be left behind if working with <300,000 cells. See Post-Hybridization Sample Washing & Recovery on page 36 for more details.

k. Resuspend each cell pellet in **0.5 ml room temperature** Post-Hyb Wash Buffer B. Pipette mix 5x.

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

m. Prepare Post-Hyb Resuspension Buffer B. Pipette mix 10x and maintain at **4°C**.

Post-Hyb Resuspension Buffer B *Add reagents in the order listed*	PN	1X* + 10% (µl)	4X* + 10% (µl)	8X* + 10% (µl)	16X* + 10% (µl)
Nuclease-free Water	-	522.5	2090.0	4180.0	8360.0
Conc. Post-Hyb Buffer B	2001308	27.5	110.0	220.0	440.0
Total	**-**	**550.0**	**2200.0**	**4400.0**	**8800.0**

*X represents the number of samples, for example, 4X = 4 samples

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

o. Remove the supernatant without disturbing the pellet.

TIPS

Up to 30 µl supernatant may be left behind if working with <300,000 cells. See Post-Hybridization Sample Washing & Recovery on page 36 for more details.

p. Resuspend cell pellet in an appropriate volume of **chilled** Post-Hyb Resuspension Buffer B. Pipette mix 20x. Maintain on ice.

The buffer volume will depend on the targeted cell recovery and the cell stock concentration.

When targeting maximum cell recovery (20,000 cells/Probe Barcode) - resuspend in **250 µl** buffer.

When not targeting maximum cell recovery - follow the steps below to determine the appropriate volume.

- See Cell Suspension Volume Calculator for Multiplexing 4 Samples on page 74 to determine the desired cell stock concentration based on targeted cell recovery.
- Calculate the volume of resuspension buffer using the cells input into probe hybridization, an estimated ~70% cell recovery* during the washing step, and the desired cell stock concentration. See example calculation on the next page.

***70% is a conservative estimate for the number of cells recovered during the Post-hybridization Wash. Cell loss will vary from sample to sample and user to user.**

Example Calculation

Volume of Resuspension Buffer

$$= \frac{\text{Cell Input into Probe Hybridization} \times \text{Expected Recovery Rate}}{\text{Desired Cell Stock Concentration}}$$

Cell Input: 250,000

Desired Cell Stock Concentration: 500 cells/µl

$$= \frac{250{,}000 \times 0.70}{500 \text{ cells/µl}}$$

=350 µl

q. Pass each sample through a 30 µm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation Filters) into a new 1.5-ml microcentrifuge tube.

Filtration is essential for optimal microfluidic performance. Hold the pipette tip at an angle and touch the filter membrane where the filter meets the wall. Slowly pipette through the filter. Tap gently or centrifuge briefly if liquid remains at the end of the filter. To maximize recovery, residual volume can be pipetted from underneath the filter.

TIPS *See Sample Filtration on page 38 for details.*

r. Determine cell concentration of each sample using an automated cell counter or a hemocytometer. See Cell Counting on page 39 for details on supported cell counters.

STOP **s.** Store the sample after resuspending in appropriate reagents (see Sample Storage on the next page) or **immediately** proceed to next step.

t. Pool an equal number of cells from different hybridization reactions into a 5-ml (for pooling 4 samples) or 15-ml (for pooling 4, 8, or 16 samples) centrifuge tube. See table below on how to calculate the volume of each sample to be added.

Example calculation for pooling an equal number of cells from each of 4 samples

Four fixed samples hybridized with unique probes for multiplexing, washed, and counted post-hybridization wash

Probe Barcode	Post-Hybridization Cell Conc.	Total Cells in Sample	Cells per Sample Added to the Pool	Sample Volume to be Added (µl)
	Cells in 490 µl Post-Hyb Resuspension Buffer	Post-Hybridization Cell Conc. * 490	Cell Count of Sample with Lowest Concentration	Cells per Sample Added to the Pool/Post-Hybridization Cell Conc.)
BC001	660 cells/µl	323,400	242,550	242,550/660 = 367.5
BC002	815 cells/µl	399,350	242,550	242,550/815 = 297.6
BC003	495 cells/µl	242,550	242,550	242,550/495 = 490.0
BC004	995 cells/µl	487,550	242,550	242,550/995 = 243.7

Sample Volume = Sample in 500 µl Post-Hyb Resuspension Buffer B - 10 µl for counting

u. Determine cell concentration using an automated cell counter or a hemocytometer. See Tips & Best Practices for Cell Counting on page 39.

v. Optional. If the sample concentration is not sufficient to achieve the desired target cells recovery, concentrate the sample as follows:

- Centrifuge a known sample volume at **850 rcf** for **5 min** at **4°C**.
- Carefully remove only a fraction of the supernatant, and pipette thoroughly to resuspend the cell pellet in the remaining volume. The amount of supernatant removed should be proportional to the desired increase in concentration. For example, to increase the concentration 4-fold from a starting volume of 400 µl, centrifuge, then remove 300 µl supernatant, and finally resuspend the cell pellet in the remaining 100 µl (400/100 = 4).
- Recount to confirm final concentration. The concentrated sample may need dilution before counting to avoid loss of sample and going over the limit of the counter.

w. Proceed **immediately** to Prepare GEM Master Mix + Sample Dilution on page 73.

Sample Storage

- Add 0.1 volume Enhancer to sample in Post-Hyb Resuspension Buffer B. For example, add 50 µl Enhancer to 500 µl of sample in Post-Hyb Resuspension Buffer B.
- Add 50% glycerol (freshly prepared) for a final concentration of 10%. For example, add 137.5 µl 50% Glycerol to 550 µl of sample in Post-Hyb Resuspension Buffer B and Enhancer.
- Store at **–80°C** for up to **12 months**.

Using Stored Samples

- When ready to use samples stored at −80°C from this step, thaw at room temperature until no ice remains and then continue from step m of this section.

10x GENOMICS

Step 3:

GEM Generation and Barcoding

3.0 Get Started

Firmware Version 2.0 or higher is required in the Chromium X/iX used for this GEM-X Flex protocol.

Item		10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐	Single Cell TL v1 Gel Beads	2000538	Equilibrate to room temperature 30 min before loading the chip.	–80°C
☐	Reducing Agent B	2000087	Vortex, verify no precipitate, centrifuge briefly.	–20°C
Thaw & Keep Warm				
☐	Enhancer	2000482	Thaw 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.	–20°C
Place on Ice				
☐	GEM Enzyme Mix B	2001302	Centrifuge briefly before adding to the mix.	–20°C
☐	GEM Reagent Mix	2000491	Thaw at room temperature. Vortex, verify no precipitate, centrifuge briefly. Keep on ice.	–20°C
☐	Post-Hyb Resuspension Buffer B *Use the Post-Hyb Resuspension Buffer B prepared at the previous step (2.1) for sample dilution.*	—	Additional buffer can be prepared using the buffer preparation table in step 2.1.	4°C
Obtain				
☐	Partitioning Oil B	2001213	—	Ambient
☐	GEM-X FX Chip	2001257	See Tips & Best Practices.	Ambient
☐	Chromium X Series Chip Holder	3000598	See Tips & Best Practices.	Ambient

Item		10x PN	Preparation & Handling	Storage
☐	X/iX Chip Gasket	3000656	See Tips & Best Practices.	Ambient
☐	10x Vortex Adapter	330002	See Tips & Best Practices.	Ambient
☐	50% glycerol solution *For adding to unused wells*	—	See Tips & Best Practices.	
☐	Glycerol for molecular biology, ≥99% *Prepare fresh 50% glycerol solution for sample storage*	—	See Tips & Best Practices.	—

3.1 Prepare GEM Master Mix + Sample Dilution

Before preparing GEM Master Mix, ensure that the Gel Beads are properly thawed and ready to use.

a. Prepare Master Mix on ice. Pipette mix 15x and centrifuge briefly.

GEM Master Mix *Add reagents in the order listed*		PN	1X* + 10% (µl)	4X* + 10% (µl)
●	GEM Reagent Mix	2000491	19.9	79.8
○	Reducing Agent B	2000087	1.6	6.4
●	GEM Enzyme Mix B	2001302	11.8	47.3
	Total	–	**33.3**	**133.5**

*1X = 1 well/GEM reaction, 4X = 4 wells/GEM reactions

b. Add the appropriate volume of Post-Hyb Resuspension Buffer B* to the appropriate volume of sample into each tube of a PCR 8-tube strip on ice. Refer to the Cell Suspension Volume Calculator on the next page for the volumes.

*Use the Post-Hyb Resuspension Buffer B prepared at the previous step (2.1) for sample dilution. Additional buffer can be prepared using the buffer preparation table in step 2.1.

Place remaining undiluted sample on ice. These samples can be stored later after GEM incubation. Guidelines for storage of remaining samples are provided in step 3.5 *GEM Incubation on page 83*.

c. Add **30.3 µl** of prepared GEM Master Mix into each tube containing diluted sample and **immediately** proceed to the next step.

Pipette mixing at this step is not required, and will be performed prior to loading into the chip.

Cell Suspension Volume Calculator for Multiplexing 4 Samples

Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery								
	2000	4000	8000	16000	32000	40000	56000	64000	80000
2000	1.5	2.9	5.8	11.6	23.2	29.0			
	33.2	31.8	28.9	23.1	11.5	5.7			
2500	1.2	2.3	4.6	9.3	18.6	23.2	32.5		
	33.5	32.4	30.1	25.4	16.1	11.5	2.2		
3000	1.0	1.9	3.9	7.7	15.5	19.3	27.1	30.9	
	33.7	32.8	30.8	27.0	19.2	15.4	7.6	3.8	
3500	0.8	1.7	3.3	6.6	13.3	16.6	23.2	26.5	33.1
	33.9	33.0	31.4	28.1	21.4	18.1	11.5	8.2	1.6
4000	0.7	1.5	2.9	5.8	11.6	14.5	20.3	23.2	29.0
	34.0	33.2	31.8	28.9	23.1	20.2	14.4	11.5	5.7
4500	0.6	1.3	2.6	5.2	10.3	12.9	18.0	20.6	25.8
	34.1	33.4	32.1	29.5	24.4	21.8	16.7	14.1	8.9
5000	0.6	1.2	2.3	4.6	9.3	11.6	16.2	18.6	23.2
	34.1	33.5	32.4	30.1	25.4	23.1	18.5	16.1	11.5
5500	0.5	1.1	2.1	4.2	8.4	10.5	14.8	16.9	21.1
	34.2	33.6	32.6	30.5	26.3	24.2	19.9	17.8	13.6
6000	0.5	1.0	1.9	3.9	7.7	9.7	13.5	15.5	19.3
	34.2	33.7	32.8	30.8	27.0	25.0	21.2	19.2	15.4
6500	0.4	0.9	1.8	3.6	7.1	8.9	12.5	14.3	17.8
	34.3	33.8	32.9	31.1	27.6	25.8	22.2	20.4	16.9
7000	0.4	0.8	1.7	3.3	6.6	8.3	11.6	13.3	16.6
	34.3	33.9	33.0	31.4	28.1	26.4	23.1	21.4	18.1
7500	0.4	0.8	1.5	3.1	6.2	7.7	10.8	12.4	15.5
	34.3	33.9	33.2	31.6	28.5	27.0	23.9	22.3	19.2
8000	0.4	0.7	1.5	2.9	5.8	7.3	10.2	11.6	14.5
	34.3	34.0	33.2	31.8	28.9	27.4	24.5	23.1	20.2
8500	0.3	0.7	1.4	2.7	5.5	6.8	9.6	10.9	13.6
	34.4	34.0	33.3	32.0	29.2	27.9	25.1	23.8	21.1
9000	0.3	0.6	1.3	2.6	5.2	6.4	9.0	10.3	12.9
	34.4	34.1	33.4	32.1	29.5	28.3	25.7	24.4	21.8
9500	0.3	0.6	1.2	2.4	4.9	6.1	8.5	9.8	12.2
	34.4	34.1	33.5	32.3	29.8	28.6	26.2	24.9	22.5
10000	0.3	0.6	1.2	2.3	4.6	5.8	8.1	9.3	11.6
	34.4	34.1	33.5	32.4	30.1	28.9	26.6	25.4	23.1
Yellow boxes	Indicate a low transfer volume that may result in higher cell load variability								

Cell Suspension Volume Calculator for Multiplexing 8 Samples

Volume of Cell Suspension Stock per reaction (µl) | Volume of Post-Hyb Resuspension Buffer B per reaction (µl)

Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery								
	4000	8000	16000	32000	64000	80000	96000	120000	160000
2000	2.9	5.8	11.6	23.2					
	31.8	28.9	23.1	11.5					
2500	2.3	4.6	9.3	18.6					
	32.4	30.1	25.4	16.1					
3000	1.9	3.9	7.7	15.5	30.9				
	32.8	30.8	27.0	19.2	3.8				
3500	1.7	3.3	6.6	13.3	26.5	33.1			
	33.0	31.4	28.1	21.4	8.2	1.6			
4000	1.5	2.9	5.8	11.6	23.2	29.0			
	33.2	31.8	28.9	23.1	11.5	5.7			
4500	1.3	2.6	5.2	10.3	20.6	25.8	30.9		
	33.4	32.1	29.5	24.4	14.1	8.9	3.8		
5000	1.2	2.3	4.6	9.3	18.6	23.2	27.8		
	33.5	32.4	30.1	25.4	16.1	11.5	6.9		
5500	1.1	2.1	4.2	8.4	16.9	21.1	25.3	31.6	
	33.6	32.6	30.5	26.3	17.8	13.6	9.4	3.1	
6000	1.0	1.9	3.9	7.7	15.5	19.3	23.2	29.0	
	33.7	32.8	30.8	27.0	19.2	15.4	11.5	5.7	
6500	0.9	1.8	3.6	7.1	14.3	17.8	21.4	26.8	
	33.8	32.9	31.1	27.6	20.4	16.9	13.3	7.9	
7000	0.8	1.7	3.3	6.6	13.3	16.6	19.9	24.9	33.1
	33.9	33.0	31.4	28.1	21.4	18.1	14.8	9.8	1.6
7500	0.8	1.5	3.1	6.2	12.4	15.5	18.6	23.2	30.9
	33.9	33.2	31.6	28.5	22.3	19.2	16.1	11.5	3.8
8000	0.7	1.5	2.9	5.8	11.6	14.5	17.4	21.8	29.0
	34.0	33.2	31.8	28.9	23.1	20.2	17.3	12.9	5.7
8500	0.7	1.4	2.7	5.5	10.9	13.6	16.4	20.5	27.3
	34.0	33.3	32.0	29.2	23.8	21.1	18.3	14.2	7.4
9000	0.6	1.3	2.6	5.2	10.3	12.9	15.5	19.3	25.8
	34.1	33.4	32.1	29.5	24.4	21.8	19.2	15.4	8.9
9500	0.6	1.2	2.4	4.9	9.8	12.2	14.7	18.3	24.4
	34.1	33.5	32.3	29.8	24.9	22.5	20.0	16.4	10.3
10000	0.6	1.2	2.3	4.6	9.3	11.6	13.9	17.4	23.2
	34.1	33.5	32.4	30.1	25.4	23.1	20.8	17.3	11.5
Yellow boxes	Indicate a low transfer volume that may result in higher cell load variability								

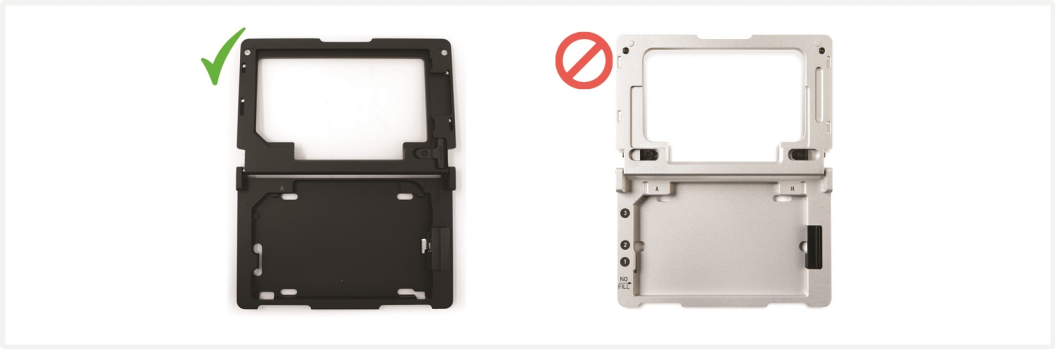
Cell Suspension Volume Calculator for Multiplexing 16 Samples

Volume of Cell Suspension Stock per reaction (µl) | Volume of Post-Hyb Resuspension Buffer per reaction (µl)

Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery								
	8000	16000	32000	48000	96000	128000	192000	256000	320000
2000	5.8	11.6	23.2						
	28.9	23.1	11.5						
3000	3.9	7.7	15.5	23.2					
	30.8	27.0	19.2	11.5					
4000	2.9	5.8	11.6	17.4					
	31.8	28.9	23.1	17.3					
5000	2.3	4.6	9.3	13.9	27.8				
	32.4	30.1	25.4	20.8	6.9				
6000	1.9	3.9	7.7	11.6	23.2	30.9			
	32.8	30.8	27.0	23.1	11.5	3.8			
7000	1.7	3.3	6.6	9.9	19.9	26.5			
	33.0	31.4	28.1	24.8	14.8	8.2			
8000	1.5	2.9	5.8	8.7	17.4	23.2			
	33.2	31.8	28.9	26.0	17.3	11.5			
9000	1.3	2.6	5.2	7.7	15.5	20.6	30.9		
	33.4	32.1	29.5	27.0	19.2	14.1	3.8		
10000	1.2	2.3	4.6	7.0	13.9	18.6	27.8		
	33.5	32.4	30.1	27.7	20.8	16.1	6.9		
11000	1.1	2.1	4.2	6.3	12.7	16.9	25.3	33.7	
	33.6	32.6	30.5	28.4	22.0	17.8	9.4	1.0	
12000	1.0	1.9	3.9	5.8	11.6	15.5	23.2	30.9	
	33.7	32.8	30.8	28.9	23.1	19.2	11.5	3.8	
13000	0.9	1.8	3.6	5.4	10.7	14.3	21.4	28.6	
	33.8	32.9	31.1	29.3	24.0	20.4	13.3	6.1	
14000	0.8	1.7	3.3	5.0	9.9	13.3	19.9	26.5	33.1
	33.9	33.0	31.4	29.7	24.8	21.4	14.8	8.2	1.6
15000	0.8	1.5	3.1	4.6	9.3	12.4	18.6	24.7	30.9
	33.9	33.2	31.6	30.1	25.4	22.3	16.1	10.0	3.8
16000	0.7	1.5	2.9	4.4	8.7	11.6	17.4	23.2	29.0
	34.0	33.2	31.8	30.3	26.0	23.1	17.3	11.5	5.7
17000	0.7	1.4	2.7	4.1	8.2	10.9	16.4	21.8	27.3
	34.0	33.3	32.0	30.6	26.5	23.8	18.3	12.9	7.4
18000	0.6	1.3	2.6	3.9	7.7	10.3	15.5	20.6	25.8
	34.1	33.4	32.1	30.8	27.0	24.4	19.2	14.1	8.9
Yellow boxes	Indicate a low transfer volume that may result in higher cell load variability								

Assemble GEM-X FX Chip

GEM-X FX Chip is only compatible with Chromium X Series Chip Holder (PN-3000598). DO NOT use any other holder.



See Tips & Best Practices on page 31 for chip handling instructions.

a. Close the holder lid. Attach the gasket by holding the tongue (curved end, to the right) and hook the gasket on the left-hand tabs of the holder. Gently pull the gasket toward the right and hook it on the two right-hand tabs.

b. DO NOT touch the smooth side of the gasket.

c. Open the chip holder.

d. Remove the chip from the sealed bag. Use the chip within ≤ 24 h.

e. Align notch on the chip (upper left corner) and the open holder with the gasket attached.

f. Slide the chip to the left until the chip is inserted under the guide on the holder. Depress the right hand side of the chip until the spring-loaded clip engages.

g. Keep the assembled unit with the attached gasket open until ready for and while dispensing reagents into the wells.

h. DO NOT touch the smooth side of the gasket.

i. The assembled chip is ready for loading the indicated reagents. See Load GEM-X Chip on page 79 for reagent volumes and loading order.

j. After loading reagents, close the chip holder. DO NOT press down on the top of the gasket.

For GEM generation, load the indicated reagents only in the specified rows, starting from row labeled 1, followed by rows labeled 2 & 3.

DO NOT load reagents in the top row labeled NO FILL.

Representative chip image

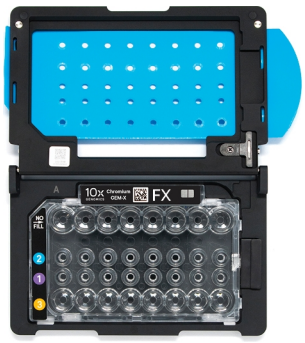
3.2 Load GEM-X Chip

TIPS

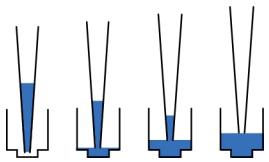
- After removing chip from the sealed bag, use in **≤24 h**.
- Open the lid (gasket attached) of the assembled chip and lay flat for loading.
- Ensure that the Gel Beads are properly thawed and ready to use.
- When loading the chip, raising and depressing the pipette plunger should each take **~5 sec**. When dispensing, raise the pipette tips at the same rate as the liquid is rising, keeping the tips slightly submerged.

Color Legend
2: Gel beads
1: Sample
3: Oil

GEM-X FX Chip, gasket attached
Representative chip image



Pipette technique

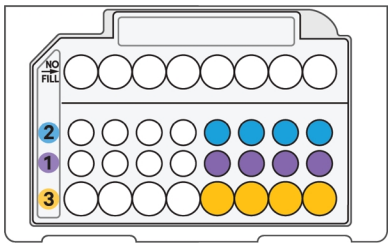


The Chromium X Series Chip Holder, Chip Gasket X/iX, and GEM-X chip images shown below are representative and do not show the specific color & label. Chip holder and gasket should be black and blue in color, respectively. Refer to Assemble GEM-X FX Chip on page 77 for details.

a. If loading less than 8 samples/chip, add 50% glycerol solution to each unused well in row 1, 2, and 3

- **60 µl** in each unused well in row labeled 1
- **60 µl** in each unused well in row labeled 2
- **250 µl** in each unused well in row labeled 3
  by pipetting two aliquots of 125 µl each.

Glycerol
in GEM-X FX Chip

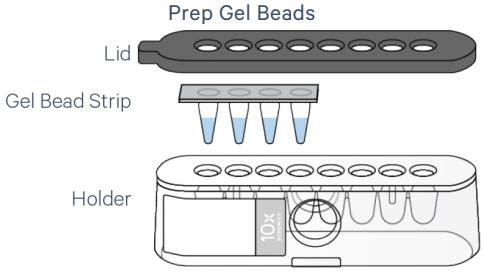


DO NOT add 50% glycerol solution to the wells in top row labeled NO FILL.
DO NOT use any substitute for 50% glycerol solution.

b. Prepare Gel Beads

- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**.
- Centrifuge the Gel Bead strip for **~5 sec**.
- Confirm there are no bubbles at the bottom of the tubes & the liquid levels are even.
- Place the Gel Bead strip back in the holder. Secure the holder lid.

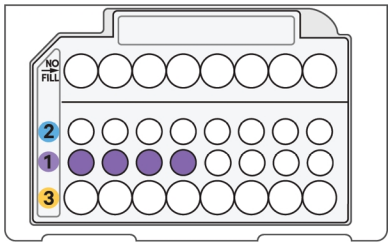
Prep Gel Beads



c. Load Row Labeled 1

- With pipette set to 60 µl, gently pipette mix the GEM Master Mix + Sample 15x.
- Using the same pipette tip, dispense **60 µl** Master Mix + Cell Suspension into the bottom center of each well in **row labeled 1** without introducing bubbles.
- Wait **30 sec**.

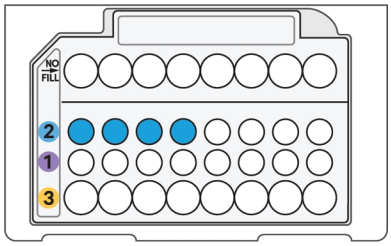
60 µl Master Mix + Cell Suspension in GEM-X FX Chip



d. Load Row Labeled 2

- Puncture the foil seal of the Gel Bead tubes.
- Slowly aspirate **60 µl** Gel Beads.
- Dispense into the bottom center of each well in **row labeled 2** without introducing bubbles.
- Wait **30 sec**.

60 µl Gel Beads in GEM-X FX Chip

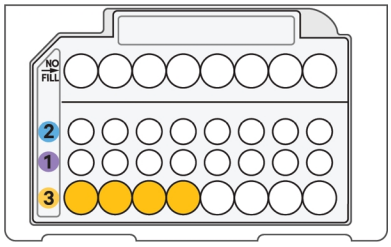


e. Load Row Labeled 3

- Dispense **250 µl** Partitioning Oil B into the wells in **row labeled 3** by pipetting two aliquots of **125 µl** from a reagent reservoir.

Failure to add Partitioning Oil B to the row labeled 3 will prevent GEM generation and can damage the instrument.

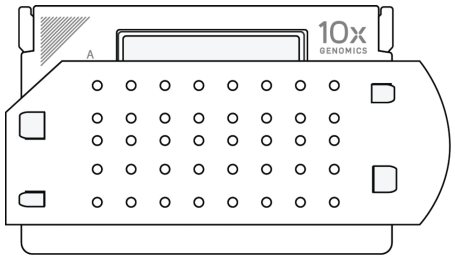
250 µl Partitioning Oil B in GEM-X FX Chip



f. Prepare for Run

- Close the lid (gasket already attached). DO NOT touch the smooth side of the gasket. DO NOT press down on the top of the gasket.
- Keep the chip horizontal and be careful when moving/setting down the chip to avoid wetting the gasket with oil or spilling oil over the outside of the wells.*

GEM-X FX Chip, closed



*Run the chip in Chromium X/iX **immediately** after loading the Partitioning Oil B.*

*If the chip was tilted, oil spillage may appear as fluid between the chip and surface of the chip holder. It is recommended to proceed with the run in such cases. If the recovered emulsion volume appears normal, proceed with the rest of the assay. If enough oil is spilled out of the well, it can result in <100 µl recovered emulsion volume.

3.3 Run the Chromium X/iX

Consult the Chromium X Series Instrument User Guide with Readiness Test (CG000396) for detailed instrument operation instructions and follow the instrument touchscreen prompts for execution. Run time for GEM-X FX Chip is ~6 min.

a. Press the eject button on the Chromium X/iX to eject the tray. If the eject button is not touched within **1 min**, tray will close automatically. System requires a few seconds before the tray can be ejected again.

b. Place the assembled chip with the gasket in the tray, ensuring that the chip stays horizontal. Press the button to retract the tray.

c. Confirm GEM-X FX program on screen. Press the play button.

d. At completion of the run **(~6 min)**, Chromium X/iX will chime. **Immediately** proceed to the next step.

Run Chromium X/iX

3.4 Transfer GEMs

a. Place a tube strip on ice.

b. Press the eject button of the Chromium X/iX and remove the chip.

c. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees. Be careful when opening the chip holder at 45 degrees to avoid wetting the gasket with oil or spilling oil.

d. Visually compare the remaining volume in rows labeled 1-2. Abnormally high volume in one well relative to other wells may indicate a clog.

Take a picture of the GEMs in the pipette tips and/or tube strips.

e. **Slowly** aspirate **100 µl** GEMs from the lowest points of the recovery wells in the top NO FILL row without creating a seal between the tips and the bottom of the wells.

f. Withdraw pipette tips from the wells. GEMs should appear opaque and uniform across all channels. Excess Partitioning Oil (clear) in the pipette tips indicates a potential clog.

g. Over the course of **~20 sec**, dispense GEMs into the tube strip on ice with the pipette tips against the sidewalls of the tubes.

h. It is recommended to start GEM incubation within **5 min**. If multiple chips are run back-to-back, place the GEMs from the subsequent chips in separate thermal cyclers.

Representative chip image

3.5 GEM Incubation

Use a thermal cycler that can accommodate at least 100 µl volume. A volume corresponding to the maximum allowable amount, for a given supported thermal cycler, is the preferred setting. For example a volume of 100 µl is the preferred setting on Bio-Rad C1000 Touch. In alternate thermal cyclers, use highest reaction volume setting.

a. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
80°C	100 µl	~125 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	25°C	00:60:00
2	60°C	00:45:00
3	80°C	00:20:00
Hold	4°C	Hold

b. Store at **4°C** for up to **a week**, or proceed to the next step.

DO NOT store the GEMs at −20°C.

c. Undiluted sample placed on ice at step 3.1 Prepare GEM Master Mix + Sample Dilution on page 73 can either be discarded or stored at **−80°C** for up to **12 months**. See Sample Storage below:

Sample Storage of Undiluted Sample

- Add 0.1 volume Enhancer to sample in Post-Hyb Resuspension Buffer B. For example, add 50 µl Enhancer to 500 µl of sample in Post-Hyb Resuspension Buffer B.
- Add 50% glycerol (freshly prepared) for a final concentration of 10%. For example, add 137.5 µl 50% glycerol to 550 µl sample in Post-Hyb Resuspension Buffer B and Enhancer.
- Store at **−80°C** for up to **12 months**.

  When ready to use samples stored at −80°C, thaw at room temperature until no ice remains. Then continue from step 2.1A-n (for Pooled Wash workflow) or 2.1B-m (for Individual Wash workflow) of Post-Hybridization Wash step to wash the sample once before proceeding to the step 3.1. Samples may undergo a color change during storage (e.g. black, light gray, or green), however this will not impact assay performance.

10x GENOMICS

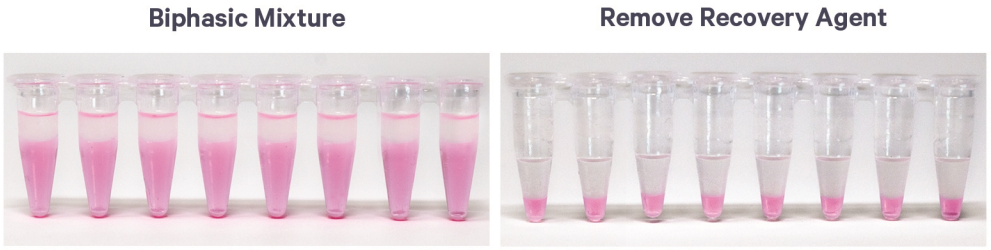
Step 4:

GEM Recovery and Pre-Amplification

4.0 Get Started

Item			10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
☐	◯	Reducing Agent B	2000087	Thaw, vortex, verify no precipitate, centrifuge briefly.	−20°C
☐		Pre-Amp Primers C *Verify name & PN* *Retrieve from Flex Feature Barcode Kit, 64 rxns, PN-1000628.*	2000953	Thaw, vortex, centrifuge briefly.	−20°C
☐		Beckman Coulter SPRIselect Reagent	—	Manufacturer's recommendations.	Ambient
Place on Ice					
☐	◯	Amp Mix C	2001311	Vortex and centrifuge briefly.	−20°C
Obtain					
☐	◯	Recovery Agent	220016	—	Ambient
☐		Qiagen Buffer EB	—	Manufacturer's recommendations.	Ambient
☐		10% Tween 20	—	Manufacturer's recommendations.	Ambient
☐		10x Magnetic Separator/10x Magnetic Separator B	230003/ 2001212	—	Ambient
☐		Prepare 80% Ethanol *Prepare 2.5 ml for 4 GEM reactions.*	—	Prepare fresh.	—

4.1 Post-GEM Incubation – Recovery



Only 4 reactions can be run per kit. Image shows 8 reactions derived from two kits.

a. Add **125 µl** Recovery Agent to each sample at **room temperature**. DO NOT pipette mix or vortex the biphasic mixture.

b. Firmly secure the cap on the tube strip, ensuring that no liquid is trapped between the cap and the tube rim. Mix by inverting the capped tube strip 5x.

DO NOT invert without firmly securing the caps.

c. Wait **2 min**.

The resulting biphasic mixture contains Recovery Agent/Partitioning Oil (pink) and aqueous phase (translucent/opaque).

A smaller aqueous phase volume indicates a clog during GEM generation.

d. Centrifuge briefly.

e. Slowly remove and discard **125 µl** Recovery Agent/Partitioning Oil (pink) from the bottom of the tube. DO NOT aspirate any aqueous sample.

f. Proceed directly to Pre-Amplification PCR. No cleanup step is required.

4.2 Pre-Amplification PCR

a. Prepare Pre-Amplification Mix on ice. Vortex and centrifuge briefly.

Pre-Amplification Mix *Add reagents in the order listed*		PN	1X (µl)	4X + 10% (µl)
○	**Amp Mix C**	2001311	25.0	110.0
●	**Pre-Amp Primers C** Verify name & PN ⚠	2000953	10.0	44.0
	Total		**35.0**	**154.0**

b. Add **35 µl** Pre-Amplification Mix to aqueous sample from the previous step.

c. Cap firmly and invert 8x to mix. Centrifuge briefly.

d. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~30-45 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:03:00
2	98°C	00:00:15
3	63°C	00:00:20
4	72°C	00:01:00
5	Go to Step 2, 7x (total 8 cycles)	
6	72°C	00:01:00
7	4°C	Hold

STOP **e.** Store at **4°C** for up to **72 h** or **–20°C** for **≤1 week**, or proceed to the next step.

4.3 DNA Cleanup – SPRIselect

a. Prepare Elution Solution. Vortex and centrifuge briefly.

Elution Solution *Add reagents in the order listed*		PN	1000 µl
	Buffer EB		980
	10% Tween 20	-	10
◯	**Reducing Agent B**	2000087	10
	Total		**1000**

b. Centrifuge the sample (PCR product) for 30 sec in a microcentrifuge and transfer **70 µl** of the upper layer to a new tube.

Presence of a cloudy precipitate at the interface between phases is normal. Avoid transferring the precipitate when transferring 70 µl at this step.

c. Vortex to resuspend the SPRIselect reagent. Add **126 µl** SPRIselect reagent **(1.8X)** to each sample and pipette mix 15x (pipette set to 180 µl).

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

e. Place on the magnet•**High** until the solution clears.

f. Remove the supernatant. DO NOT discard any beads.

g. With the tube still in the magnet, add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.

h. Remove the ethanol.

i. **Repeat** steps g and h for a total of 2 washes.

j. Centrifuge briefly and place on the magnet•**Low**.

k. Remove any remaining ethanol. DO NOT let the sample dry to ensure maximum elution efficiency.

l. Remove from the magnet. Add **101 µl** Elution Solution. Wait **1 min** before resuspending. Pipette mix 15x.

m. Incubate **2 min** at **room temperature**.

n. Place the tube strip on the magnet•**High** until the solution clears.

o. Transfer **100 µl** sample to a new tube strip.

STOP **p.** Store at **4°C** for **≤72 h** or at **−20°C** for **≤4 weeks**, or proceed to the next step.

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Step 5:

GEM-X Flex – Gene Expression Library Construction

5.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
□	Dual Index Plate TS Set A *Verify name & PN. Use indicated plate only*	3000511	Vortex and centrifuge briefly.	−20°C
□	Beckman Coulter SPRIselect Reagent	—	Manufacturer's recommendations.	—
□	Agilent Bioanalyzer High Sensitivity Kit *If used for QC*	—	Manufacturer's recommendations.	—
□	Agilent TapeStation ScreenTape & Reagents *If used for QC*	—	Manufacturer's recommendations.	—
Place on Ice				
□	Amp Mix C	2001311	Vortex and centrifuge briefly.	−20°C
□	Pre-Amp Primers B *Verify name & PN* *Needed for optional Cycle Number Determination using qPCR*	2000529	Thaw, vortex, centrifuge briefly.	−20°C
□	KAPA Library Quantification Kit for Illumina Platforms	—	Manufacturer's recommendations.	—
Obtain				
□	Qiagen Buffer EB	—	Manufacturer's recommendations.	Ambient
□	EvaGreen Plus Dye, 20X in Water *Needed for optional Cycle Number Determination using qPCR*	—	Manufacturer's recommendations.	Ambient
□	10x Magnetic Separator/ 10x Magnetic Separator B	230003/ 2001212	See Tips & Best Practices.	Ambient
□	Prepare 80% Ethanol *Prepare 2.5 ml for 4 GEM reactions.*	—	Prepare fresh.	Ambient

5.1 Sample Index PCR

a. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x sample index name (PN-3000511 Dual Index Plate TS Set A well ID) used.

b. Determine sample index PCR cycle numbers using one of the following methods (See Sample Index PCR Cycles on page 47 for guidance on which method to use):

Using qPCR: See Optional: Gene Expression Library Cycle Number Determination Using qPCR on page 116

Using Targeted cell recovery: Use the table below for guidance on cycle numbers.

Total Cycles*

Targeted Cell Recovery	Cell Lines	PBMCs
500-2,000	11	15
2,000-4,000	10	14
4,000-7,000	9	13
7,000-12,000	8	12
12,000-25,000	7	11
25,000-50,000	6	10
50,000-128,000	5	9
128,000-250,00	4	8
250,000-320,000	3	7

**Cycle number optimization may be needed based on the total RNA content of the sample. The ideal target library concentration is 50 - 200 nM. However, if the concentration is between 10-50 nM or between 200-500 nM and if the libraries do not contain low or high molecular weight peaks, sequencing can still be performed. If optimization is needed, utilize the optional Gene Expression Library Cycle Number Determination using qPCR step.*

c. Prepare Sample Index PCR Mix on ice.

Sample Index PCR Mix *Add reagents in the order listed*		PN	1X (µl)	1X + 10% (µl)	4X + 10% (µl)
◯	**Amp Mix C**	2001311	50.0	55.0	220.0
	Nuclease-free Water	—	10.0	11.0	44.0
	Total		**60.0**	**66.0**	**264.0**

d. Transfer **ONLY 20 µl** sample from the step DNA Cleanup – SPRIselect on page 88 to a new tube strip. The remaining sample can be stored at −20°C for up to 4 weeks, for generating additional libraries.

e. Add **60 µl** Sample Index PCR Mix to **20 µl** sample.

f. Add **20 µl** of an individual Dual Index TS Set A to each sample. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.

g. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25-40 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	54°C	00:00:30
4	72°C	00:00:20
5	Go to step 2, use the cycle numbers determined at step b	
6	72°C	00:01:00
7	4°C	Hold

h. Store at **4°C** for ≤**72 h**, or proceed to the next step.

5.2 Post Sample Index PCR Size Selection – SPRIselect

a. Vortex to resuspend the SPRIselect reagent. Add **100 µl** SPRIselect Reagent **(1.0X)** to each sample. Pipette mix 15x (pipette set to 180 µl).

b. Incubate **5 min** at **room temperature**.

c. Place on the magnet•**High** until the solution clears.

d. Remove the supernatant. DO NOT discard any beads.

e. With the tube still in the magnet, add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.

f. Remove the ethanol.

g. **Repeat** steps e and f for a total of 2 washes.

h. Centrifuge briefly and place on the magnet•**Low**.

i. Remove any remaining ethanol. DO NOT let the sample dry to ensure maximum elution efficiency.

j. Remove from the magnet. Add **41 µl** Buffer EB. Pipette mix 15x.

k. Incubate **2 min** at **room temperature**.

l. Place on the magnet•**Low** until the solution clears.

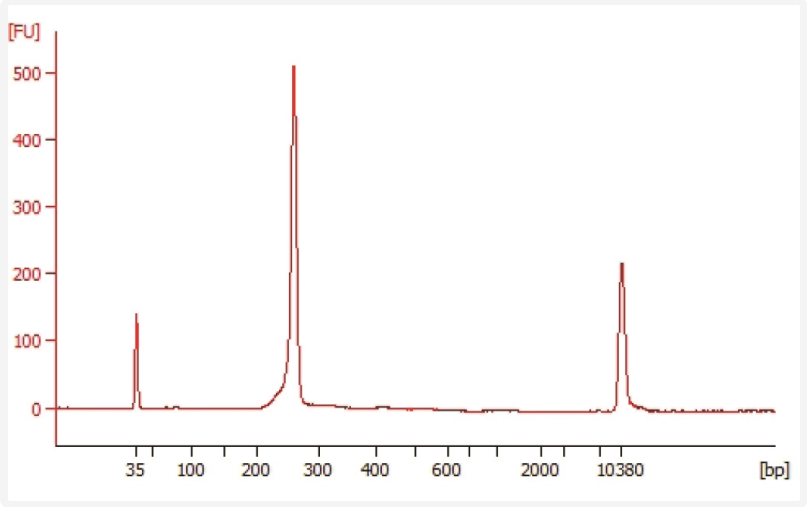
m. Transfer **40 µl** to a new tube strip.

n. Store at **4°C** for up to **72 h** or at **−20°C** for **long-term** storage.

5.3 Post Library Construction QC

Run **1 µl** sample at **1:80** dilution on an Agilent Bioanalyzer High Sensitivity chip. Select the region between 150-300 bp to determine average size of the library.

Representative Trace



Alternate QC Method

Agilent TapeStation

LabChip

Fragment Analyzer

See Appendix for:

- Post Library Construction Quantification on page 121
- Agilent TapeStation Traces on page 122
- LabChip Traces on page 122
- Fragment Analyzer Traces on page 123

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Step 6:

GEM-X Flex – Protein Expression Library Construction

6.0 Get Started

Item			10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
☐	⚠	Dual Index Plate TN Set A *Verify name & PN.* *Use indicated plate only*	3000510	Vortex and centrifuge briefly.	–20°C
☐		Beckman Coulter SPRIselect Reagent	—	Manufacturer's recommendations.	—
☐		Agilent Bioanalyzer High Sensitivity Kit *If used for QC*	—	Manufacturer's recommendations.	—
☐		Agilent TapeStation ScreenTape & Reagents If used for QC	—	Manufacturer's recommendations.	—
Place on Ice					
☐	◯	Amp Mix	2000047	Vortex and centrifuge briefly.	–20°C
☐	◯	Amp Mix C *Needed for optional Cycle Number Determination using qPCR*	2001311	Vortex and centrifuge briefly.	–20°C
☐		Forward Primer CTACACGACGCTCTTCCGATCT Reverse Primer CTCGTGGGCTCGGAGATGTGTAT AAGAGACAG *Needed for optional Cycle Number Determination using qPCR*	—	Manufacturer's recommendations.	—
☐		EvaGreen Plus Dye, 20X in Water *Needed for optional Cycle Number Determination using qPCR*	—	Manufacturer's recommendations.	Ambient
☐		KAPA Library Quantification Kit for Illumina Platforms	—	Manufacturer's recommendations.	—
Obtain					
☐		Qiagen Buffer EB	—	Manufacturer's recommendations.	Ambient
☐		10x Magnetic Separator/ 10x Magnetic Separator B	230003/ 2001212	See Tips & Best Practices.	Ambient
☐		Prepare 80% Ethanol *Prepare 2.5 ml for 4 GEM reactions.*	—	Prepare fresh.	Ambient

6.1 Sample Index PCR

a. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x sample index name (PN-3000510 Dual Index Plate TN Set A well ID) used.

b. Determine sample index PCR cycle numbers using one of the following methods (See Sample Index PCR Cycles on page 47 for guidance on which method to use):

Using qPCR: See Optional: Protein Expression Library Cycle Number Determination Using qPCR on page 118

Using Targeted cell recovery: Use the table below for guidance on cycle numbers.

Targeted Cell Recovery	Total Cycles*
500-2,000	14-15
2,000-4,000	13-14
4,000-7,000	12-13
7,000-12,000	11-12
12,000-25,000	10-11
25,000-50,000	9-10
50,000-128,000	8-9
128,000-250,000	7-8
250,000-320,000	6-7

**One additional cycle may be added if performing intracellular protein labeling only protocol, including with the MultiPro Human Fixed Cell Immune Profiling Antibody cocktail. Not required if also labeling with cell surface (e.g. the Human Discovery Panel from PTG), or if not titrating the antibodies being used.*

c. Prepare Sample Index PCR Mix.

Sample Index PCR Mix *Add reagents in the order listed*		PN	1X (µl)	1X + 10% (µl)	4X + 10% (µl)
◯	**Amp Mix**	2000047	50.0	55.0	220.0
	Nuclease-free Water	—	10.0	11.0	44.0
	Total		**60.0**	**66.0**	**264.0**

d. Transfer **ONLY 20 µl** sample (pre-amplified DNA) from the DNA Cleanup – SPRIselect on page 88 to a new tube strip.

e. Add **60 µl** Sample Index PCR Mix to **20 µl** sample.

f. Add **20 µl** of an individual Dual Index TN Set A to each sample and record the well ID used. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.

g. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25-40 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	54°C	00:00:30
4	72°C	00:00:20
5	Go to step 2, use the cycle numbers determined at step b	
6	72°C	00:01:00
7	4°C	Hold

**Optimization of cycle number may be needed based on target protein expression levels and number of antibodies used for labeling.*

STOP

h. Store at **4°C** for ≤**72 h**, or proceed to the next step.

6.2 Post Sample Index PCR Size Selection – SPRIselect

a. Vortex to resuspend the SPRIselect reagent. Add **100 µl** SPRIselect Reagent **(1.0X)** to each sample. Pipette mix 15x (pipette set to 180 µl).

b. Incubate **5 min** at **room temperature**.

c. Place on the magnet•**High** until the solution clears.

d. Remove the supernatant. DO NOT discard any beads.

e. With the tube still in the magnet, add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.

f. Remove the ethanol.

g. **Repeat** steps e and f for a total of 2 washes.

h. Centrifuge briefly and place on the magnet•**Low**.

i. Remove any remaining ethanol. DO NOT let the sample dry to ensure maximum elution efficiency.

j. Remove from the magnet. Add **41 µl** Buffer EB. Pipette mix 15x.

k. Incubate **2 min** at **room temperature**.

l. Place on the magnet•**Low** until the solution clears.

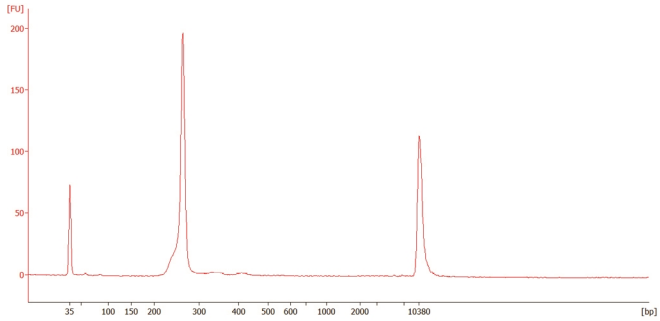
m. Transfer **40 µl** to a new tube strip.

n. Store at **4°C** for up to **72 h** or at **−20°C** for **long-term** storage.

6.3 Post Library Construction QC

Run **1 µl** sample at **1:80** dilution on an Agilent Bioanalyzer High Sensitivity chip. Select the region between 150-300 bp to determine the average size of the library.

Representative Trace



Determine the average fragment size from the Bioanalyzer trace.

Alternate QC Method

Agilent TapeStation

LabChip

Fragment Analyzer

See Appendix for:

- Post Library Construction Quantification on page 121
- Agilent TapeStation Traces on page 122
- LabChip Traces on page 122
- Fragment Analyzer Traces on page 123

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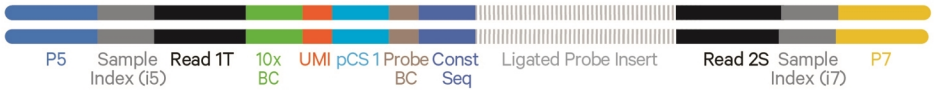
Step 7:

Sequencing

Sequencing Libraries

GEM-X Flex – Gene Expression and Protein Expression libraries comprise standard Illumina paired-end constructs, which begin with P5 and end with P7. These libraries include 16 bp 10x GEM Barcodes (10x Barcode) encoded at the start of TruSeq Read 1 (Read 1T). Sample index sequences are incorporated as the i5 and i7 index reads.

GEM-X Flex – Gene Expression Library



TruSeq Read 1 (Read 1T) and Small RNA Read 2 (Read 2S) are used in paired-end sequencing of Gene Expression libraries. Read 2 sequences the ligated probe sequence, constant sequence, and the 8 bp Probe Barcode that identifies the probe set used to hybridize the sample.

Probe Barcode can be sequenced in both Read 1 and Read 2 sequencing configurations. See GEM-X Flex – Library Sequencing Parameters on page 103 for more details.

GEM-X Flex – Protein Expression Library

TruSeq Read 1 (Read 1T) and Nextera Read 2 (Read 2N) are used for paired-end sequencing of GEM-X Flex – Protein Expression library. Read 2 sequences the Feature Barcode sequence, constant sequence, and Antibody Multiplexing Barcode that identifies the sample.

A single library contains reads derived from up to 16 samples, with cell barcodes composed of a Probe Barcode or an Antibody Multplexing Barcode and a 10x GEM Barcode (10x Barcode).

Antibody Multiplexing Barcode can be sequenced in both Read 1 and Read 2 sequencing configurations. See GEM-X Flex – Library Sequencing Parameters on page 103 for more details.

Sequencing these libraries produces a standard Illumina BCL data output folder.

Illumina Sequencer Compatibility

The compatibility of the listed sequencers has been verified by 10x Genomics. Some variation in assay performance is expected based on sequencer choice. For more information about performance variation, visit the 10x Genomics Support website.

- MiSeq
- NextSeq 500/550
- NextSeq 1000/2000
- NovaSeq 6000
- NovaSeq X series

Sample Indices

Each sample index in the Dual Index Kit TS Set A (PN-1000251) or Dual Index Kit TN Set A (PN-1000250) is a mix of one unique i7 and one unique i5 sample index. If multiple samples are pooled in a flow cell lane, the sample index name (i.e. the Dual Index TS Set A plate well ID, SI-TS-) is needed in the sample sheet used for generating FASTQs with “cellranger mkfastq”. Samples utilizing the same sample index should not be pooled together, or run on the same flow cell lane, as this would not enable correct sample demultiplexing.

Color balance in the index read is important for optimal quality and demultiplexing results. For example, when pooling samples on the NovaSeq X Series instruments, Illumina recommends avoiding index combinations which only have signals from A bases, G bases, or G+A bases in any given cycle. Refer to Illumina’s guidance on index color balancing.

GEM-X Flex – Library Sequencing Parameters

Read 2 Sequencing Configuration: This configuration reads the sequences required for sample demultiplexing in Read 2.

Parameter	Description
Sequencing Depth	Minimum 10,000 read pairs per cell for Gene Expression library Minimum 5,000 read pairs per cell for Protein Expression library
Sequencing Type	Paired-end, dual indexing

Parameter	Description
Sequencing Read	**Recommended Number of Cycles**
Read 1	28 cycles
i7 Index	10 cycles
i5 Index	10 cycles
Read 2	90 cycles*
	**Minimum required Read 2 length is 76 bp*

Alternate Sequencing Parameters

Read 1 Sequencing Configuration: This alternative configuration reads the sequences required for sample demultiplexing in Read 1. This read scheme is utilized if the sequences required for sample demultiplexing do not occur in the same positions when read in Read 2.

Parameter	Description
Sequencing Depth	Minimum 10,000 read pairs per cell for Gene Expression library Minimum 5,000 read pairs per cell for Protein Expression library
Sequencing Type	Paired-end, dual indexing
Sequencing Read	**Recommended Number of Cycles**
Read 1	48 cycles
i7 Index	10 cycles
i5 Index	10 cycles
Read 2	50 cycles

When sequencing GEM-X Flex Gene Expression libraries on a NovaSeq 6000 instrument (Standard workflow) with a loading concentration of 150 pM, a comparison of Read 1 (Read 1: 48 cycles, i7 index: 10 cycles, i5 index: 10 cycles, Read 2: 50 cycles) and Read 2 (Read 1: 28 cycles, i7 index: 10 cycles, i5 index: 10 cycles, Read 2: 90 cycles) schemes indicates that their performance is similar. In most cases, the two schemes can be used interchangeably. However, for effective data analysis, it may be important to understand the specific conditions under which Cell Ranger extracts information from Read 1 versus Read 2, as well as the rationale behind these selections. See the Technical Note Sequencing Metrics & Base Composition of Chromium Flex Libraries (CG000677) for more information.

Library Loading

Library quantification should be done with the KAPA DNA Quantification Kit using the average insert size determined by Agilent Bioanalyzer, Revvity LabChip, or Agilent TapeStation QC. Alternate methods to KAPA qPCR for final

library quantification may result in under quantification, and consequently overloading.

Once quantified and normalized, the libraries should be denatured and diluted as recommended for Illumina sequencing platforms. See Illumina documentation for denaturing and diluting libraries. See the 10x Genomics Support website for more information.

The following table provides library loading concentrations that are recommended as general guidelines based on internal testing. Libraries might need to be titrated for optimal performance.

Library Loading

Instrument	Loading Concentration (pM)	PhiX (%)
MiSeq	12	5
NextSeq 500/550	2.5	5
NextSeq 1000/2000	650	5
NovaSeq 6000 standard*	100-150	10
NovaSeq 6000 Xp workflow*	150-200	10
NovaSeq X series	150-200	10

These recommendations are based on qPCR quantification. Alternative quantification methods may affect optimal loading concentration.

*The NovaSeq 6000 standard workflow permits loading one library pool across all lanes of the flow cell; whereas the Xp workflow enables sequencing various library pools in each lane of the NovaSeq 6000 flow cell.

Library Pooling

GEM-X Flex – Gene Expression and Protein Expression libraries may be pooled for sequencing, taking into account the differences in cell number and per-cell read depth requirements between each library. Samples utilizing the same sample index should not be pooled together or run on the same flow cell lane, as this would not enable correct sample demultiplexing.

Library Pooling Example

Libraries	Sequencing Depth (read pairs per cell)	Library Pooling Ratio
GEM-X Flex– Gene Expression library	10,000	2
GEM-X Flex – Protein Expression library	5,000	1

Data Analysis and Visualization

Sequencing data may be analyzed using Cell Ranger or 10x Genomics Cloud Analysis (see supported pipelines and products webpage) and visualized using Loupe Browser. Key features for these tools are listed below. For detailed product-specific information, visit the 10x Genomics Support website.

Cell Ranger

Cell Ranger is a set of analysis pipelines that processes Chromium Single Cell Gene Expression and Gene Expression Flex data to align reads, generate Feature Barcode matrices, and perform clustering and gene expression analysis.

- Input: Base call (BCL) to generate FASTQ files
- Output: BAM, MEX, CSV, HDF5, Web Summary, .cloupe
- Operating System: Linux

Loupe Browser

Loupe Browser is an interactive data visualization tool that requires no prior programming knowledge.

- Input: .cloupe
- Output: Data visualization, including t-SNE and UMAP projections, custom clusters, differentially expressed genes
- Operating System: MacOS, Windows

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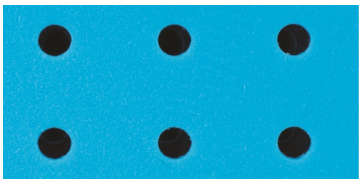
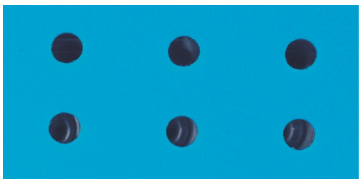
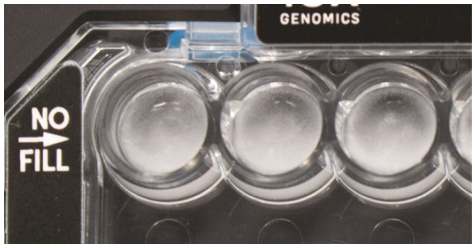
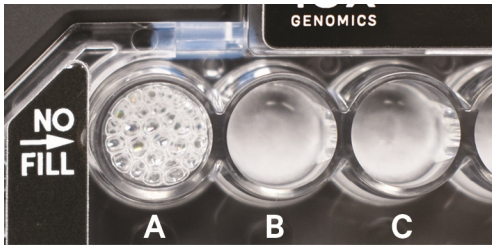
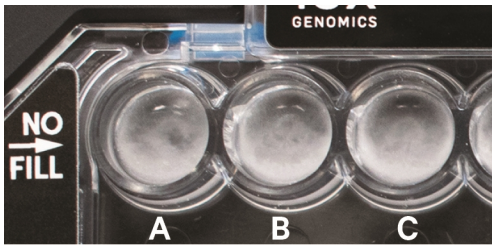
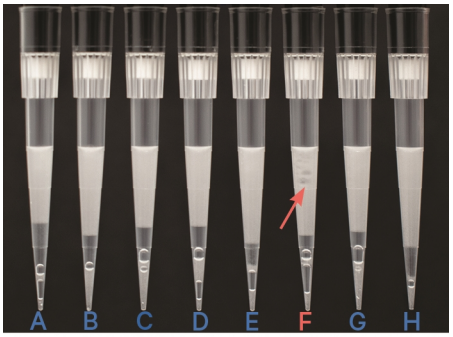
Troubleshooting

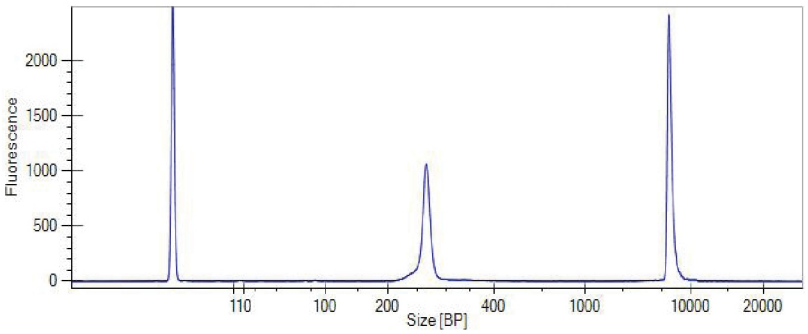
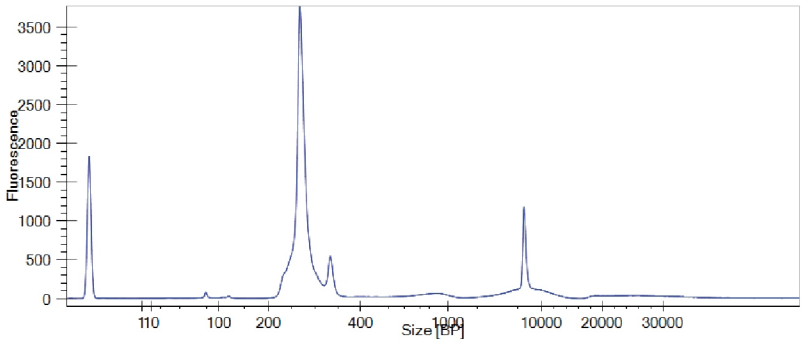
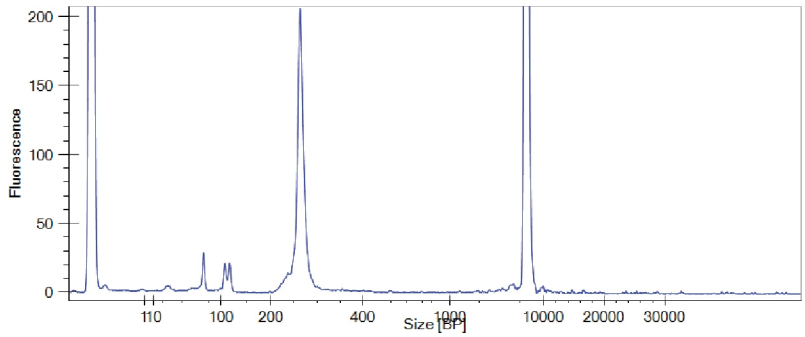
GEM Generation & Barcoding

STEP	Normal	Impacted
3.2 **Load Chromium GEM-X Chip**	 Gasket holes are aligned with the sample and gel bead wells.	 Gasket holes are misaligned with the gel bead wells. Open and close the chip holder slowly once. *One of the rows of Chromium X/iX gasket will not align with the GEM-X chip. This is normal and will not impact the assay.*
3.4 **After chip is removed from the instrument and the wells are exposed.**	 All recovery wells are similar in volume and opacity.	 Recovery well A indicates a wetting failure. Not all wetting failures may present themselves with excess bubbles (foam).  Recovery wells A-C show normal emulsions. Not all normal emulsions may have small (in size and number) bubbles in the recovery wells. **Inspecting emulsions in pipette tips is recommended for diagnosing emulsion failures.**

STEP	Normal	Impacted
3.4 **Transfer GEMs from chip**		
	All liquid levels are similar in volume and opacity without air trapped in the pipette tips.	Pipette tip A shows wetting failure. Pipette tips B-C show uniform emulsions & slightly low volumes. (~95 µl) Most wetting failures will not impact emulsion volumes of other sample run on the chip. Occasionally, wetting failures may impact the emulsion volumes recovered from other lanes. This is expected and does not indicate an emulsion failure if the samples are uniform in volume and opacity (B-C).
		
		In pipette tips A-E & G-H, a portion of the emulsion displays separate layers of oil, which indicates clog. Pipette tip F indicates a clog (separate oil layers) and wetting failure (non-uniform emulsion).
	Emulsion failures are not expected to be observed across the entire chip. The above images are for illustrative purposes to show a range in emulsion failures. A clog is not expected to impact other lanes on the chip.	

If a channel clogs or wetting failure occurs during GEM generation, it is recommended that the sample be remade. If any of the listed issues occur, take a picture and send it to support@10xgenomics.com for further assistance. Replacement reagents and chips may be provided for properly documented clogs or wetting failures if they are associated with runs of unexpired reagents and chips, and are reported within 30 days of the expiration date.

Post Library Construction QC

Step	
GEM-X Flex – Gene Expression Library Correct Sample Index PCR cycling	 The ideal target library concentration is 50-200 nM. However, if the concentration is between 10-50 nM or between 200-500 nM and if the libraries do not contain low or high molecular weight peaks, sequencing can still be performed.
Over cycling	 Additional higher molecular weight peaks present in the library trace indicate over cycling.
Under cycling	 Higher proportion of low molecular weight peaks present in the library trace indicate under cycling.

Chromium X Series Errors

The Chromium X touchscreen will guide the user through recoverable errors. If the error continues, or if the instrument has seen critical or intermediate errors, email support@10xgenomics.com with the displayed error code. Support will request a troubleshooting package. Upload pertinent logs to 10x Genomics by navigating to the Logs menu option on screen.

There are two types of errors:

Critical Errors – When the instrument has seen a critical error, the run will immediately abort. Do not proceed with any further runs. Contact support@10xgenomics.com with the error code.

a. System Error

b. Pressure Error

c. Chip Error

d. Run Error

e. Temperature Error

f. Software Error

User Recoverable Errors – Follow error handling instructions through the touchscreen and continue the run.

a. Gasket Error

b. Tray Error

c. Chip Error

d. Unsupported Chip Error

e. Network Error

f. Update Error

Consult the Chromium X Series (X/iX) User Guide (CG000396) for additional information and follow the Chromium X touchscreen prompts for execution. The Chromium X touchscreen will guide the user through recoverable errors.

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Appendix

Alternate Multiplexing Configurations & Pooling Strategies

It is possible to pool fewer than 4 or 16 samples, for example if a sample is lost during hybridization or post-hybridization washing or if fewer than the maximum number of samples is desired. In such cases, pool the remaining samples post-hybridization and continue with the protocol.

Expected Cell Concentration at Different PBS Dilution

This table provides expected approximated cell concentration after diluting in PBS at step 2.1A-e. Note that diluting with PBS will improve the signal to noise when counting with fluorescent dyes at this step. Choose an appropriate dilution based on starting cell number in hybridization that will not result in the cell concentration being out of range for the cell counting instrument. Most automated cell counters have a lower limit at 100 cells/µl.

The concentrations shown in this table do not account for any cell loss that might occur during hybridization step. Cell loss may vary from 10-30% and should be accounted for when choosing a dilution.

For 8-strip tubes

PBS Dilution	~ Concentration at Different Starting Cell # in Hybridization			
	50,000	**100,000**	**300,000**	**500,000**
No Dilution	180 cells/µl	360 cells/µl	1,080 cells/µl	1,800 cells/µl
1:2 Dilution (10 µl diluted sample + 10 µl PBS)	90 cells/µl	180 cells/µl	540 cells/µl	900 cells/µl
1:4 Dilution (10 µl diluted sample + 30 µl PBS)	45 cells/µl	90 cells/µl	270 cells/µl	450 cells/µl

For 1.5-ml microcentrifuge tubes

PBS Dilution	~ Concentration at Different Starting Cell # in Hybridization			
	50,000	**100,000**	**300,000**	**500,000**
No Dilution	110 cells/µl	220 cells/µl	660 cells/µl	1,100 cells/µl
1:2 Dilution (10 µl diluted sample + 10 µl PBS)	55 cells/µl	110 cells/µl	330 cells/µl	550 cells/µl
1:4 Dilution (10 µl diluted sample + 30 µl PBS)	25 cells/µl	50 cells/µl	150 cells/µl	250 cells/µl

Post-Hyb Wash Buffer B Preparation

For Pooling Samples into 4 GEM Wells

Prepare Post-Hyb Wash Buffer B. Vortex briefly and keep at **room temperature**.

DO NOT keep at 4°C.

Volumes needed when pooling 4, 8, or 16 samples into 4 GEM wells

Post-Hyb Wash Buffer B *Add reagents in the order listed*	PN	Pooling 4 samples (ml)*	Pooling 8 samples (ml)*	Pooling 16 samples (ml)*
Nuclease-free Water	-	19.80	31.68	55.44
Conc. Post-Hyb Buffer B	2001308	1.10	1.76	3.08
Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.*	2000482	1.10	1.76	3.08
Total	**-**	**22.0**	**35.2**	**61.6**

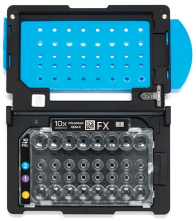
**Volumes are in ml and include 10% overage*

GEM-X Flex: Chip Loading Overview

This section provides a quick overview to the GEM-X FX Chip loading and does not include detailed instructions. See Load GEM-X Chip on page 79 for details.

Steps

Open the lid (gasket attached) of the assembled chip and lay flat for loading.



Color Legend

- **2: Gel beads**
- **1: Sample**
- **3: Oil**

a. Add 50% glycerol solution to each unused well

- Load 60 µl to row labeled 1
- Load 60 µl to row labeled 2
- Load 250 µl to row labeled 3

b. Prepare Gel Beads

- Vortex for 30 sec
- Centrifuge for 5 sec

c. Load Row Labeled 1

- Mix GEM Master Mix + Sample
- Load 60 µl to row labeled 1
- Wait 30 sec

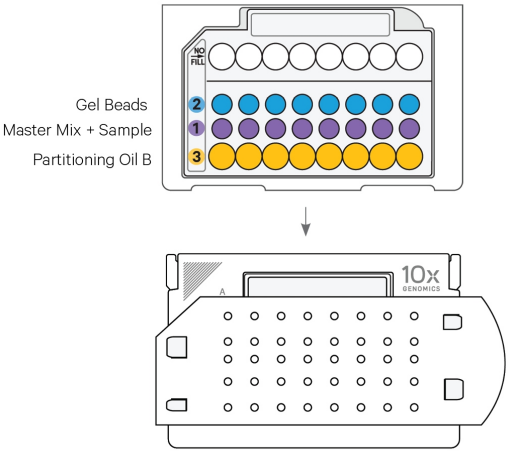
d. Load Row Labeled 2

- Aspirate Gel Beads
- Load 60 µl to row labeled 2
- Wait 30 sec

e. Load Row Labeled 3

- Load 250 µl Partitioning Oil B to row labeled 3 by pipetting two aliquots of 125 µl from a reagent reservoir.

f. Close the lid and prepare for run.



Representative Images.
Chip holder and gasket should be black and blue in color, respectively.

Optional: Gene Expression Library Cycle Number Determination Using qPCR

This step provides guidance on performing qPCR assay to determine the appropriate number of PCR cycles for the construction of GEM-X Flex - Gene Expression library. qPCR assay is recommended when working with an unknown tissue type or sample preparation method for the first time. Running qPCR to determine sample index PCR cycles will lead to more reliable library yields, and will reduce the probability of under or over-cycling the final libraries.
The qPCR guidance provided here was developed on the Bio-Rad CFX96 Real-time System (PN-1855096) using the Biotium, 31000-T or 31077-T reagent. Other instruments or dyes may also be compatible but have not been tested.

a. Dilute Pre-Amp Primers B 1:10 in nuclease-free water.

See the qPCR mix table below to determine the amount of diluted primer needed.

b. Prepare qPCR Mix on ice according to the table below. Add reagents in the order listed. Vortex and centrifuge briefly. Maintain on ice.

qPCR Mix	PN	Stock	Final	1X (µl)	2X* + 10% (µl)	5X* + 10% (µl)
Amp Mix C Up to 8 qPCR reactions can be performed using the amount provided in the reagent tube.	2001311	-	-	5.0	11.0	27.5
Diluted Pre-Amp Primers B	2000529	-	-	1.25	2.75	6.875
EvaGreen, EvaGreenPlus (Biotium, 31000-T or 31077-T) *Minimize light exposure*	-	20X	1X	0.5	1.10	2.75
Nuclease-free Water	-	-	-	2.25	4.95	12.375
Total	**-**	**-**	**-**	**9.0**	**19.8**	**49.5**

*Includes 1 negative control

c. Add **9 µl** qPCR Mix to one well per sample in a qPCR plate (a well for negative control may be included).

d. Dilute **2 µl** sample from Pre-Amplification Cleanup - SPRIselect in **8 µl** (1:5) nuclease-free water. For target cell recovery >20,000 cells, dilute further according to the table below. Pipette mix, centrifuge briefly.

Targeted Cell Recovery	1:5 Diluted Pre-amp DNA (µl)	Nuclease-free Water (µl)	Total Volume (µl)
20,000-128,000	5	15	20
128,000-320,000	2	78	80

e. Transfer **1 µl** diluted sample to each qPCR plate well containing qPCR Mix. For the negative control, add **1 µl** nuclease-free water to the corresponding well. Pipette mix.

f. Apply seal and centrifuge briefly. Record which sample is in which well of the qPCR plate.

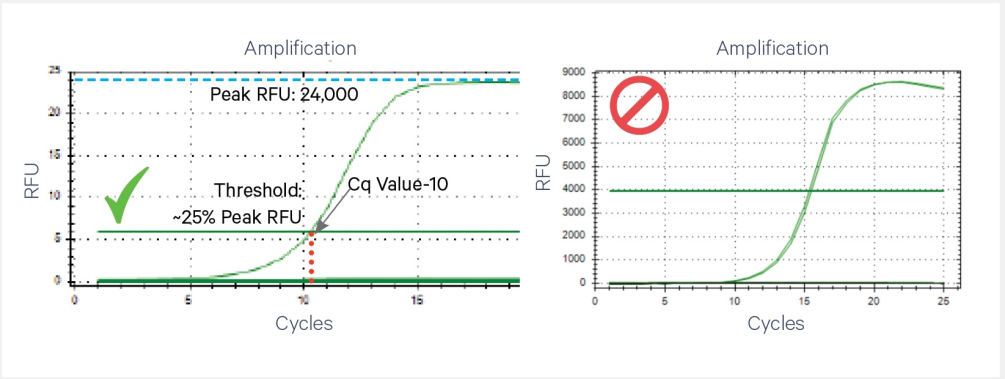
g. Prepare a qPCR system with the following protocol, place the plate in the qPCR system, and start the program.

Lid Temperature	Reaction Volume	Run Time
105°C	10 µl	35 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:03:00
2	98°C	00:00:05
3	63°C	00:00:30
	Read signal	
4	Go to step 2, 29x (total of 30 cycles)	-

h. Record the Cq value for each sample.

Selecting the correct cycle number may require manually adjusting the RFU or ΔRn threshold depending on the software's settings for the qPCR system. Follow the instructions below for manual adjusting.

Set the y-axis to a linear scale. Plot RFU on the y-axis if not using a reference dye, or ΔRn if using a reference dye. The threshold for determining the Cq value should be set along the exponential phase of the amplification plot, at ~25% of the peak fluorescence value.



i. Round up Cq values determined at the previous step to the nearest whole number and use the table below for guidance on cycle numbers.

Targeted Cell Recovery	Total Cycles	Example
<20,000	Cq value	Cq value = 13.7 Rounded Cq value = 14 Cycle no. = 14
20,000-128,000	Cq value-1	Cq value = 9.8 Rounded Cq value = 10 Cycle no. = 9 (10-1)
128,000-320,000	Cq value-5	Cq value = 11.4 Rounded Cq value = 12 Cycle no. = 7 (12-5)

Optional: Protein Expression Library Cycle Number Determination Using qPCR

This step provides guidance on performing qPCR assay to determine the appropriate number of PCR cycles for the construction of GEM-X Flex - Protein Expression libraries. qPCR assay is recommended when working with an unknown tissue type or sample preparation method for the first time. Running qPCR to determine sample index PCR cycles will lead to more reliable library yields, and will reduce the probability of under or over-cycling the final libraries.

The qPCR guidance provided here was developed on the Bio-Rad CFX96 Real-time System (PN-1855096) using the Biotium, 31000-T or 31077-T reagent. Other instruments or dyes may also be compatible but have not been tested.

a. Prepare 1 µM stock of forward and reverse primer by diluting in nuclease-free water.

See the qPCR mix table below to find out the amount of diluted primer needed.

b. Prepare qPCR Mix on ice according to the table below. Add reagents in the order listed. Vortex and centrifuge briefly. Maintain on ice.

qPCR Mix	PN	Stock	Final	1X (µl)	2X* + 10% (µl)	5X* + 10% (µl)
Amp Mix C Up to 8 qPCR reactions can be performed using the amount provided in the reagent tube.	2001311	-	-	5.0	11.0	27.5
Forward Primer CTACACGACGCTCTTCCGATCT	-	1 µM	0.125 µM	1.25	2.75	6.875

qPCR Mix	PN	Stock	Final	1X (µl)	2X* + 10% (µl)	5X* + 10% (µl)
Reverse Primer CTCGTGGGCTCGGAGATGTGTATAAGAGACAG	-	1 µM	0.125 µM	1.25	2.75	6.875
EvaGreen, EvaGreenPlus (Biotium, 31000-T or 31077-T) *Minimize light exposure*	-	20X	1X	0.5	1.1	2.75
Nuclease-free Water	-	-	-	1.0	2.2	5.5
Total	**-**	**-**	**-**	**9.0**	**19.8**	**49.5**

*Includes 1 negative control

c. Add **9 µl** qPCR Mix to one well per sample in a qPCR plate (a well for negative control may be included).

d. Dilute **2 µl** sample from Pre-Amplification Cleanup - SPRIselect in **8 µl** (1:5) nuclease-free water.

For target cell recovery >20,000 cells, dilute further according to the table below. Pipette mix, centrifuge briefly.

Targeted Cell Recovery	Pre-amp DNA (µl)	Nuclease-free Water (µl)	Total Volume (µl)
20,000-128,000	5	15	20
128,000-320,000	2	78	80

e. Transfer **1 µl** diluted sample from Pre-Amplification Cleanup - SPRIselect to each qPCR plate well containing qPCR Mix. For the negative control, add **1 µl** nuclease-free water to the corresponding well. Pipette mix.

f. Apply seal and centrifuge briefly. Record which sample is in which well of the qPCR plate.

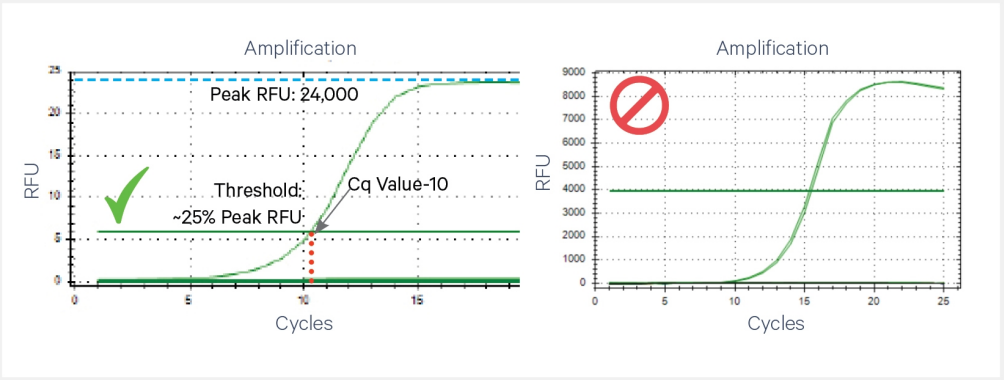
g. Prepare a qPCR system with the following protocol, place the plate in the qPCR system, and start the program.

Lid Temperature	Reaction Volume	Run Time
105°C	10 µl	35 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:03:00
2	98°C	00:00:05
3	63°C	00:00:30
	Read signal	
4	Go to step 2, 29x (total of 30 cycles)	-

h. Record the Cq value for each sample.

Selecting the correct cycle number may require manually adjusting the RFU or ΔRn threshold depending on the software's settings for the qPCR system. Follow the instructions below for manual adjusting.

Set the y-axis to a linear scale. Plot RFU on the y-axis if not using a reference dye, or ΔRn if using a reference dye. The threshold for determining the Cq value should be set along the exponential phase of the amplification plot, at ~25% of the peak fluorescence value.



i. Round up Cq values determined at the previous step to the nearest whole number and use the table below for guidance on cycle numbers.

Targeted Cell Recovery	Total Cycles	Example
<20,000	Cq value	Cq value = 13.7 Rounded Cq value = 14 Cycle no. = 14
20,000-128,000	Cq value-1	Cq value = 9.8 Rounded Cq value = 10 Cycle no. = 9 (10-1)
128,000-320,000	Cq value-5	Cq value = 11.4 Rounded Cq value = 12 Cycle no. = 7 (12-5)

Post Library Construction Quantification

a. Thaw KAPA Library Quantification Kit for Illumina Platforms.

b. Dilute **2 µl** sample with deionized water to appropriate dilutions that fall within the linear detection range of the KAPA Library Quantification Kit for Illumina Platforms. (For more accurate quantification, make the dilution(s) in duplicate).

c. Make enough Quantification Master Mix for the DNA dilutions per sample and the DNA Standards (plus 10% excess) using the guidance for 1 reaction volume below.

Quantification Master Mix	1X (µl)
SYBR Fast Master Mix + Primer	12
Water	4
Total	**16**

d. Dispense **16 µl** Quantification Master Mix for sample dilutions and DNA Standards into a 96 well PCR plate.

e. Add **4 µl** sample dilutions and **4 µl** DNA Standards to appropriate wells. Centrifuge briefly.

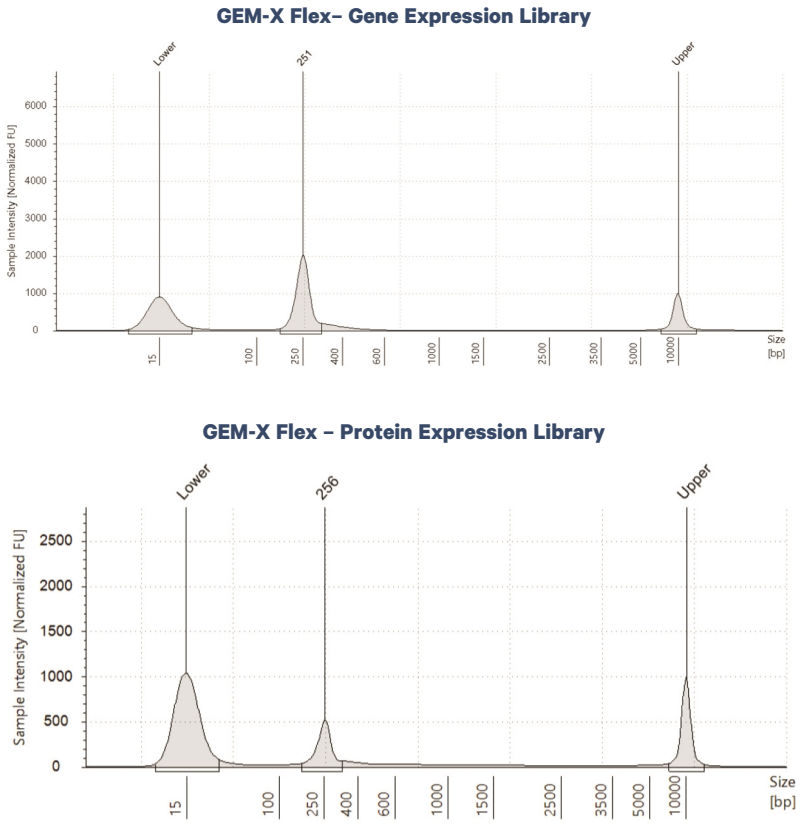
f. Incubate in a thermal cycler with the following protocol.

Step	Temperature	Run Time
1	95°C	00:03:00
2	95°C	00:00:05
3	67°C Read Signal	00:00:30
4	Go to Step 2, 29X (Total 30 cycles)	

g. Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on insert size derived from the Bioanalyzer/TapeStation trace.

Agilent TapeStation Traces

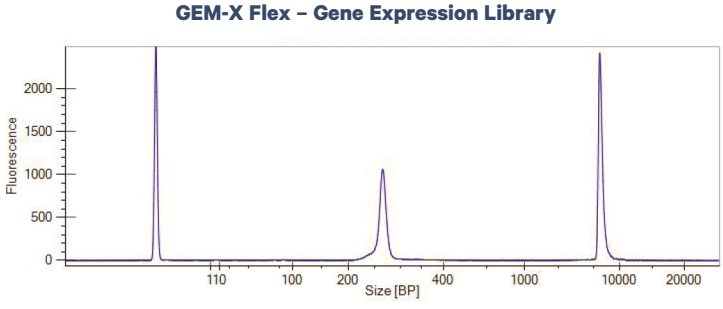
Agilent TapeStation High Sensitivity D5000 ScreenTape was used.



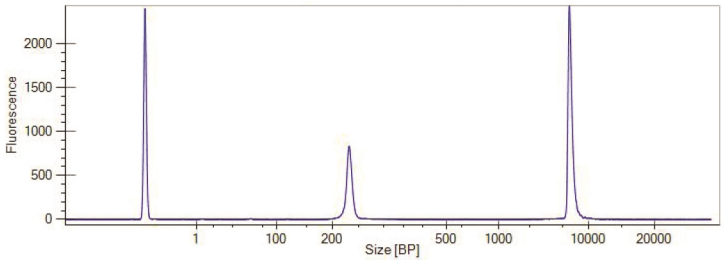
All traces are representative. Samples were run at 1:80 dilution.

LabChip Traces

DNA High Sensitivity Reagent Kit was used.



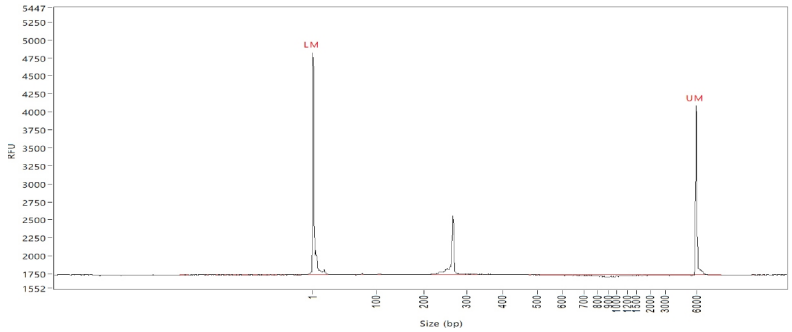
GEM-X Flex – Protein Expression Library



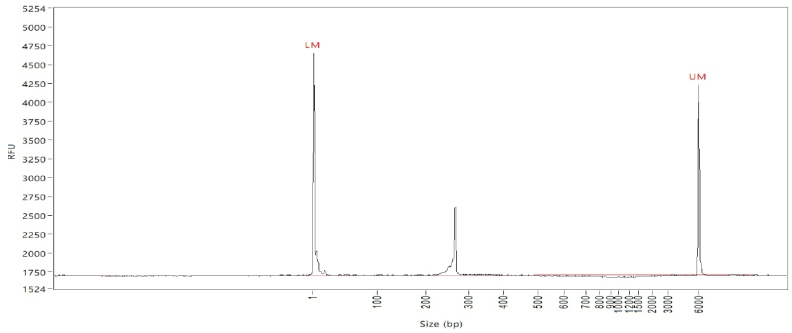
All traces are representative. Samples were run at 1:80 dilution.

Fragment Analyzer Traces

GEM-X Flex – Gene Expression Library



GEM-X Flex – Protein Expression Library



All traces are representative. Samples were run at 1:40 dilution.

Oligonucleotide Sequences

Gel Bead Primer

Gel Bead Primers

5’-CTACACGACGCTCTTCCGATCT-N16-N12-TTGCTAGGACCG-3’

Pre-Amp Primers B

Forward Primers

5’-CTACACGACGCTCTTCCGATCT-3’

Reverse Primer

5'-CCTTGGCACCCGAGAATTCCA-3'

Dual Index Kit TS Set A

Forward Primers

5’-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTC-3'

Reverse Primers

5'-CAAGCAGAAGACGGCATACGAGAT-N10-GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA-3'

Pre-Amp Primers C

Forward Primers

5’-CTACACGACGCTCTTCCGATCT-3’

Reverse Primers - Gene Expression

5'- CCTTGGCACCCGAGAATTCCA-3'

Reverse Primers - Protein Expression

5’-CTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3’

Dual Index Kit TN Set A

Forward Primers

5’-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTC-3'

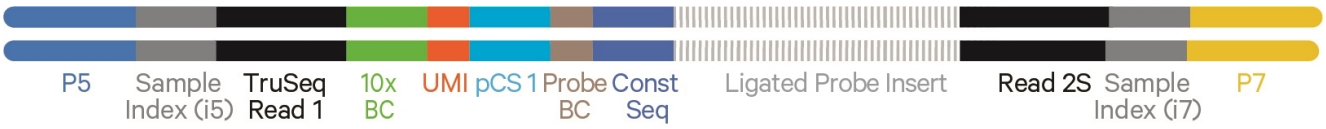
Reverse Primer

5'-CAAGCAGAAGACGGCATACGAGAT-N10-GTCTCGTGGGCTCGG-3'

GEM-X Flex– Gene Expression Library

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N12-TTGCTAGGACCG-BC8-NN-TACGTGCTAACCGCGT-Ligated_Probe_insert-TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3’

3’-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12-AACGATCCTGGC-BC8-NN-ATGCACGATTGGCGCA-Ligated_Probe_insert-ACCTTAAGAGCCCACGGTTCCTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5’



Protein Feature Barcode

5'-CGGAGATGTGTATAAGAGACAG-N10-N15-N9-CCCATATAAGAAA-3'

GEM-X Flex – Protein Expression Library

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N12-TTGCTAGGACCG-AB8-N5-AACCGCGTATTCAGGT-TTTCTTATATGGG-N9-N15-N10-CTGTCTCTTATACACATCTCCGAGCCCACGAGAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'

3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12-AACGATCCTGGC-AB8-N5-TTGGCGCATAAGTCCA-AAAGAATATACCC-N9-N15-N10-GACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'