10x GENOMICS

User Guide | CG000834 | Rev C

GEM-X Flex v2

for Multiplexed Samples

For use with:

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

GEM-X Flex GEM & Library Kit v2, *4 rxns PN-1000918*

GEM-X Flex Hybridization & Wash Kit v2, *96 rxns PN-1000904*

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

GEM-X Flex Human Transcriptome Probe Kit v2, *16 samples PN-1000900 | 96 samples PN-1000901*

GEM-X Flex Mouse Transcriptome Probe Kit v2, *16 samples PN-1000902 | 96 samples PN-1000903*

GEM-X Flex Rat Transcriptome Probe Kit v2, *16 samples PN-1001117 | 96 samples PN-1001118*

GEM-X Flex Barcode Oligo Plate v2, 96 samples *Set A PN-1000894 | Set B PN-1000897 | Set C PN-1000898 | Set D PN-1000899*

GEM-X Flex Gel Bead Kit v2, *4 rxns PN-1000896*

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

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

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

Document Revision Summary

Document Number

CG000834 | Rev C

Title

GEM-X Flex v2 for Multiplexed Samples

Revision

Rev B to Rev C

Revision Date

May 15, 2026

Description of Changes

- Updated to include RAT WTA Probes v2
- Added gel removal guidance in Probe Hybridization on page 50 (step e) and Post-Storage Gel Formation in Fixed Samples on page 110
- Updated Conc. Post-Hyb Buffer B handling in Get Started on page 54 and Get Started on page 59
- Additional tips regarding subpooled samples in Probe & Barcode Oligo Hybridization for Subpooling on page 112
- Updated for general minor consistency of language, format, and terms throughout

Notices

Document Number

CG000834 | Rev C

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GEM-X Flex v2 Reagent Kits

10x reagents are listed in the same order as their placement in the kits.

Refer to SDS for handling and disposal information.

GEM-X Flex Sample Preparation v2 Kit PN-1000781

GEM-X Flex Sample Preparation v2 Kit – Module 1
PN-1000781
Shipped on dry ice; Store at -20°C

Cap Color	Reagent	PN	Cap Color	Reagent	PN
●	Conc. Fix & Perm Buffer B	2001301	●	Conc. Fix & Perm Buffer B	2001301
○	Enhancer	2000482	○	Enhancer	2000482
○	Enhancer	2000482	-	*empty*	-
●	Conc. Quench Buffer B	2001300	●	Conc. Quench Buffer B	2001300
●	Conc. Quench Buffer B	2001300	●	Conc. Quench Buffer B	2001300
●	Conc. Quench Buffer B	2001300	●	Conc. Quench Buffer B	2001300

GEM-X Flex Sample Preparation v2 Kit – Module 2
PN-1000781
Shipped on dry ice; Store at -20°C

Cap Color	Reagent	PN	Cap Color	Reagent	PN
●	Additive C	2001332	●	Additive C	2001332
●	Additive C	2001332	●	Additive C	2001332
-	*empty*	-			

The following table provides the number of samples that can be processed using this sample preparation kit:

Protocol Name	CG Number	Sample Supported	Format
Fixation of Cells & Nuclei for GEM-X Flex Gene Expression	CG000782	48 samples	Tube based
Plate-based Sample Fixation for GEM-X Flex Gene Expression	CG000833	96 samples	Plate based
Tissue Fixation & Dissociation for GEM-X Flex Gene Expression	CG000783	24 samples	Tube based
Sample Preparation from FFPE Tissue Sections for GEM-X Flex Gene Expression	CG000784	96 samples	Tube and plate based
Blood Fixation and Cell Isolation for GEM-X Flex Gene Expression	CG000785	48 samples	Tube based

GEM-X Flex GEM & Library Kit v2, 4 rxns, PN-1000918

GEM-X Flex GEM & Library Kit v2
4 rxns, PN-1000918
Shipped on dry ice; Store at -20°C

Cap Color	Reagent	PN	Cap Color	Reagent	PN
●	GEM Enzyme Mix B	2001302	●	GEM Reagent Mix	2000491
○	Reducing Agent B	2000087	-	*empty*	-
●	Pre-Amp Primers B	2000529	●	Pre-Amp Primers C	2000953
○	Amp Mix C	2001311	○	Amp Mix C	2001311
-	*empty*	-	-	*empty*	-

GEM-X Flex Hybridization & Wash Kit v2, 96 rxns, PN-1000904

GEM-X Flex Hybridization & Wash Kit – Module 1
96 rxns, PN-1000904
Shipped on dry ice; Store at -20°C

Cap Color	Reagent	PN	Cap Color	Reagent	PN
●	Hyb Buffer B	2000485	●	Hyb Buffer B	2000485
●	Hyb Buffer B	2000485	●	Hyb Buffer B	2000485
●	Hyb Buffer B	2000485	●	Hyb Buffer B	2000485
●	Hyb Buffer B	2000485	-	*empty*	-
○	Additive A	220093	-	*empty*	-

GEM-X Flex Hybridization & Wash Kit – Module 2
96 rxns, PN-1000904
Shipped on dry ice; Store at -20°C

Cap Color	Reagent	PN	Cap Color	Reagent	PN
○	Enhancer	2000482	○	Enhancer	2000482
○	Enhancer	2000482	○	Enhancer	2000482
-	*empty*	-	-	*empty*	-
●	Conc. Post-Hyb Buffer B	2001308	●	Conc. Post-Hyb Buffer B	2001308
●	Conc. Post-Hyb Buffer B	2001308	●	Conc. Post-Hyb Buffer B	2001308

GEM-X Flex Human Transcriptome Probe Kits v2

GEM-X Flex
Human Transcriptome Probe Kit v2
16 samples, PN-1000900
Shipped on dry ice
Store at -20°C

Cap	Reagent	#	PN
●	Human WTA Probes v2	1	2001492

GEM-X Flex
Human Transcriptome Probe Kit v2
96 samples, PN-1000901
Shipped on dry ice
Store at -20°C

Cap	Reagent	#	PN
●	Human WTA Probes v2	1	2001490

GEM-X Flex Mouse Transcriptome Probe Kit v2

GEM-X Flex
Mouse Transcriptome Probe Kit v2
16 samples, PN-1000902
Shipped on dry ice
Store at -20°C

Cap	Reagent	#	PN
●	Mouse WTA Probes v2	1	2001493

GEM-X Flex
Mouse Transcriptome Probe Kit v2
96 samples, PN-1000903
Shipped on dry ice
Store at -20°C

Cap	Reagent	#	PN
●	Mouse WTA Probes v2	1	2001491

GEM-X Flex Rat Transcriptome Probe Kit v2

GEM-X Flex
Rat Transcriptome Probe Kit v2
16 samples, PN-1001117
Shipped on dry ice
Store at -20°C

Cap	Reagent	#	PN
●	Rat WTA Probes v2	1	2001885

GEM-X Flex
Rat Transcriptome Probe Kit v2
96 samples, PN-1001118
Shipped on dry ice
Store at -20°C

Cap	Reagent	#	PN
●	Rat WTA Probes v2	1	2001886

GEM-X Flex Gel Bead Kit v2, 4 rxns, PN-1000896

GEM-X Flex
Gel Bead Kit v2
4 rxns, PN-1000896
Shipped on dry ice
Store at -80°C

Reagent	#	PN
GEM-X RTL Gel Beads v2 (4 rxns)	1	2001482

GEM-X Flex Supplemental Wash Kit, PN-1000828

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

Cap Color	Reagent	PN	Cap Color	Reagent	PN
●	Conc. Post-Hyb Buffer B	2001308	●	Conc. Post-Hyb Buffer B	2001308
●	Conc. Post-Hyb Buffer B	2001308	●	Conc. Post-Hyb Buffer B	2001308
○	Enhancer	2000482	○	Enhancer	2000482
○	Enhancer	2000482	-	*empty*	-
-	*empty*	-	-	*empty*	-

A supplemental wash kit (one per 96 samples) is required when purchasing the 96 or 384 sample kit bundle under the following conditions:

- Following the Individual Wash workflow or
- Planning to store samples post-hybridization in >4 batches

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

Partitioning Oil B
Shipped at ambient temperature
Store at ambient temperature

Cap	Reagent	#	PN
●	Partitioning Oil B	4	2001213

Recovery Agent
Shipped at ambient temperature
Store at ambient temperature

Cap	Reagent	#	PN
○	Recovery Agent	4	220016

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

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

GEM-X Flex Barcode Oligo Plate v2

GEM-X Flex Barcode Oligo Plate v2, Set A
96 samples PN-1000894
Shipped on dry ice
Store at -20°C

Reagent	#	PN
Barcoding Oligo Plate, Set A	1	3002863

GEM-X Flex Barcode Oligo Plate v2, Set B
96 samples PN-1000897
Shipped on dry ice
Store at -20°C

Reagent	#	PN
Barcoding Oligo Plate, Set B	1	3002876

GEM-X Flex Barcode Oligo Plate v2, Set C
96 samples PN-1000898
Shipped on dry ice
Store at -20°C

Reagent	#	PN
Barcoding Oligo Plate, Set C	1	3002877

GEM-X Flex Barcode Oligo Plate v2, Set D
96 samples PN-1000899
Shipped on dry ice
Store at -20°C

Reagent	#	PN
Barcoding Oligo Plate, Set D	1	3002878

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

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

Reagent	#	PN
Dual Index Plate TS Set A	1	3000511

GEM-X Flex v2 Core Reagent Bundle

Kit Bundle	PN	Components	PN	Quantity
GEM-X Flex Gel Bead & Library Kit v2, 4 rxns	1000919	GEM-X Flex Gel Bead Kit v2, 4 rxns	1000896	1
		GEM-X Flex GEM & Library Kit v2, 4 rxns	1000918	1
GEM-X Flex Core Reagent Bundle v2, 96 rxns	1000937	GEM-X Flex Gel Bead Kit v2, 4 rxns	1000896	1
		GEM-X Flex Hybridization & Wash Kit v2, 96 rxns	1000904	1
		Barcode Oligo Plate v2, Set A, 96 samples	1000894	1
		GEM-X Flex GEM & Library Kit v2, 4 rxns	1000918	1

GEM-X Flex v2 Reagent Bundle for Multiplexing - Human

Kit Bundle	PN	Components	PN	Quantity
GEM-X Flex v2 Human, 16 samples	1000927	GEM-X Flex Human Transcriptome Probe Kit v2, 16 samples	1000900	1
		GEM-X Flex Gel Bead Kit v2, 4 rxns	1000896	1

Kit Bundle	PN	Components	PN	Quantity
		GEM-X Flex Hybridization & Wash Kit v2, 96 rxns	1000904	1
		Barcode Oligo Plate v2, Set A, 96 samples	1000894	1
		GEM-X Flex GEM & Library Kit v2, 4 rxns	1000918	1
GEM-X Flex v2 Human, 96 samples	1000928	GEM-X Flex Human Transcriptome Probe Kit v2, 96 samples	1000901	1
		GEM-X Flex Gel Bead Kit v2, 4 rxns	1000896	1
		GEM-X Flex Hybridization & Wash Kit v2, 96 rxns	1000904	1
		Barcode Oligo Plate v2, Set A, 96 samples	1000894	1
		GEM-X Flex GEM & Library Kit v2, 4 rxns	1000918	1
GEM-X Flex v2 Human, 384 samples	1000929	GEM-X Flex Human Transcriptome Probe Kit v2, 96 samples	1000901	4
		GEM-X Flex Gel Bead Kit v2, 4 rxns	1000896	1
		GEM-X Flex Hybridization & Wash Kit v2, 96 rxns	1000904	4
		GEM-X Flex Barcode Oligo Plate v2, Set A, 96 samples	1000894	1
		GEM-X Flex Barcode Oligo Plate v2, Set B, 96 samples	1000897	1
		GEM-X Flex Barcode Oligo Plate v2, Set C, 96 samples	1000898	1
		GEM-X Flex Barcode Oligo Plate v2, Set D, 96 samples	1000899	1
		GEM-X Flex GEM & Library Kit v2, 4 rxns	1000918	1

GEM-X Flex v2 Reagent Bundle for Multiplexing - Mouse

Kit Bundle	PN	Components	PN	Quantity
GEM-X Flex v2 Mouse, 16 samples	1000931	GEM-X Flex Mouse Transcriptome Probe Kit v2, 16 samples	1000902	1
		GEM-X Flex Gel Bead Kit v2, 4 rxns	1000896	1
		GEM-X Flex Hybridization & Wash Kit v2, 96 rxns	1000904	1
		Barcode Oligo Plate v2, Set A, 96 samples	1000894	1
		GEM-X Flex GEM & Library Kit v2, 4 rxns	1000918	1
GEM-X Flex v2 Mouse, 96 samples	1000932	GEM-X Flex Mouse Transcriptome Probe Kit v2, 96 samples	1000903	1
		GEM-X Flex Gel Bead Kit v2, 4 rxns	1000896	1
		GEM-X Flex Hybridization & Wash Kit v2, 96 rxns	1000904	1
		Barcode Oligo Plate v2, Set A, 96 samples	1000894	1
		GEM-X Flex GEM & Library Kit v2, 4 rxns	1000918	1
GEM-X Flex v2 Mouse, 384 samples	1000933	GEM-X Flex Mouse Transcriptome Probe Kit v2, 96 samples	1000903	4
		GEM-X Flex Gel Bead Kit v2, 4 rxns	1000896	1
		GEM-X Flex Hybridization & Wash Kit v2, 96 rxns	1000904	4
		GEM-X Flex GEM & Library Kit v2, 4 rxns	1000918	1
		GEM-X Flex Barcode Oligo Plate v2, Set A, 96 samples	1000894	1
		GEM-X Flex Barcode Oligo Plate v2, Set B, 96 samples	1000897	1
		GEM-X Flex Barcode Oligo Plate v2, Set C, 96 samples	1000898	1
		GEM-X Flex Barcode Oligo Plate v2, Set D, 96 samples	1000899	1

GEM-X Flex v2 Reagent Bundle for Multiplexing - Rat

Kit Bundle	PN	Components	PN	Quantity
GEM-X Flex v2 Rat, 16 samples	1001114	GEM-X Flex Rat Transcriptome Probe Kit v2, 16 samples	1001117	1
		GEM-X Flex Gel Bead Kit v2, 4 rxns	1000896	1
		GEM-X Flex Hybridization & Wash Kit v2, 96 rxns	1000904	1
		Barcode Oligo Plate v2, Set A, 96 samples	1000894	1
		GEM-X Flex GEM & Library Kit v2, 4 rxns	1000918	1
GEM-X Flex v2 Rat, 96 samples	1001115	GEM-X Flex Rat Transcriptome Probe Kit v2, 96 samples	1001118	1
		GEM-X Flex Gel Bead Kit v2, 4 rxns	1000896	1
		GEM-X Flex Hybridization & Wash Kit v2,	1000904	1

Kit Bundle	PN	Components	PN	Quantity
		96 rxns		
		Barcode Oligo Plate v2, Set A, 96 samples	1000894	1
		GEM-X Flex GEM & Library Kit v2, 4 rxns	1000918	1
GEM-X Flex v2 Rat, 384 samples	1001116	GEM-X Flex Rat Transcriptome Probe Kit v2, 96 samples	1001118	4
		GEM-X Flex Gel Bead Kit v2, 4 rxns	1000896	1
		GEM-X Flex Hybridization & Wash Kit v2, 96 rxns	1000904	4
		GEM-X Flex GEM & Library Kit v2, 4 rxns	1000918	1
		GEM-X Flex Barcode Oligo Plate v2, Set A, 96 samples	1000894	1
		GEM-X Flex Barcode Oligo Plate v2, Set B, 96 samples	1000897	1
		GEM-X Flex Barcode Oligo Plate v2, Set C, 96 samples	1000898	1
		GEM-X Flex Barcode Oligo Plate v2, Set D, 96 samples	1000899	1

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 v2 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 v2 - Protocol Planner (CG000832) 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 Fixation	Variable*	STOP 4°C ≤1 week/-80°C ≤12 months
	*Refer to the appropriate Demonstrated Protocols for details.	
Step 1: Probe Hybridization (page 47)		
1.1 Probe Hybridization (page 50)	16-24 h	
Step 2: Barcode Oligo Hybridization (page 53)		
2.1 Barcode Oligo Hybridization (page 55)	150 min	
Step 3: Post-Hybridization Washing (page 58)		
Option 3.1 A: Pooled Wash Workflow (page 61)	90 min	STOP -80°C ≤12 months
Option 3.1 B: Individual Wash Workflow (page 71)	90 min	
Step 4: GEM Generation and Barcoding (page 75)		
4.1 Prepare GEM Master Mix + Sample Dilution (page 78)	30 min	
4.2 Load GEM-X Chip (page 83)	10 min	
4.3 Run the Chromium X/iX (page 85)	6 min	
4.4 Transfer GEMs (page 86)	5 min	
4.5 GEM Incubation (page 86)	125 min	STOP 4°C ≤1 week (GEMs) -80°C ≤12 months (washed undiluted sample from step 3.1)
Step 5: GEM Recovery and Pre-Amplification (page 88)		
5.1 Post-GEM Incubation – Recovery (page 90)	10 min	
5.2 Pre-Amplification PCR (page 91)	55 min	STOP 4°C ≤72 h/-20°C ≤1 week
5.3 DNA Cleanup – SPRIselect (page 92)	30 min	STOP 4°C ≤72 h/-20°C ≤4 weeks
Step 6: GEM-X Flex v2 – Gene Expression Library Construction (page 93)		
6.1 Sample Index PCR (page 95)	40 min	STOP 4°C ≤72 h
6.2 Post Sample Index PCR Size Selection – SPRIselect (page 98)	30 min	STOP 4°C ≤72 h/-20°C long term
6.3 Post Library Construction QC (page 99)	60 min	

Sample Preparation

This User Guide is compatible with samples prepared in tubes as well as in 96-well plates. See below for the documents needed to prepare samples for this User Guide. Consult GEM-X Flex v2 - Protocol Planner (CG000832) for additional details.

1 Sample Fixation

Sample Fixation in Tubes
Fix single cell & nuclei suspensions.
Demonstrated Protocol CG000782

Sample Fixation in 96-well Plates
Fix single cell & nuclei suspensions.
Demonstrated Protocol CG000833

Tissue Fixation
Fix and dissociate tissues.
Demonstrated Protocol CG000783

Sample Preparation - FFPE Samples
Isolate nuclei from FFPE sections.
Demonstrated Protocol CG000784

Blood Fixation
Fix blood and isolate PBMCs & leukocytes.
Demonstrated Protocol CG000785

Consult the 10x Genomics support website for additional documentation.

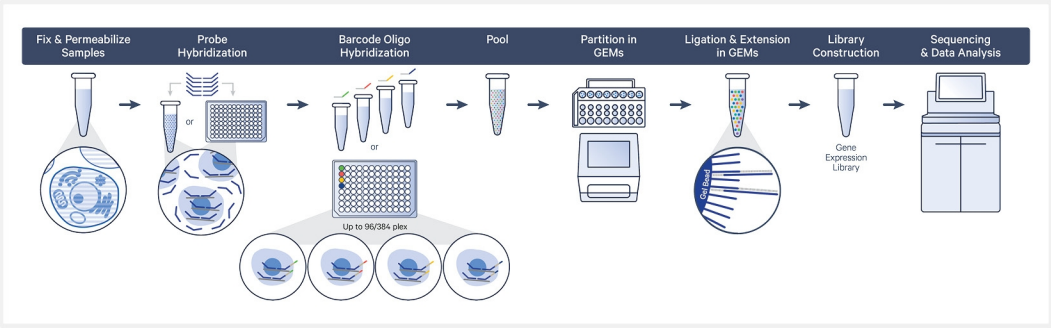
Stepwise Objectives

GEM-X Flex v2 assay offers comprehensive, scalable solutions to measure gene expression from up to 384 formaldehyde fixed samples to be run within a single GEM reaction. Gene expression is measured using probe pairs designed to hybridize to mRNA specifically.

For this workflow, fixed samples are first hybridized with whole transcriptome probe panel to enable binding to the target mRNA. This is followed by hybridization with barcoding oligos, each containing a specific Sample Barcode, to enable multiplexing of up to 384 samples. Using a microfluidic chip, the hybridized and pooled samples (sample multiplexing) 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.

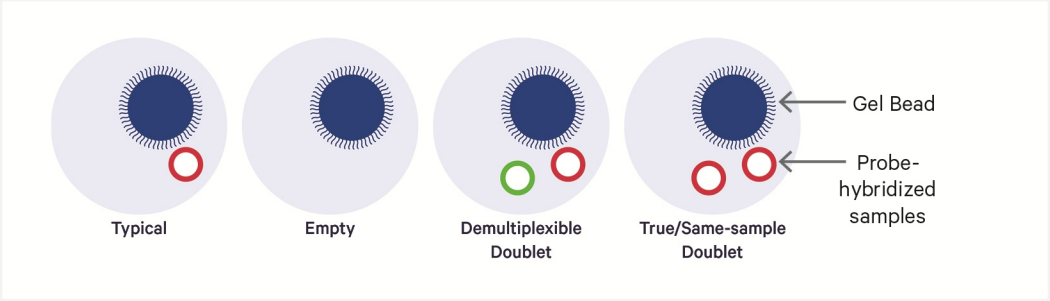
Inside the GEMs, probe pairs and the barcoding oligos are ligated and the 10x GEM Barcode is added, and all ligated probes within a GEM share a common 10x GEM Barcode but different Sample Barcode. These probes are then pre-amplified in bulk, after which gene expression libraries are generated and sequenced.

Workflow Overview



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

GEMs Generated During Multiplexing

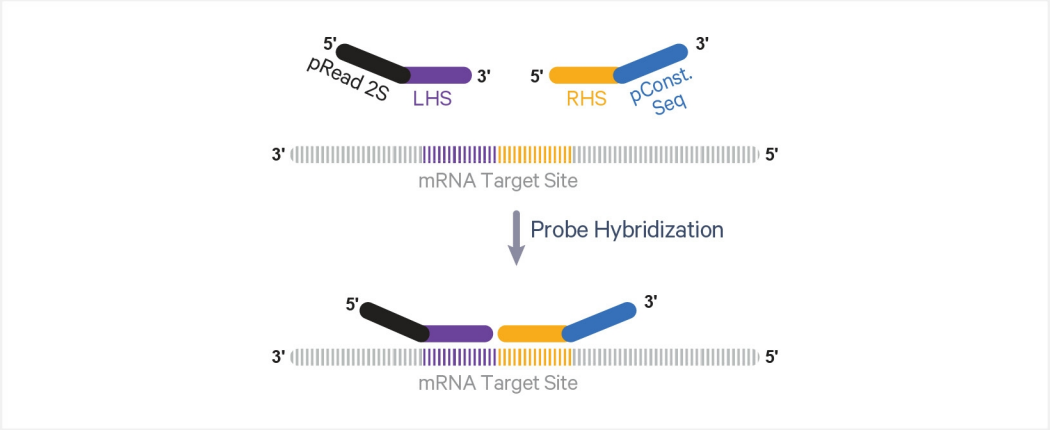


A high-level overview of each step in this User Guide, including gene expression library construction, is provided in the following sections.

Step 1: Probe Hybridization

During 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.

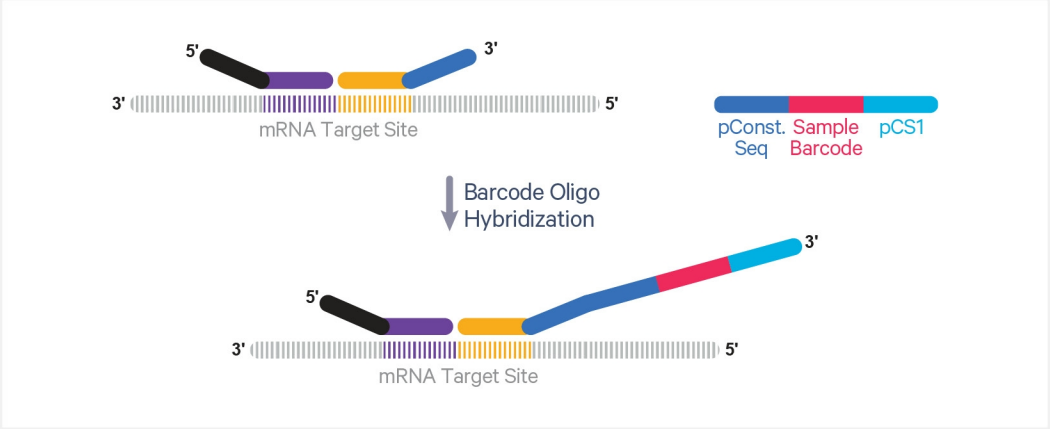
Probe Hybridization



Step 2: Barcode Oligo Hybridization

Barcoding oligos are introduced to the sample for a second hybridization after overnight incubation. These oligos, comprising a partial capture sequence, a Sample Barcode, and a partial constant sequence, enable sample multiplexing.

Barcode Oligo Hybridization



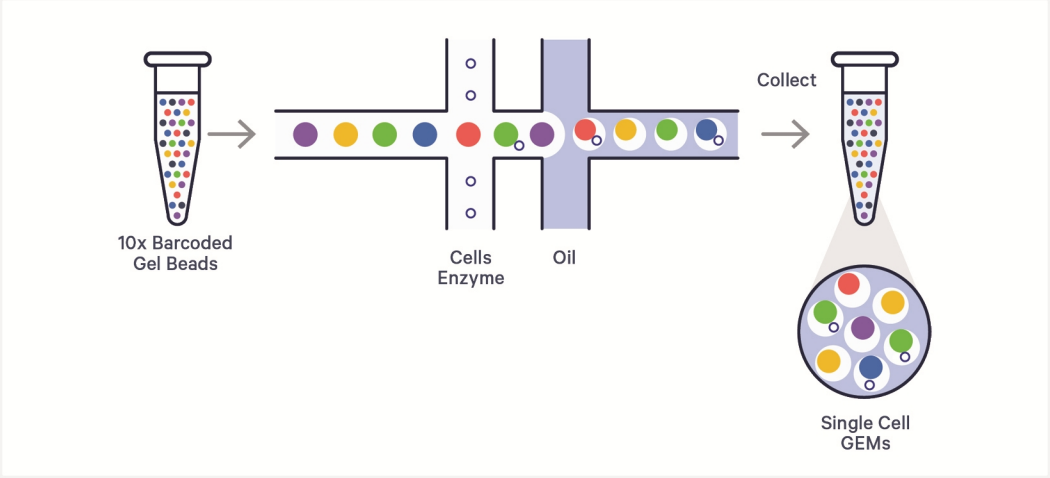
Step 3: Post-Hybridization Washing & Pooling

Samples hybridized with unique Sample 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).

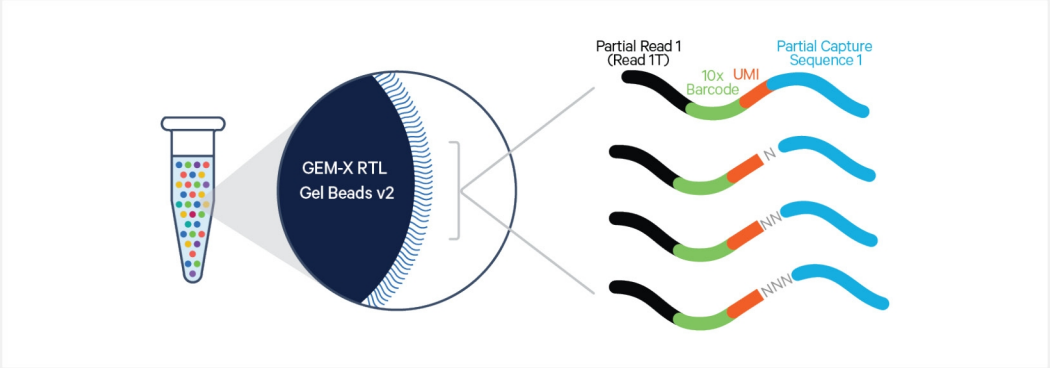
Step 4: GEM Generation & Barcoding

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. The primers also contain Ns (0-3) between 12 nt UMI and capture sequence to increase base diversity in the Sample Barcode region when reading from R1 direction.

GEM Generation



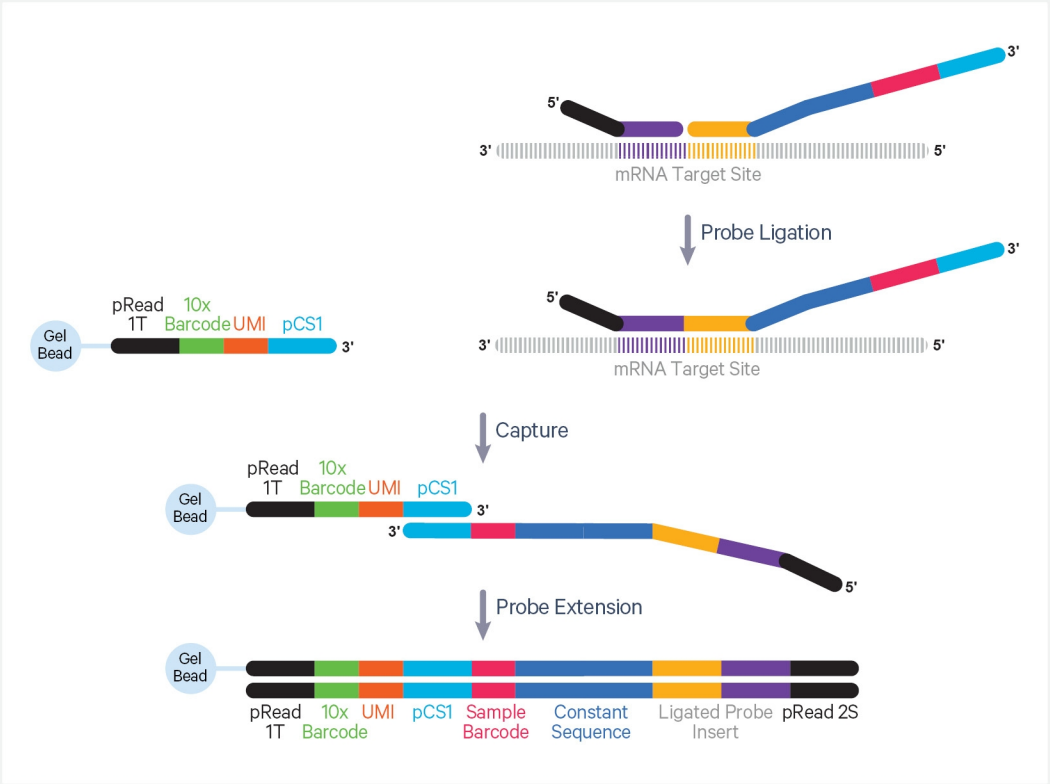
Gel Bead



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 probes and is extended by a polymerase to add the UMI, 10x GEM Barcode, and partial Read 1T.

Inside Individual GEMs

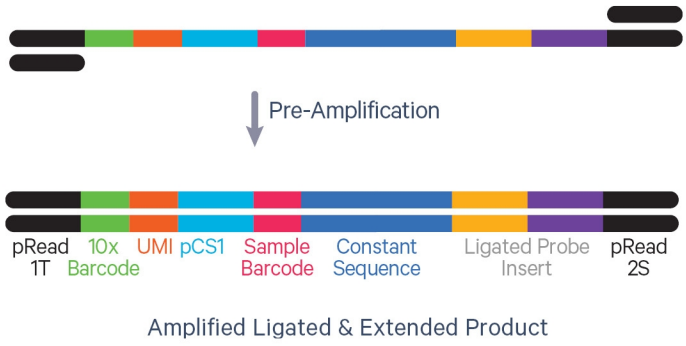


Finally, a heat denaturation step inactivates the enzymes in the GEM reaction.

Step 5: 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. The pre-amplified products are then cleaned up by SPRIselect.

DNA Pre-Amplification - Gene Expression

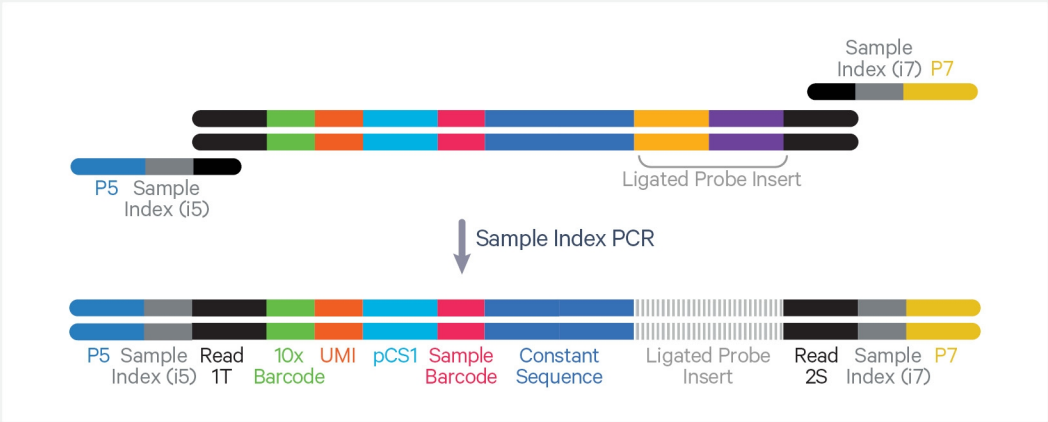


Step 6: GEM-X Flex v2 – Gene Expression Library Construction

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

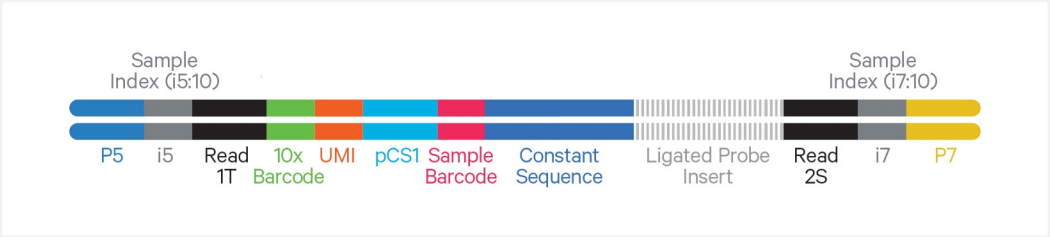


Step 7: Sequencing

A GEM-X Flex v2 – 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 10 bp Sample Barcode that identifies the sample.

Sample Barcode can be sequenced in both Read 1 and Read 2 sequencing configurations. See 10x Genomics support website for library sequencing parameters.

GEM-X Flex v2 – Gene Expression Library

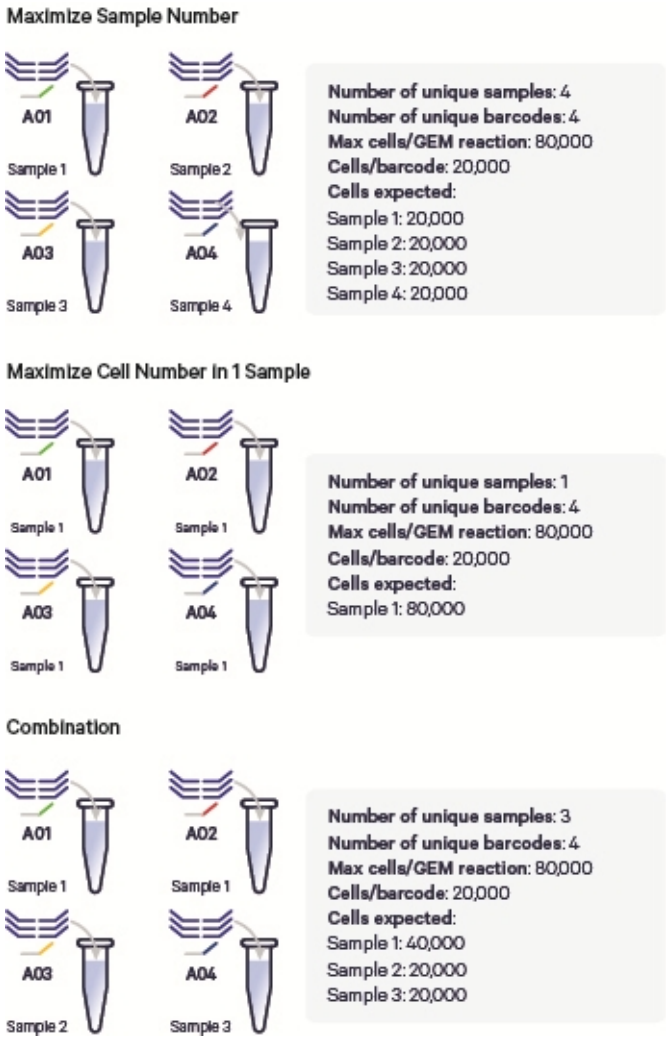


A single library contains reads derived from up to 384 samples, with Sample Barcodes and 737,000 10x GEM Barcodes (10x Barcodes).

See Appendix for Oligonucleotide Sequences on page 121

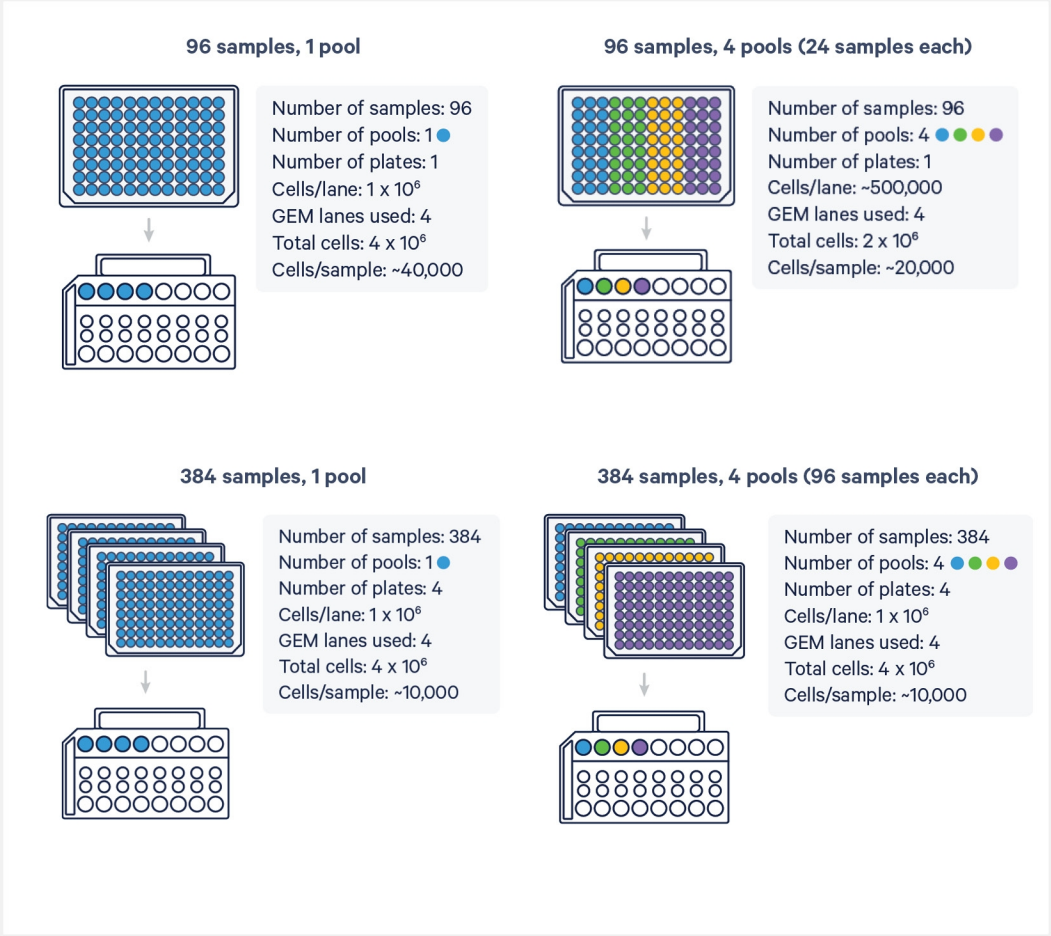
GEM-X Flex v2 Multiplexing Experiment Design

The GEM-X Flex v2 assay enables multiplexing of up to 384 samples through hybridization with barcoding oligos, each containing a specific Sample Barcode. For instance, in a 4-plex workflow, experiments can be designed to either maximize the number of samples, increase the cell count within a sample (sample subpooling), or use a combination of both strategies.



Subpooling a single sample across multiple Sample Barcodes enables the capture of more cells from that sample, with a lower undetected multiplet rate. In Cell Ranger analysis, these uniquely barcoded subpools can be combined bioinformatically to create a single sample. This approach allows for an increased target cell count for a specific sample, all while maintaining a low undetected multiplet rate. For details on setting up hybridization when subpooling, see* *Probe & Barcode Oligo Hybridization for Subpooling on page 112.*

GEM-X Flex v2 assay supports plate-based multiplexing workflows, further enabling scaling of cell load and plexy. Below are a few examples of 96-plex and 384-plex multiplexing workflow setups in 96-well plates using 96 and 384 Barcode Oligos, respectively.



Tips & Best Practices

TIPS

Icons

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

Version specific steps

Emulsion-safe Plastics

- Use validated emulsion-safe plastics and other consumables when handling GEMs as some plastics can destabilize GEMs.

Reagent Reservoirs

- For plate-based workflow, a divided reagent reservoir can be used in case of pooling 16 or more samples.
- Note that reagent tubes have only sufficient volumes for divided reservoirs. DO NOT use undivided reservoirs.
- See GEM-X Flex v2 Protocol Planner (CG000832) for some recommended reservoirs.

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 after use.
- 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.

96-well Plates and Incubation

- Use deep-well (≥350 µl) V-bottom 96-well plates for GEM-X Flex v2 workflow. Other plate types can also be used as an alternative. See GEM-X Flex v2 Protocol Planner (CG000832) for the recommended plates and for the important consideration when using alternative plates.
- Pre-heat the thermal cycler, oven, or thermomixer to the appropriate temperature before placing the plate for incubation. Ensure that the lid temperature is also set to the same temperature.
- Use consistent heating methods across experiments/conditions to reduce variability.
- When using a thermal cycler, ensure that the plate wells are in alignment with the thermal block.

Placement of the Plate in the Thermal Cycler

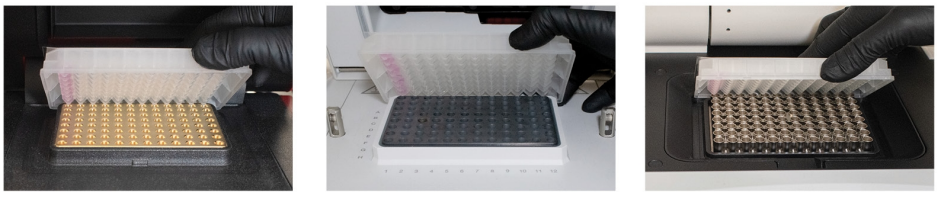
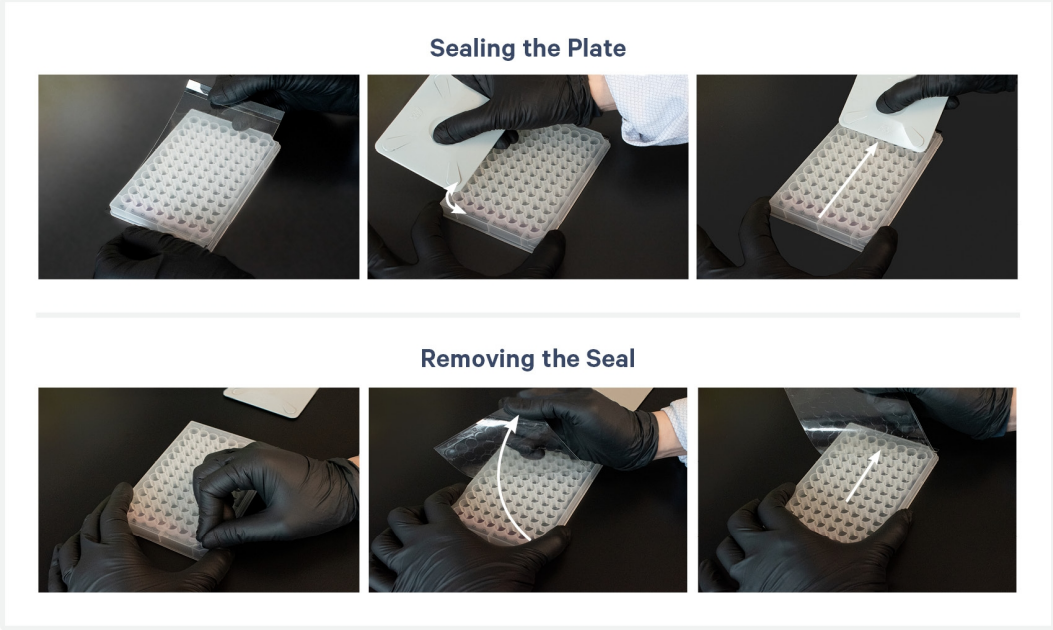


Plate Sealing & Removal

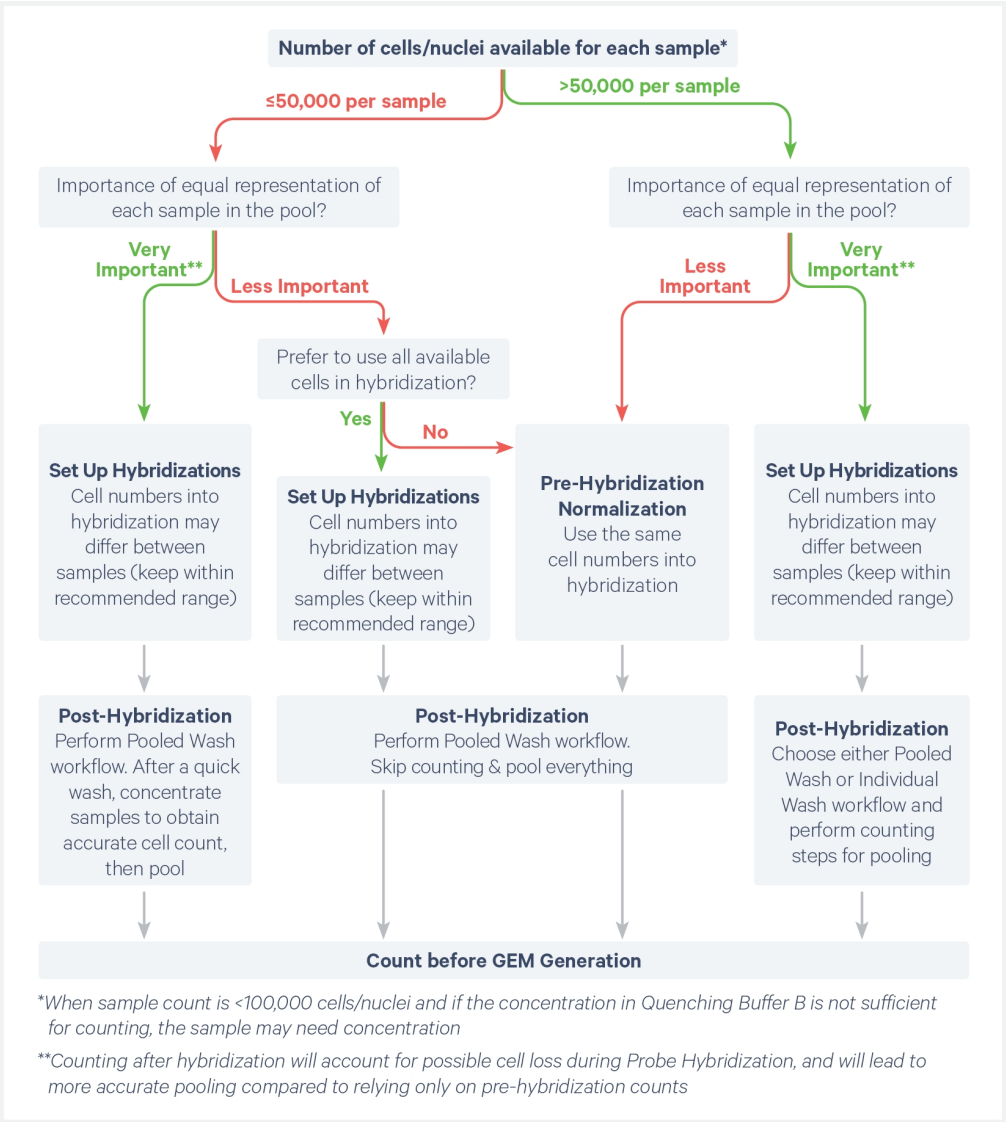
- Use the recommended seals for sealing the plate.
- To apply the seal, place the plate flat on a clean work surface. Peel the backing from the adhesive seal. Align the seal with the plate and apply while firmly holding the plate with one hand. Press on the seal around the edge of each well to ensure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



Probe Hybridization

- Start with fixed cells for Probe Hybridization and use appropriate number of cells per hybridization reaction. For guidance, see Sample Input for Probe Hybridization on page 49.
- **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 post-hybridization counting before pooling can be skipped and the entire

volume of each sample can be pooled (see the flowchart below).

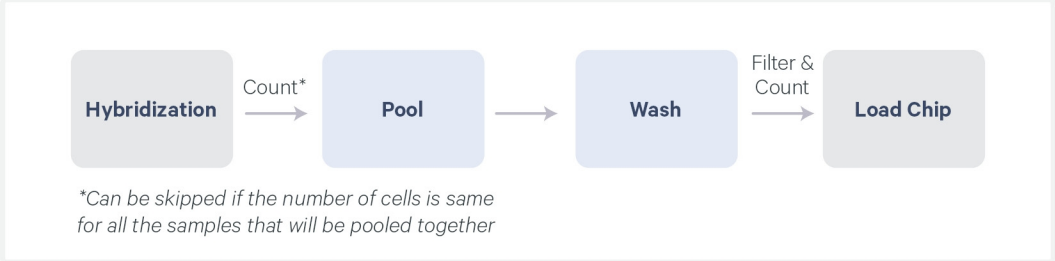


- **Example**: During a 4-plex workflow, with 300,000 to 2 x 10^6 post-fixation count, all the samples are counted and the cell numbers are normalized into probe hybridization (300,000 cells for each sample). In this case, the post-hybridization wash counting can be skipped and all the volume can be pooled together.
- **Incubation Time:** Recommended incubation time for probe hybridization is 16-24 h. Keep the incubation time the same length for all the samples within an experiment.

Post-Hybridization Pooling & Washing Guidance

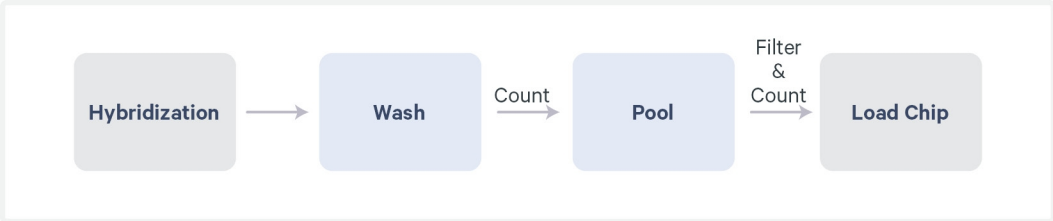
- Samples hybridized with unique barcoding oligos can be pooled immediately after hybridization and washed as a pool (**Pooled Wash workflow**). Alternatively, samples can be washed individually and pooled after the washing is complete (**Individual Wash workflow**).
- No difference in data quality is expected between Pooled Wash and Individual Wash workflows.
- 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 cell loss due to a more robust pellet compared to the Individual Wash workflow.
- The cell counting is done after Barcode Oligo 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).
- 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 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.

Centrifugation & Supernatant Removal

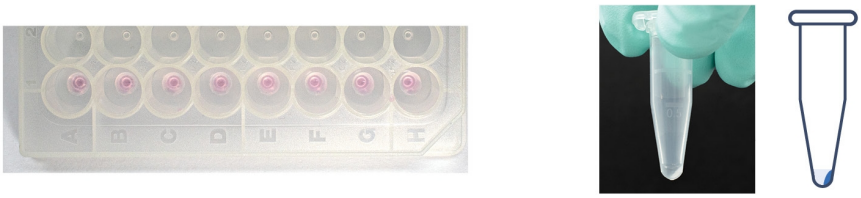
- Using a swinging bucket centrifuge can increase cell recovery during centrifugation.
- When working with samples with low cell numbers (i.e. <300,000 cells), complete removal of the supernatant is not required.
- During Probe and Barcode Oligo Hybridization, up to 15 µl supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.

  For plate-based steps, removing a fixed volume can help with cell recovery.

  - For supernatant removal during Probe Hybridization steps, set up a P200 multichannel pipette to 200 µl, and remove 200 µl supernatant before adding Probe Hyb Mix.
  - For supernatant removal during Barcode Oligo Hybridization steps, set up a P200 multichannel pipette to 125 µl, and pipette twice to remove 250 µl supernatant before adding Barcode Oligo Hyb Mix (or set up a P1000 multichannel pipette and remove 250 µl supernatant).

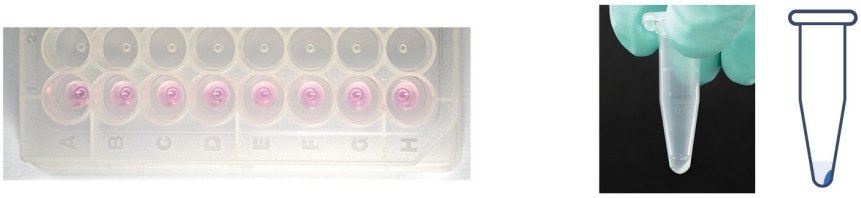
    Using a plate stand/holder can also help with more stable multichannel pipetting and supernatant removal.
- During Post-Hybridization Pooling and Washing, up to 30 µl supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.

Supernatant Removal during Hybridization



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

Supernatant Removal during Post-Hybridization Washes

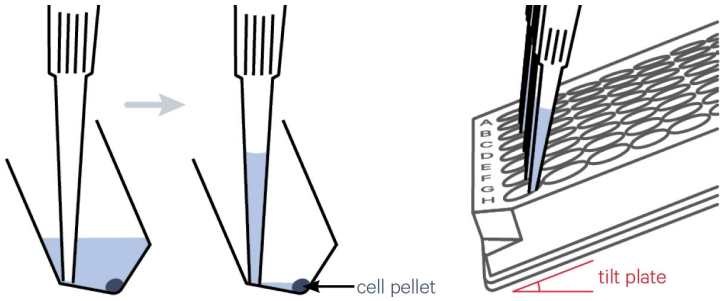


During post-hybridization washes, up to 30 µl supernatant may be left behind

Images shown above are for illustrative purposes.

- If uncertain about the volume remaining in the wells/tubes, add 15 or 30 µl water/PBS to an empty well/tube and use it for a visual estimation.
- After each buffer addition step, gently mix cells/nuclei 5x, or until the pellet is completely resuspended, without introducing bubbles.
- Centrifugation speed and time may need optimization depending upon sample type. Optimize sample-specific centrifugation conditions before moving to plates. The conditions optimized in tubes should apply to plates as well.
- Tilt the plate when removing the supernatant. Rest pipette tips on the bottom seam of the well to aspirate supernatant (see below) without disturbing the pellet.

Pipette Technique for Supernatant Removal from Plates



Cell Counts for Chip Loading

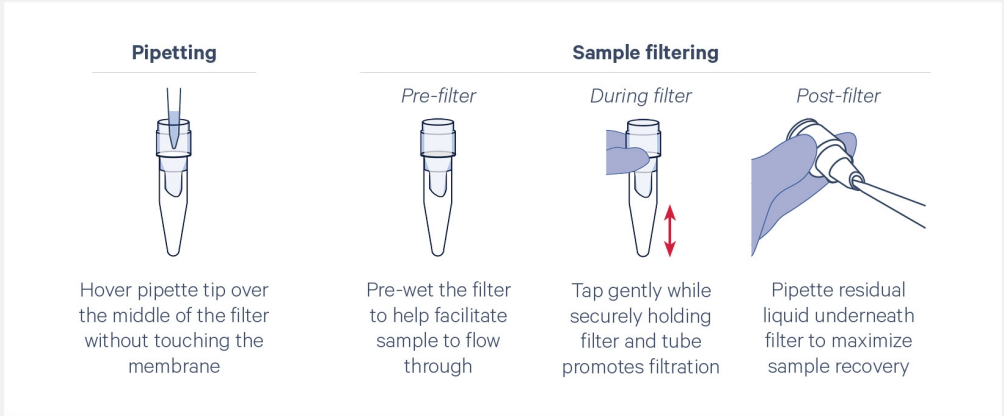
- The GEM-X Flex v2 assay can target up to 1 x 10^6 cells per GEM reaction. The number of cells targeted per Sample Barcode will depend upon the number of samples multiplexed. The solution can target up to 20,000 cells per Sample Barcode if using a ≤48-plex reaction.
- For each multiplexed sample, assuming 1 unique Sample Barcode is used per sample, the undetected (i.e. with same Barcode) cell multiplet rate for GEM-X FX Chip is approximately 0.4% multiplets per 1,000 cells recovered.

Undetectable Multiplet Rate (%)	Cells Loaded/ Sample Barcode	Cells Recovered/ Sample Barcode	Number of Sample Barcodes *(cells equally distributed; cells/well)*									
			4		16		24		96		384	
			Loaded	Recovered	Loaded	Recovered	Loaded	Recovered	Loaded	Recovered	Loaded	Recovered
~0.2	725	500	2,900	2,000	11,600	8,000	17,400	12,000	69,600	48,000	278,400	192,000
~0.32	1,160	800	4,640	3,200	18,560	12,800	27,840	19,200	111,360	76,800	445,440	307,200
~0.4	1,450	1,000	5,800	4,000	23,200	16,000	34,800	24,000	139,200	96,000	556,800	384,000
~0.72	2,610	1,800	10,440	7,200	41,760	28,800	62,640	43,200	250,560	172,800	1,002,240	691,200
~0.8	2,900	2,000	11,600	8,000	46,400	32,000	69,600	48,000	278,400	192,000	1,113,600	768,000
~1.04	3,770	2,600	15,080	10,400	60,320	41,600	90,480	62,400	361,920	249,600	1,447,680	998,400
~1.6	5,800	4,000	23,200	16,000	92,800	64,000	139,200	96,000	556,800	384,000		
~2.4	8,700	6,000	34,800	24,000	139,200	96,000	208,800	144,000	835,200	576,000		
~3.2	11,600	8,000	46,400	32,000	185,600	128,000	278,400	192,000	1,113,600	768,000		
~4.0	14,500	10,000	58,000	40,000	232,000	160,000	348,000	240,000	1,392,000	960,000		
~4.2	15,225	10,500	60,900	42,000	243,600	168,000	365,400	252,000	1,461,600	1,008,000		
~5.0	18,125	12,500	72,500	50,000	290,000	200,000	435,000	300,000				
~5.32	19,285	13,300	77,140	53,200	308,560	212,800	462,840	319,200				
~6.0	21,750	15,000	87,000	60,000	348,000	240,000	522,000	360,000				
~7.0	25,375	17,500	101,500	70,000	406,000	280,000	609,000	420,000				
~8.0	29,000	20,000	116,000	80,000	464,000	320,000	696,000	480,000				

Sample Filtration

Post-pooling Filtration

- After resuspension in Post-Hyb Resuspension Buffer, pass the sample through an appropriate 30 µm or 40 µm filter. See GEM-X Flex v2 Protocol Planner CG000832 for recommended filters. DO NOT use any unsupported filters.
- For detail filtration guidance, see Post-Hybridization Washing on page 58.
- Filtration is essential for optimal microfluidic performance.



Cell Counting

- Accurate counting is critical for optimal assay performance.
- Combination of counters and dyes tested for counting fixed cells/nuclei, post-hybridization and post-hybridization wash.

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/nuclei 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.*

- It is strongly recommended that the fixed sample be stained with a fluorescent dye such as PI staining solution and counted using an automated fluorescent cell counter or hemocytometer. Fluorescent staining enables accurate counting even in the presence of sub-cellular debris.
- Focus cells/nuclei 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.

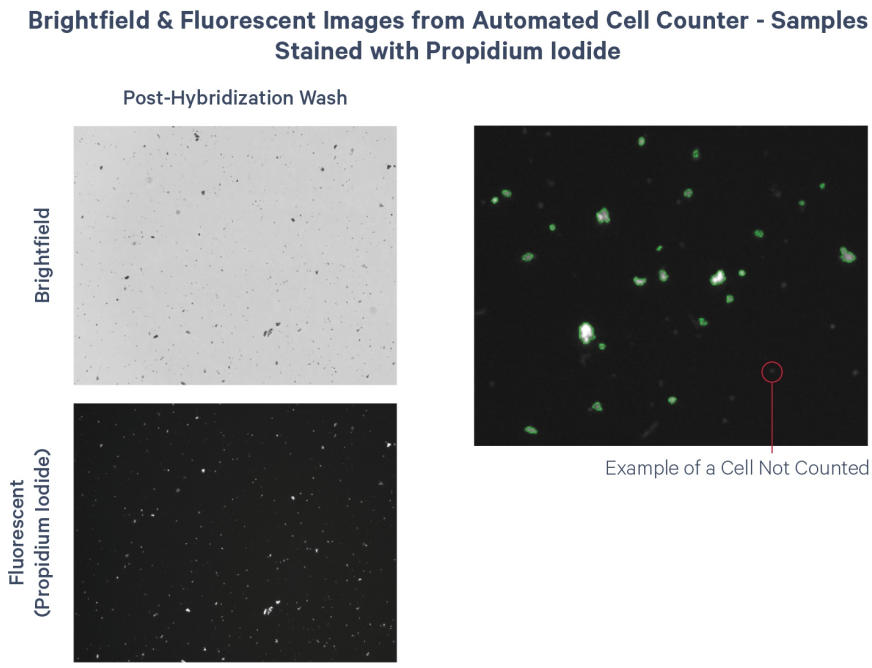
  Some sample or cell resuspension buffers may have background autofluorescence that impacts counting accuracy; changing the fluorescence threshold/gating might not fix the background issue. In such cases, diluting the sample leads to stable counts over a broader range of fluorescence thresholds.

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.



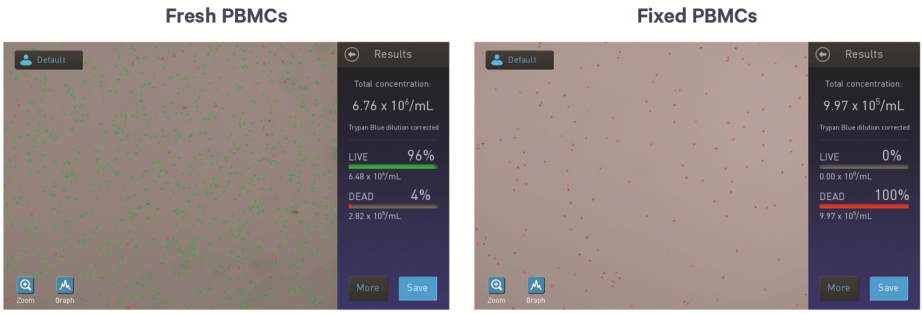
Counting Using Trypan Blue (Only for Debris-Free Samples)

Debris-free samples, for example, cell lines, can also be counted using trypan blue. Samples that are more like to have debris, for example, fixed & dissociated tissue samples, should be counted using fluorescent dyes.

This protocol below 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. If using hemocytometer, count fixed cells by placing hemocytometer under the microscope.
- The majority of fixed cells or nuclei suspensions will be stained with trypan blue stain and appear 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.

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 83 for specific instructions.

Chromium X/iX Firmware

- To run the GEM-X chips in the Chromium X/iX instrument, firmware version 2.0.0 or higher is required; however the most updated version is recommended for best performance.
- 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

- Use 50% glycerol solution. See GEM-X Flex v2 protocol Planner (CG000832) for recommended vendor and PN.

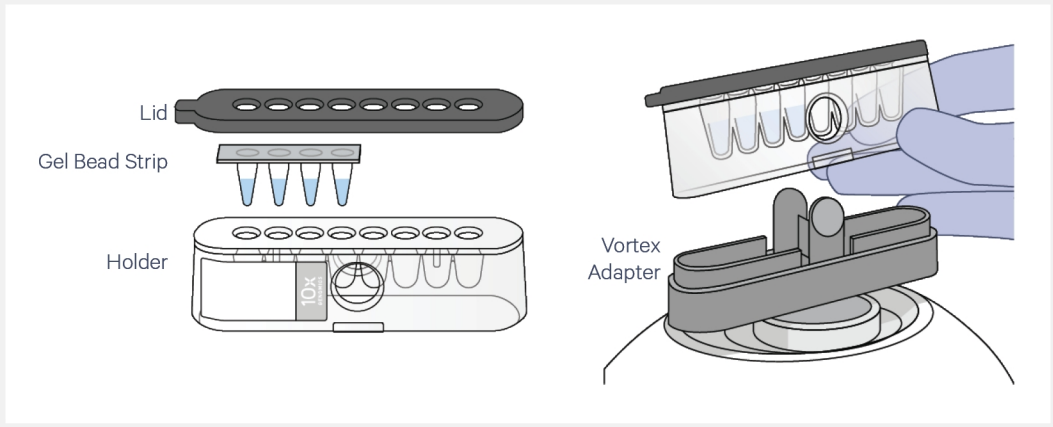
  OR

- 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 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



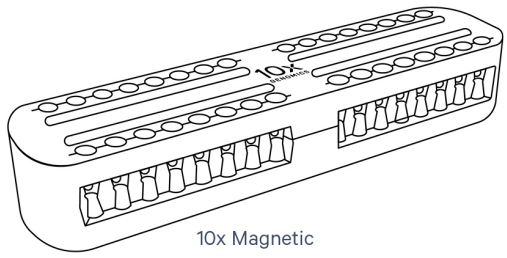
- Use one tube of Gel Beads per chip well. DO NOT puncture the foil seals of tubes not used at the time.
- After removing the Gel Bead strip from the packaging, equilibrate the Gel Bead strip to **room temperature** for at least **30 min** before use.
- Store unused Gel Beads at **−80°C** and avoid more 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**.

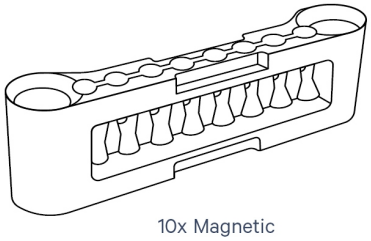
- Centrifuge the Gel Bead strip for ~5 sec after removing from the holder. Confirm there are no bubbles at the bottom of tubes and the liquid levels look even. Place Gel Bead strip back in the holder and secure the holder lid.
- If the required volume of beads cannot be recovered, place the pipette tips 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

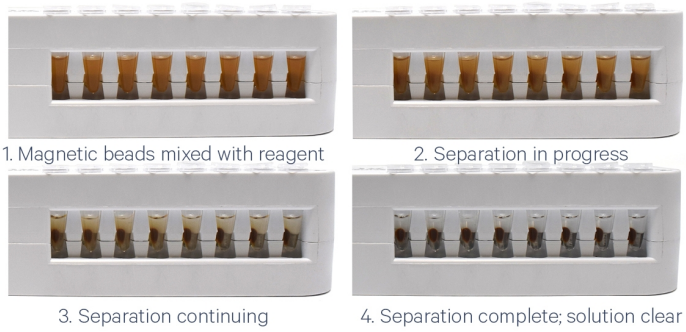


10x Magnetic Separator

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).

Example Ratio: $\frac{\text{= Volume of SPRIselect reagent added to the sample}}{\text{Volume of DNA sample}} \frac{\text{= 50 µl}}{\text{100 µl}}$ = **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 and general sample type.

- For new tissue types or sample preparation methods, qPCR assay is recommended to determine sample index PCR cycles. This ensures more reliable library yields and reduces the chance of under or over cycling.
- For 320,000–1,000,000 cells, split the sample into four SI-PCR reactions using unique sample indexes for each PCR to generate four libraries per pool.
- See the 10x Genomics support site for guidance on index combinations when sequencing low plex pools.

Pooling Samples with Different RNA Content

In a multiplexing pool, sequencing reads are distributed among samples based on their RNA content, resulting in consistent sequencing saturation across all samples. Samples with higher RNA content receive more reads per cell, whereas samples with low RNA content will have proportionally fewer reads per cell. Since read distribution 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

Step 1:

Probe Hybridization

1.0 Get Started 48

Sample Input for Probe Hybridization 49

1.1 Probe Hybridization 50

1.0 Get Started

Item			10x PN	Preparation & Handling	Storage
Thaw & Keep Warm					
☐	●	Hyb Buffer B	2000485	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 maintained at 42°C for up to 10 min. If not used within 10 min, reheat at 65°C to ensure enhancer is fully dissolved.	−20°C
Place on Ice					
☐	●	Additive A	220093	Thaw at room temperature. Vortex, verify no precipitate, centrifuge briefly. Maintain on ice.	−20°C
☐	⚠	Fixed Cell Suspension	—	Consult GEM-X Flex v2 - Protocol Planner (CG000832) for details on applicable Demonstrated Protocols.	—
☐	●	Human WTA Probes v2 OR Mouse WTA Probes v2 OR Rat WTA Probes v2	2001492/ 2001490 OR 2001493/ 2001491 OR 2001885/ 2001886	Thaw on ice. Vortex and centrifuge briefly.	−20°C

Sample Input for Probe Hybridization

Sample Type	Input into Hybridization		Notes
	Minimum	Recommended	
Cells/nuclei suspension Other samples types, for example, samples derived from fixed and dissociated tissue, fixed blood, or FFPE tissue sections	*25,000	300,000 (if sufficient cells available)	Using <50,000 cells/barcode may make it difficult to target maximum load. DO NOT exceed 500,000 cells in one hybridization reaction.
Leukocytes from fresh or fixed blood (CG000785), splenocytes (CG000782), bone marrow mononuclear cells, and cells from fixed & dissociated spleens and pancreas (CG000783)	*25,000	≤100,000	It is recommended to use ≤100,000 as higher cell loads may lead to a slight decrease in data quality.

*If starting with 25,000 input into fixation, ≤25,000 fixed cells will be moved forward into hybridization. See key considerations below if using <25,000 cells.

Key considerations if using <25,000 cells
Issues
• Loss of pellet/a pellet that is not visible • Not enough cells for storage • Difficulty in pooling samples in equal number • Not enough cells left after washing to target maximum cell load (20,000 cells/Sample 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
• Follow better sample preparation practices, including using a swinging bucket rotor. • During hybridization steps, 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.

1.1 Probe Hybridization

- Ensure that the samples have been appropriately fixed and quenched. Consult GEM-X Flex v2 - Protocol Planner (CG000832) for details on the protocols to use.
- Hybridization can be performed in either tubes (1.5-ml or strip tubes) or 96-well plates, depending upon experimental needs and preferences. This section provides instructions for both methods.
- If samples were fixed in tubes and if plate-based hybridization is preferred, transfer **200 µl** of the resuspended sample (in Quenching Buffer B) to each well of an appropriate 96-well plate before starting. Consult Demonstrated Protocols CG000833 or CG000784 regarding compatible 96-well plates.
- Determine the number of cells for each hybridization reaction.

TIPS

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

TIPS

See Probe Hybridization on page 31 for guidance on sample input.

- Incubation can be performed in a thermomixer with heated lid and no shaking, an oven, or a thermal cycler.

  If using a thermal cycler, ensure that the lid temperature is also set at the appropriate temperature. If using a thermal cycler for plates, ensure that the plate wells are in alignment with the thermal block. See 96-well Plates and Incubation on page 30 for more information.

  If using an oven, ensure that it is properly equilibrated to appropriate temperature before starting incubation.

a. Set a thermomixer with **heated lid** and no shaking or oven 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 Probe Hyb Mix at **room temperature**. Add Reagents in the order listed. Vortex and centrifuge briefly.

	Probe Hyb Mix	PN	1X + 10% (µl)*	16X + 10% (µl)*	96X + 10% (µl)*	384X + 10% (µl)*
●	**Hyb Buffer B** *Thaw at 42°C. Add warm to the mix & if appears milky keep it back on 42°C.*	2000485	38.5	616.0	3,696.0	14,784.0
●	**Enhancer** *Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.*	2000482	5.5	88.0	528.0	2,112.0
○	**Additive A**	220093	2.75	44.0	264.0	1,056.0
●	**Human WTA Probes v2** **OR** **Mouse WTA Probes v2** **OR** **Rat WTA Probes v2**	2001492/ 2001490 OR 2001493/ 2001491 OR 2001885/ 2001886	8.25	132.0	792.0	3,168.0
	Total		**55.0**	**880.0**	**5,280.0**	**21,120.0**

**Volumes are in µl. X = number of sample tubes/wells.*

A calculator for the above table is included in the web version of this document.

c. Incubate Probe 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**.

If using the 96-well plate, seal the plate containing samples with a plate seal before centrifugation.

TIPS If performing pre-hybridization normalization, ensure that the same number of cells are present in each tube/well.

e. Remove the supernatant.

When removing the supernatant, if gel formation is observed (significantly larger than expected size of the cell pellet), remove the gel as per the guidance in Post-Storage Gel Formation in Fixed Samples on page 110.

If using the plate, carefully remove the seal before removing the supernatant. See Plate Sealing & Removal on page 30.

See Centrifugation & Supernatant Removal on page 34 for tips on supernatant removal from plates & tubes.

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. Up to 15 µl of supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.

f. Add **50 µl** Probe Hyb Mix to each sample tube/well and gently pipette mix 5x with pipette set at 40 µl. When using a 96-well plate, a divided reagent reservoir and a multichannel pipette can be used.

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

g. Incubate for **16-24 h** at **42°C**.

If using a plate, firmly seal the plate with a new micro film seal using a film sealing adapter or roller before incubation. See Plate Sealing & Removal on page 30.

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

Step 2:

Barcode Oligo Hybridization

2.0 Get Started 54

2.1 Barcode Oligo Hybridization 55

2.0 Get Started

Item			10x PN	Preparation & Handling	Storage
Thaw & Keep Warm					
☐		Hyb Buffer B	2000485	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 maintained 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 maintained at 42°C for up to 10 min. If not used within 10 min, reheat at 65°C to ensure enhancer is fully dissolved.	−20°C
Place on Ice					
☐		Conc. Post-Hyb Buffer B	2001308	Thaw at room temperature. Vortex and centrifuge briefly. Maintain on ice until needed during Step 2.1.	−20°C
☐		Barcoding Oligo Plate, Set A/B/C/D	3002863/ 3002876/ 3002877/ 3002878	Thaw on ice. Maintain on ice until needed. Right before use, vortex and centrifuge at 850 rcf for 5 min.	−20°C

2.1 Barcode Oligo Hybridization

- Incubation can be performed in a thermomixer with heated lid and no shaking, an oven, or a thermal cycler.
  If using a thermal cycler, ensure that the lid temperature is also set at the appropriate temperature. If using a thermal cycler for plates, ensure that the plate wells are in alignment with the thermal block. See 96-well Plates and Incubation on page 30 for more information.
  If using an oven, ensure that it is properly equilibrated to appropriate temperature before starting incubation.
- Ensure that the plate seal is properly removed and applied in between buffer addition/supernatant removal and centrifugation steps. See Plate Sealing & Removal on page 30.

a. Set a thermomixer with **heated lid** and no shaking or oven 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	2 h
Step	**Temperature**	**Time**
Pre-equilibrate	42°C	Hold
Barcode Oligo Hybridization	42°C	2 h

b. Prepare Post-Hyb Wash Buffer B. Add reagents in the order listed. Vortex briefly and keep at **room temperature**. DO NOT keep at 4°C.

	Post-Hyb Wash Buffer B	PN	1X + 10% (µl)*	16X + 10% (µl)*	96X + 10% (µl)*	384X + 10% (µl)*
	Nuclease-free Water	-	198.0	3,168.0	19,008.0	76,032.0
	Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate.* *Add warm to the mix.*	2000482	11.0	176.0	1,056.0	4,224.0
	Conc. Post-Hyb Buffer B	2001308	11.0	176.0	1,056.0	4,224.0
	Total	-	**220.0**	**3,520.0**	**21,120.0**	**84,480.0**

**Volumes are in µl. X = number of sample tubes/wells.*

A calculator for the above table is included in the web version of this document.

c. Prepare Barcode Oligo Hyb Mix at **room temperature**. Add reagents in the order listed. Vortex and centrifuge briefly.

	Barcode Oligo Hyb Mix	PN	1X* + 10% (µl)	16X* + 10% (µl)	96X* + 10% (µl)	384X* + 10% (µl)
	Hyb Buffer B *Thaw at 42°C. Add warm to the mix and if appears milky keep it back on 42°C.*	2000485	38.5	616.0	3,696.0	14,784.0
	Enhancer** *Heat at 65°C for 10 min. Vortex and verify no precipitate.* *Add warm to the mix.*	2000482	5.5	88.0	528.0	2,112.0
	Total	-	**44.0**	**704.0**	**4,224.0**	**16,896.0**

**Volumes are in µl. X = number of sample tubes/wells.*

A calculator for the above table is included in the web version of this document.

***Thawed Enhancer can be kept on ice until needed during the next step (Post-Hybridization Washing). It should be pre-heated to 65°C for 10 min before using to make Post-Hyb Wash Buffer B.*

d. Incubate Barcode Oligo Hyb Mix at **42°C** for at least **5 min**.

e. Remove tubes/plates from overnight incubation. Keep at room temperature for **10 min** before proceeding to next step.

f. Add **200 µl** Post-Hyb Wash Buffer B to each tube/well and pipette mix 5x.

If using plates, remove seal before adding buffer.

TIPS

When using a 96-well plate, a divided reagent reservoir and a multichannel pipette can be used.

g. Centrifuge sample at **850 rcf** for **10 min** at **room temperature**.

If using plates, seal the plate with a new plate seal before centrifugation.

h. Remove the supernatant.

When removing the supernatant, if gel formation is observed (significantly larger than the expected size of the cell pellet), remove as much of the supernatant as possible without removing the gel and then add **200 µl** Post-Hyb Wash Buffer B. Incubate for **10 min** at **37°C**. Centrifuge briefly and remove the supernatant. If gel is still present, repeat the step to remove the gel completely.

TIPS

If using the plate, carefully remove the seal before removing the supernatant.

Up to 15 µl supernatant may be left behind to optimize cell recovery without significantly impacting assay performance. To minimize leaving behind >15 µl, a fixed volume of supernatant can be removed. See Centrifugation & Supernatant Removal on page 34

i. Add **40 µl** Barcode Oligo Hyb Mix to each tube/well. When using a 96-well plate, a divided reagent reservoir and a multichannel pipette can be used.

Keep sample at **room temperature**. DO NOT place on ice. Mixing at this step is not needed.

j. Retrieve the already thawed Barcoding Oligo plates from ice. Vortex and centrifuge at **850 rcf** for **5 min**. Without touching the bottom of the plate well, aspirate **10 µl** of an individual Barcoding Oligo (Set A, B, C or D) and add to each sample tube/well. Pipette mix 5x (pipette set to 40 µl). Record the Barcoding Oligo Plate, Set (A, B, C, or D) and the well ID used. For example, A-A01 (set A, well A01).

k. Incubate for **2 h** at **42°C**.

If using the plate, firmly seal the plate with a new micro film seal with a film sealing adapter or roller before incubation.

l. Proceed to the next step.

Step 3:

Post-Hybridization Washing

3.0 Get Started 59

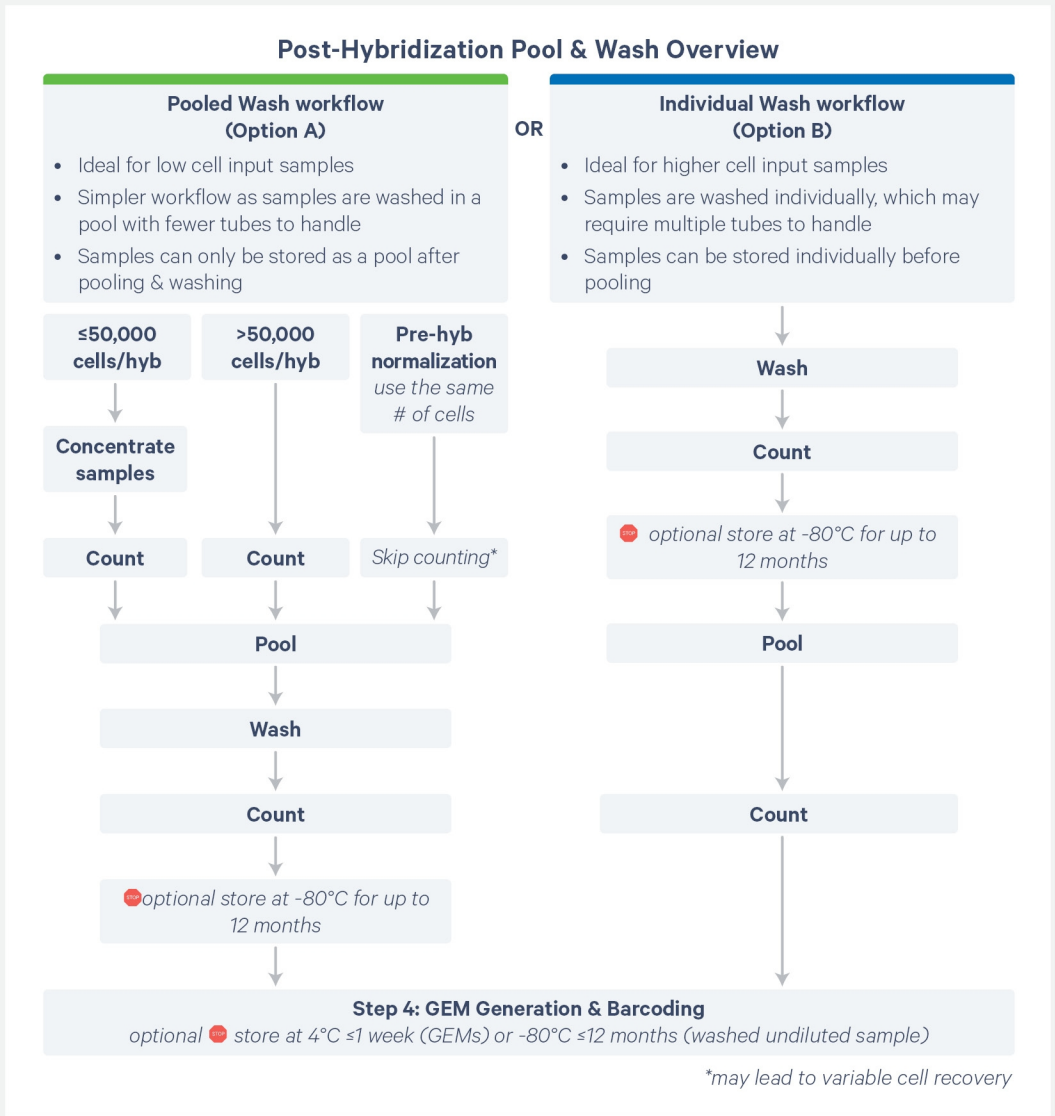
3.1 Post-Hybridization Pool & Wash 60

3.0 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 maintained at 42°C for up to 10 min. If not used within 10 min, reheat at 65°C to ensure enhancer is fully dissolved.	−20°C
Place on Ice				
☐	Conc. Post-Hyb Buffer B	2001308	Thaw at room temperature. Vortex and centrifuge briefly. Maintain on ice.	−20°C
Obtain				
☐	Sysmex Sterile Single-pack CellTrics Filters/Miltenyi Biotec Pre-Separation Filters (30 µm) OR pluriStrainer Mini 40 µm (Cell Strainer)	—	Manufacturer's recommendations.	Ambient
☐	Nacali USA Centrifugal filtration plate for 96-well, 30 µm *For optional filtration before pooling*	—	Manufacturer's recommendations.	Ambient
☐	Glycerol for molecular biology, ≥99% *Prepare fresh 50% glycerol solution for sample storage*	—	See Tips & Best Practices.	—

3.1 Post-Hybridization Pool & Wash

Samples hybridized with unique barcoding oligos 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 3.1A) and Individual Wash workflow (Option 3.1B)**.



Option 3.1 A: Pooled Wash Workflow

- During the centrifugation steps, up to 30 µl supernatant may be left behind if working with <300,000 cells. See Centrifugation & Supernatant Removal on page 34 for more details. If uncertain about the volume remaining in the tube, add 30 µl water/PBS to an empty tube/well of the same size and use it for a visual estimation.
- Incubation can be performed in a thermomixer with heated lid and no shaking, an oven, or a thermal cycler.

  If using a thermal cycler, ensure that the lid temperature is also set at the appropriate temperature. If using a thermal cycler for plates, ensure that the plate wells are in alignment with the thermal block. See 96-well Plates and Incubation on page 30 for more information.

  If using an oven, ensure that it is properly equilibrated to appropriate temperature before starting incubation.

a. Prepare Post-Hyb Wash Buffer B for Post-hybridization pooling (step c, d, or e). Add reagents in the order listed. Vortex briefly and keep at **room temperature**. DO NOT keep at 4°C.

	Post-Hyb Wash Buffer B	PN	1X* + 10% (ml)	Pooling 16 samples (ml)	Pooling 96 samples (ml)	Pooling 384 samples (ml)
	Nuclease-free Water	-	0.445	7.12	42.72	170.88
	Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.*	2000482	0.025	0.4	2.4	9.6
	Conc. Post-Hyb Buffer B	2001308	0.025	0.4	2.4	9.6
	Total	-	**0.495**	**7.92**	**47.52**	**190.08**

**X = number of sample tubes/wells. Volumes are in ml.*

A calculator for the above table is included in the web version of this document.

b. Remove tubes/plates from the thermomixer/oven/thermal cycler after Barcode Oligo Hybridization. Keep at **room temperature** for **10 min** before proceeding to next step.

Proceed to either **step c, d, or e,** depending on whether the pre-hyb normalization was performed and the cell count.

c. **Post-hybridization Pooling (Pre-hyb Normalized Samples)**:

i. Add **100 µl** Post-Hyb Wash Buffer B per sample tube/well into a 15-ml or 50-ml centrifuge tube. For example, for pooling from 96 tubes/wells, add 9.6 ml (100 µl * 96) buffer.

ii. Remove seal (for plates) and add **150 µl** Post-Hyb Wash Buffer B to each tube/well. Pipette mix 5x.

Optional (for plates): Nuclei or other sample types prone to clumping can be filtered using an appropriate plate-filter before pooling. Place the plate-filter on a new 96-well v-bottom plate, add samples to the filter, secure the lid, and centrifuge at 850 rcf for 1 min. After centrifugation, remove the plate-filter and lid. Pipette mix the filtered samples.

iii. **Pool samples**:

From Plates: Using a P200 multichannel pipette, transfer samples from the wells to a reagent reservoir. Pipette mix the pooled samples in the reservoir and using a P1000/serological pipette, transfer to the tube containing Post-Hyb Wash Buffer B.

TIPS *If using the full plate, samples might need transferring before the reservoir capacity is reached.*

From Tubes: Using a standard pipette, transfer samples from tubes to the tube containing Post-Hyb Wash Buffer B.

Optional (for tubes): Nuclei or other sample types prone to clumping can be filtered using an appropriate tube-filter during transfer. Place filter on top of the 15- or 50-ml tube containing Post-Hyb Wash Buffer B and add samples to the filter. Pipette mix the filtered samples.

TIPS *When pooling >96 samples, use separate 50-ml tubes, with each tube holding up to one 96-reaction pool.*

iv. Add **200 µl** Post-Hyb Wash Buffer B to each tube/well. Pipette mix 5x. Pipette carefully to avoid buffer overflow.

When using a 96-well plate, a divided reagent reservoir and a multichannel pipette can be used.

v. Transfer samples into the same reservoir/tube as step iii above.

TIPS *If using a reservoir for pooling, pipette mix the pooled samples in the reservoir and using a P1000/serological pipette, transfer to the tube. Use separate reservoirs & tubes if making more than one pool.*

Optional: Filter the sample again if the filtration was done at previous steps.

vi. Invert the tube to mix.

vii. Centrifuge at **850 rcf** for **10 min** at **room temperature**.

viii. Remove the supernatant without disrupting the pellet.

ix. Proceed to step f.

d. Post-hybridization Pooling (Unnormalized Samples, >50,000 cells):

i. Add **225 µl** Post-Hyb Wash Buffer B per sample tube/well into a 15-ml or 50-ml centrifuge tube.

For example, for pooling from 96 tubes/wells, add 21.6 ml (225 µl * 96) buffer.

ii. Remove seal (for plates) and add **225 µl** Post-Hyb Wash Buffer B to each tube/well. Pipette mix 5x.

Optional (for plates): Nuclei or other sample types prone to clumping can be filtered using an appropriate plate-filter before pooling. Place the plate-filter on a new 96-well v-bottom plate, add samples to the filter, secure the lid, and centrifuge at 850 rcf for 1 min. After centrifugation, remove the plate-filter and lid. Pipette mix the filtered samples.

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

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.

iv. Pool an equal number of cells from different hybridization reactions and add to the tube containing Post-Hyb Wash Buffer B. See Post-Hybridization Pooling Calculation on page 70.

Optional (for tubes): Nuclei or other sample types prone to clumping can be filtered using an appropriate tube-filter during transfer. Place filter on top of the 15- or 50-ml tube containing Post-Hyb Wash Buffer B and add samples to the filter. Pipette mix the filtered samples.

When pooling >96 samples, use separate 50-ml tubes, with each tube holding up to one 96-reaction pool.

v. Invert the tube to mix.

vi. Centrifuge at **850 rcf** for **10 min** at **room temperature**.

vii. Remove the supernatant without disrupting the pellet.

viii. Proceed to step f.

e. Post-hybridization Pooling (Unnormalized Samples, ≤50,000 cells):

i. Remove seal (for plates) and add **200 µl** Post-Hyb Wash Buffer B to each tube/well. Pipette mix 5x.

ii. Apply new seal (for plates) and centrifuge at **850 rcf** for **10 min** at **room temperature**.

iii. **Concentrate** the sample by removing ~220 µl supernatant, leaving ~30 µl behind.

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

Optional (for plates): Nuclei or other sample types prone to clumping can be filtered using an appropriate plate-filter before pooling. Place the plate-filter on a new 96-well v-bottom plate, add samples to the filter, secure the lid, and centrifuge at 850 rcf for 1 min. After centrifugation, remove the plate-filter and lid. Pipette mix the filtered samples.

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

TIPS

See Tips & Best Practices for Cell Counting on page 37.

vi. Pool an equal number of cells from different hybridization reactions and add to an appropriate tube. See Post-Hybridization Pooling Calculation on page 70.

Optional (for tubes): Nuclei or other sample types prone to clumping can be filtered using an appropriate tube-filter during transfer. Place filter on top of the tube and add samples to the filter. Pipette mix the filtered samples.

vii. Invert the tube to mix.

viii. Centrifuge at **850 rcf** for **10 min** at **room temperature**.

ix. Remove the supernatant without disrupting the pellet.

x. Proceed to step f.

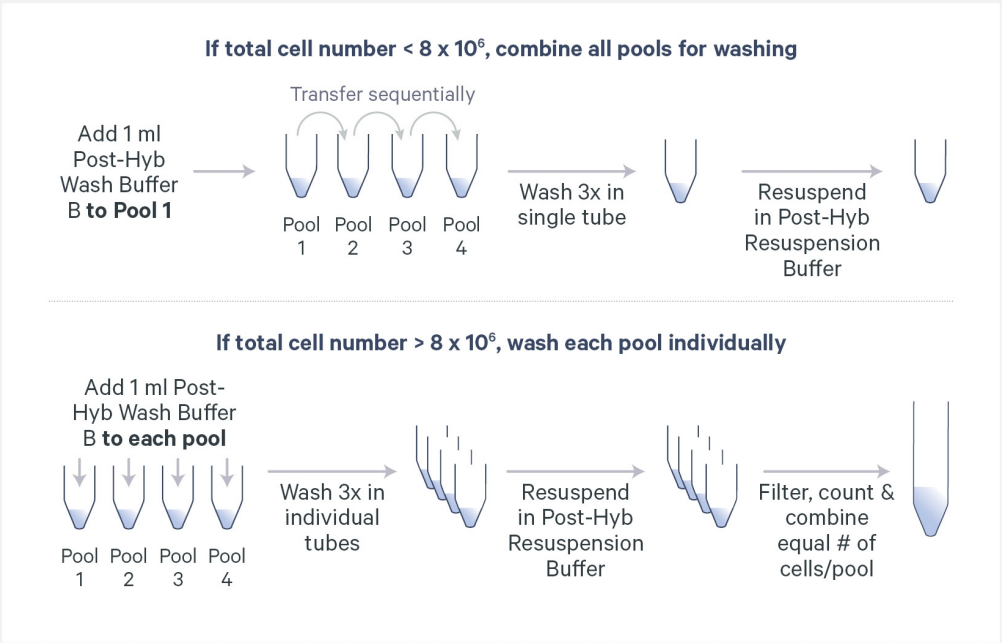
f. Prepare Post-Hyb Wash Buffer B for washing (steps g-o). Add reagents in the order listed. Vortex briefly and keep at **room temperature**. DO NOT keep at 4°C.

	Post-Hyb Wash Buffer B	PN	1 Pool + 10% (ml)	4 Pools + 10% (ml)
	Nuclease-free Water	-	2.97	11.88
	Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate.* *Add warm to the mix.*	2000482	0.165	0.66
	Conc. Post-Hyb Buffer B	2001308	0.165	0.66
	Total	-	**3.3**	**13.2**

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

When pooling >96 samples:

- If the total cell number is <8 x 10^6, add 1 ml buffer to the first pool and transfer the sample to the second pool and so on, thus combining all pools into one tube. Wash the combined samples 3x (steps h-s) and resuspend in Post-Hyb Resuspension Buffer B (step t).
- If the total cell number is >8 x 10^6, add 1 ml buffer to each pool and wash each pool 3x (steps h-s) separately. Resuspend each pool in Post-Hyb Resuspension Buffer B (step t) and combine in equal number after filtration & counting (steps u-w).



h. Incubate at **37°C** for **10 min**.

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

j. Remove the supernatant.

k. Add **1 ml** Post-Hyb Wash Buffer B to the tube. Pipette mix 5x.

l. Incubate at **37°C** for **10 min**.

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

n. Remove the supernatant without disturbing the pellet.

o. Add **1 ml** Post-Hyb Wash Buffer B to the tube. Pipette mix 5x.

p. Incubate at **37°C** for **10 min**.

q. Prepare Post-Hyb Resuspension Buffer B. Pipette mix 5x. Maintain at **4°C.** Add reagents in the order listed. The table provides volume for 1 ml buffer. If needed, additional buffer can be prepared using this table.

	Post-Hyb Resuspension Buffer B	PN	1 Pool + 10% (ml)	4 Pools + 10% (ml)
	Nuclease-free Water	-	1.045	4.18
●	**Conc. Post-Hyb Buffer B**	2001308	0.055	0.22
	Total	-	**1.1**	**4.4**

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

s. Remove the supernatant without disturbing the pellet.

t. Resuspend cell pellet in **an appropriate volume** of Post-Hyb Resuspension Buffer B. Pipette mix 5x and maintain sample on ice.

The buffer volume will depend on the targeted cell recovery and the cell stock concentration and can be calculated following the steps below:

- See Cell Suspension Volume Calculator on page 80 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 ~50% cell recovery* during the washing step, and the desired cell stock concentration. See the example calculation below.

⚠ ***50% 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
Total # of cells pooled: 10×10^6
Desired Cell Stock Concentration: 2,000 cells/µl
Volume of Resuspension Buffer
$= \frac{\text{Total \# of Cells Pooled x Expected Recovery Rate (50\%)}}{\text{Desired Cell Stock Concentration}}$
$= \frac{10 \times 10^6 \times 0.50}{2{,}000 \text{ cells/µl}}$
=2.5 ml

Version Specific

u. Pass the sample through an appropriate 30 µm/40 µm filter into a new appropriate sized tube and **place on ice**.

DO NOT use >10×10^6 cells per filter and keep the concentration below 10,000 cells/µl.

Filtration Steps:

- Pre-wet the filter membrane by adding **100 µl** Post-Hyb Resuspension Buffer B and rotating the filter until the membrane is fully saturated.
- Filter samples in at least **400 µl** Post-Hyb Resuspension Buffer B.
- Hold the pipette tip on top of the filter. DO NOT touch the filter.

  Without touching the filter, slowly pipette onto the filter. Tap gently to help initiate filtration if needed or if liquid remains at the end of the filter.
- Wash once by adding **400 µl** Post-Hyb Resuspension Buffer B onto the same filter. Tap gently to help initiate filter if needed. Collect sample in the same tube.
- To maximize recovery, residual volume can be pipetted from underneath the filter.

TIPS

See Sample Filtration on page 36 for guidance on pipetting technique.

v. Optional. If the samples need to be concentrated before counting, centrifuge at **850 rcf** for **5 min** at **4°C** and carefully remove only a fraction of the supernatant, and pipette thoroughly to resuspend the cell pellet in the remaining volume.

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

TIPS

See Tips & Best Practices for Cell Counting on page 37.

As concentrated cell suspensions are required when targeting very high cell loads, a serial dilution may be needed to accurately determine cell concentration and to be within the range of the counter being used. If cell clumping is observed, pipetting the suspension vigorously and recounting is recommended.

STOP

x. 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 78 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 **step q** (Prepare Post-Hyb Resuspension Buffer B) to wash the sample once before proceeding to step 4.1. 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 during the pooling step Pooled Wash workflow step 3.1A.

- **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

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

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

Sample 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.
A-A01	1,200 cells/µl	318,000	238,500	238,500/1,200 = 198.75
A-A02	1,600 cells/µl	424,000	238,500	238,500/1,600 = 149.06
A-A03	900 cells/µl	238,500	238,500	238,500/900 = 265.0
A-A04	1,900 cells/µl	503,500	238,500	238,500/1,900 = 125.52

Option 3.1 B: Individual Wash Workflow

- This workflow will allow an increased flexibility to store samples after washing and later change how samples are pooled before loading the chip.
- This workflow can also be used for running singleplex reactions by following all the washing steps, skipping pooling, and then continuing with the rest of the steps.
- Before starting, see Post-Hybridization Pooling & Washing Guidance on page 33.

TIPS

- During the centrifugation steps, up to 30 µl supernatant may be left behind if working with <300,000 cells. See Centrifugation & Supernatant Removal on page 34. If uncertain about the volume remaining in the tube, add 30 µl water/PBS to an empty tube/well of the same size and use it for a visual estimation.
- Incubation can be performed in a thermomixer with heated lid and no shaking, an oven, or a thermal cycler.

  If using a thermal cycler, ensure that the lid temperature is also set at the appropriate temperature. If using a thermal cycler for plates, ensure that the plate wells are in alignment with the thermal block. See 96-well Plates and Incubation on page 30 for more information.

  If using an oven, ensure that it is properly equilibrated to appropriate temperature before starting incubation.
- If using a plate, a divided reagent reservoir can also be used during washing.

a. Prepare appropriate volume of Post-Hyb Wash Buffer B. Add reagents in the order listed.

Vortex and keep at **room temperature**. DO NOT keep at 4°C.

	Post-Hyb Wash Buffer B	PN	1X* + 10% (ml)	16X* + 10% (ml)	96X* + 10% (ml)	384X* + 10% (ml)
	Nuclease-free Water	-	0.792	12.672	76.032	304.128
	Enhancer *Heat at 65°C for 10 min.* *Vortex and verify no precipitate.* *Add warm to the mix.*	2000482	0.044	0.704	4.224	16.896
	Conc. Post-Hyb Buffer B	2001308	0.044	0.704	4.224	16.896
	Total	-	**0.88**	**14.08**	**84.48**	**337.92**

**X = number of sample tubes/wells. Volumes are in ml.*

A calculator for the above table is included in the web version of this document.

b. Remove tubes/plates from the thermomixer/oven/thermal cycler after Barcode Oligo Hybridization. Keep at **room temperature** for **10 min** before proceeding to next step.

c. Remove plate seal (for plates) and add **200 µl** Post-Hyb Wash Buffer B to each tube/well. Pipette mix 5x.

d. Apply plate seal (for plates) and centrifuge at **850 rcf** for **10 min** at **room temperature**.

e. Remove the seal (for plates) and remove the supernatant without disturbing the pellet.

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

f. Add **200 µl** Post-Hyb Wash Buffer B to each tube/well. Pipette mix 5x.

g. Apply the seal (for plates) and incubate at **37°C** for **10 min**.

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

i. Remove the seal (for plates) and remove the supernatant without disturbing the pellet.

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

j. Add **200 µl** Post-Hyb Wash Buffer B to each tube/well. Pipette mix 5x.

k. Apply plate seal (for plates) and incubate at **37°C** for **10 min**.

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

m. Remove the seal (for plates) and remove the supernatant without disturbing the pellet.

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

n. Add **200 µl** Post-Hyb Wash Buffer B to each tube/well. Pipette mix 5x.

o. Apply plate seal (for plates) and incubate at **37°C** for **10 min**.

p. Prepare Post-Hyb Resuspension Buffer B. Add reagents in the order listed. Pipette mix 10x and maintain at **4°C.**

	Post-Hyb Resuspension Buffer B	PN	1X* + 10% (ml)	16X* + 10% (ml)	96X* + 10% (ml)	384X* + 10% (ml)
	Nuclease-free Water	-	1.045	16.72	100.32	401.28
●	**Conc. Post-Hyb Buffer B**	2001308	0.055	0.88	5.28	21.12
	Total	-	**1.1**	**17.6**	**105.6**	**422.4**

**X = number of samples wells/tubes. Volumes are in ml.*

A calculator for the above table is included in the web version of this document.

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

r. Remove the supernatant without disturbing the pellet.

s. Resuspend each sample pellet in **100 µl* (for multiplex)** or **400 µl (for singleplex) chilled** Post-Hyb Resuspension Buffer B. Pipette mix 5x.

*Optional: Nuclei or other sample types prone to clumping can be filtered using an appropriate 30 µm plate-filter before pooling. Place the plate-filter on a new 96-well v-bottom plate, add samples in 100 µl Post-Hyb Resuspension Buffer B to the filter, secure the lid, and centrifuge at 850 rcf for 1 min at 4°C. Rinse filter with another 100 µl chilled Post-Hyb Resuspension Buffer B using the same collection plate. Centrifuge at 850 rcf for 1 min at 4°C. After centrifugation, remove the plate-filter and lid. Pipette mix the filtered sample and proceed to the next step.

t. Store the sample (see Sample Storage below) or immediately proceed to next step.

Samples can be stored at **−80°C** for up to **12 months**.

If storing samples in the plate, a 96-well plate lid will be needed after the plate is properly sealed. See GEM-X Flex v2 - Protocol Planner (CG000832) for recommended plate lids.

For singleplex workflow, skip step u and directly proceed to step v.

u. Sample Pooling for Multiplex:

- Determine cell concentration of the sample using an automated cell counter or a hemocytometer.
  See Tips & Best Practices for Cell Counting on page 37. As concentrated cell suspensions are required when targeting very high cell loads, a serial dilution may be needed to accurately determine cell concentration.
- Pool an equal number of cells from different hybridization reactions into a new 15-ml or 50-ml tube. Invert or pipette mix the tube.

v. Pass the sample through an appropriate 30 µm/40 µm filter into a new appropriate sized tube and **place on ice**.

DO NOT use >10 x 10^6 cells per filter and keep the concentration below 10,000 cells/µl.

Filtration Steps:

- Pre-wet the filter membrane by adding **100 µl** Post-Hyb Resuspension Buffer B and rotating the filter until the membrane is fully saturated.
- Filter samples in at least **400 µl** Post-Hyb Resuspension Buffer B.

- Hold the pipette tip on top of the filter. DO NOT touch the filter.

  Without touching the filter, slowly pipette onto the filter. Tap gently to help initiate filtration if needed or if liquid remains at the end of the filter.

- Wash once by adding **400 µl** Post-Hyb Resuspension Buffer B onto the same filter. Tap gently to help initiate filter if needed. Collect sample in the same tube.

- To maximize recovery, residual volume can be pipetted from underneath the filter.

  TIPS See Sample Filtration on page 36 for guidance on pipetting technique.

w. Optional. If the pooled samples need to be concentrated before counting, centrifuge at **850 rcf** for **5 min** at **4°C** and carefully remove only a fraction of the supernatant, and pipette thoroughly to resuspend the cell pellet in the remaining volume.

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

TIPS See Tips & Best Practices for Cell Counting on page 37.

STOP **y.** 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 78 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 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 **3.1B step p** (Prepare Post-Hyb Resuspension Buffer B) to wash the sample once before proceeding to the step 4.1. Samples may undergo a color change during storage (e.g. black, light gray, or green), however this will not impact assay performance.

Step 4:

GEM Generation and Barcoding

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4.5 GEM Incubation 86

4.0 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					
☐		GEM-X RTL Gel Beads v2	2001482	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 Only needed if storing washed undiluted sample from the previous step.	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, reheat at 65°C to ensure enhancer is fully dissolved.	−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 (3.1) for sample dilution.*	—	Additional buffer can be prepared using the buffer preparation table in step 2.1.	4°C

Item		10x PN	Preparation & Handling	Storage
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
☐	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.	—

4.1 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. Add reagents in the order listed. Pipette mix 15x and centrifuge briefly.

	GEM Master Mix	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. **For targeting ≤320,000 cells:** Add 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. See Cell Suspension Volume Calculator on page 80 for details.

For targeting >320,000 cells: Depending upon the cell number, transfer an appropriate volume of the sample to a new tube. Centrifuge at **850 rcf** for **5 min** at **4°C**. Remove an appropriate volume of supernatant so that 34.7 µl is left behind. See the example calculation below.

Ensure that the samples are pipette mixed thoroughly right before loading the chip.

Example Calculation for Targeting >320,000 Cells

# of cells to be loaded = Target Cell Recovery x Recovery Factor (1.45)

= 500,000 x 1.45 = 725,000

Volume of Sample to be Centrifuged

$$= \frac{\text{\# of Cells to be Loaded}}{\text{Sample Concentration after Pooling}}$$

$$= \frac{725{,}000}{10{,}000 \text{ cells/µl}}$$

=72.5 µl

Supernatant Volume to be Removed = 72.5 µl - 34.7 µl =**37.8 µl**

*Use the Post-Hyb Resuspension Buffer B prepared at the previous step (3.1) for sample dilution. Additional buffer can be prepared using the buffer preparation table in step 3.1.

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 *GEM Incubation on page 86**.*

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.

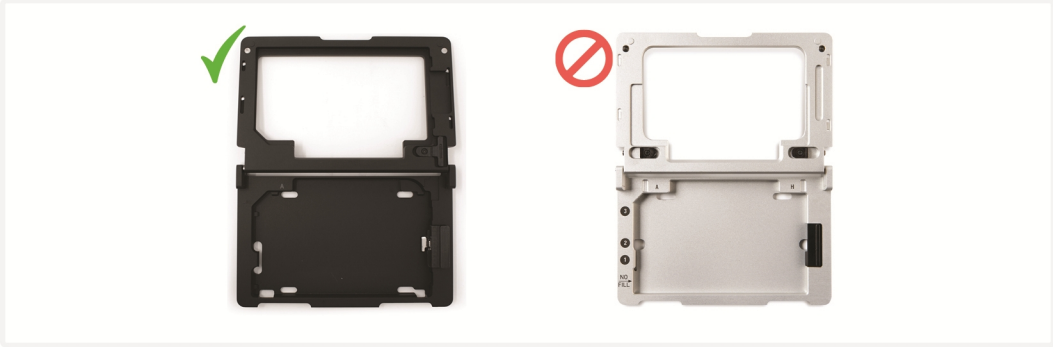
Cell Suspension Volume Calculator

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								
	10000	20000	40000	60000	80000	120000	160000	240000	320000
2000	7.3	14.5	29.0						
	27.4	20.2	5.7						
3000	4.8	9.7	19.3	29.0					
	29.9	25.0	15.4	5.7					
4000	3.6	7.3	14.5	21.8	29.0				
	31.1	27.4	20.2	12.9	5.7				
5000	2.9	5.8	11.6	17.4	23.2				
	31.8	28.9	23.1	17.3	11.5				
6000	2.4	4.8	9.7	14.5	19.3	29.0			
	32.3	29.9	25.0	20.2	15.4	5.7			
7000	2.1	4.1	8.3	12.4	16.6	24.9	33.1		
	32.6	30.6	26.4	22.3	18.1	9.8	1.6		
8000	1.8	3.6	7.3	10.9	14.5	21.8	29.0		
	32.9	31.1	27.4	23.8	20.2	12.9	5.7		
9000	1.6	3.2	6.4	9.7	12.9	19.3	25.8		
	33.1	31.5	28.3	25.0	21.8	15.4	8.9		
10000	1.5	2.9	5.8	8.7	11.6	17.4	23.2		
	33.2	31.8	28.9	26.0	23.1	17.3	11.5		
11000	1.3	2.6	5.3	7.9	10.5	15.8	21.1	31.6	
	33.4	32.1	29.4	26.8	24.2	18.9	13.6	3.1	
12000	1.2	2.4	4.8	7.3	9.7	14.5	19.3	29.0	
	33.5	32.3	29.9	27.4	25.0	20.2	15.4	5.7	
13000	1.1	2.2	4.5	6.7	8.9	13.4	17.8	26.8	
	33.6	32.5	30.2	28.0	25.8	21.3	16.9	7.9	
14000	1.0	2.1	4.1	6.2	8.3	12.4	16.6	24.9	33.1
	33.7	32.6	30.6	28.5	26.4	22.3	18.1	9.8	1.6
15000	1.0	1.9	3.9	5.8	7.7	11.6	15.5	23.2	30.9
	33.7	32.8	30.8	28.9	27.0	23.1	19.2	11.5	3.8
16000	0.9	1.8	3.6	5.4	7.3	10.9	14.5	21.8	29.0
	33.8	32.9	31.1	29.3	27.4	23.8	20.2	12.9	5.7
17000	0.9	1.7	3.4	5.1	6.8	10.2	13.6	20.5	27.3
	33.8	33.0	31.3	29.6	27.9	24.5	21.1	14.2	7.4
18000	0.8	1.6	3.2	4.8	6.4	9.7	12.9	19.3	25.8
	33.9	33.1	31.5	29.9	28.3	25.0	21.8	15.4	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.



TIPS

See Tips & Best Practices on page 28 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 to dispense 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 83 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.

Chip in Chromium X Series Chip Holder
Chromium X Series Chip Holder with Gasket
Assembled Chip
Clip
Representative chip image

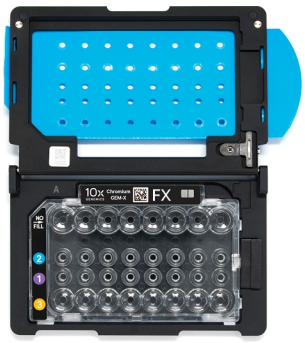
4.2 Load GEM-X Chip

TIPS

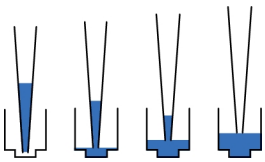
- After removing chip from the sealed bag, use in **≤24 h**.
- Open the lid (gasket attached) of the assembled chip and lay flat for loading.
- Ensure that the Gel Beads are properly thawed and ready to use.
- When loading the chip, raising and depressing the pipette plunger should each take **~5 sec**. 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



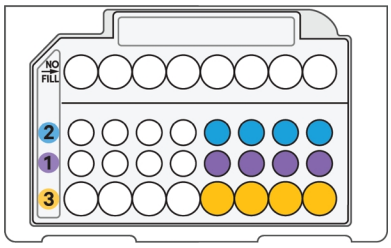
The Chromium X Series Chip Holder, Chip Gasket X/iX, and GEM-X chip images shown below are representative and do not show the specific color & label. Chip holder and gasket should be black and blue in color, respectively. Refer to *Assemble GEM-X FX Chip on page 81* *for details.*

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.

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

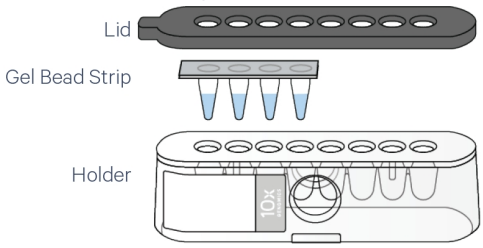
Glycerol
in GEM-X FX Chip



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.

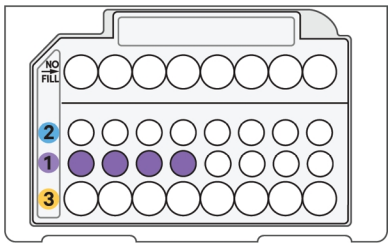
Prep Gel Beads



c. Load Row Labeled 1

- With pipette set to 60 µl, gently pipette mix the GEM Master Mix + Sample 15x. *For >320,000 cells, pipette mix additional 10x.*
- 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**.

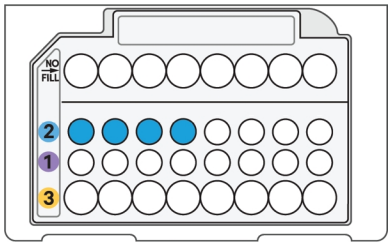
60 µl Master Mix + Cell Suspension in GEM-X FX Chip



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

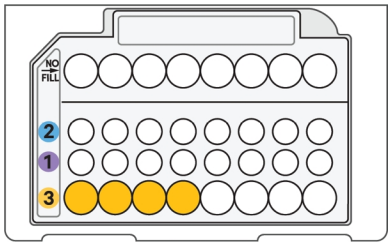


e. Load Row Labeled 3

- 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.

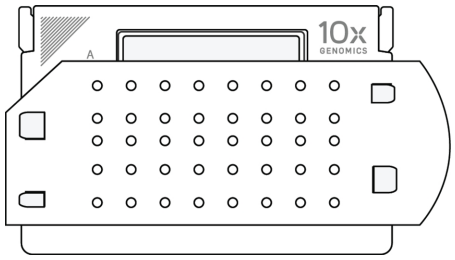
250 µl Partitioning Oil B in GEM-X FX Chip



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.* DO NOT hold the chip holder by its lid when moving to and from the instrument.

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.

4.3 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.

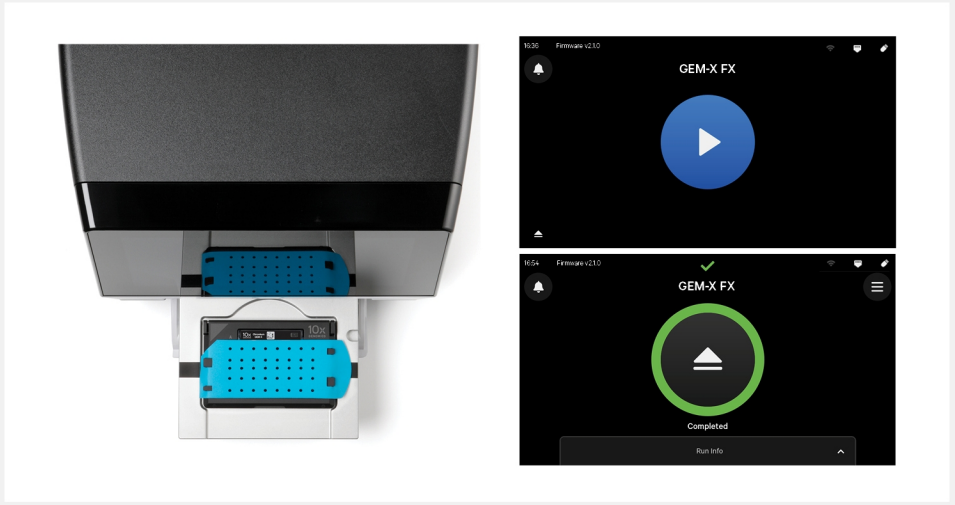
a. Press the eject button on the Chromium X/iX to eject the tray.
If the eject button is not touched within **1 min**, tray will close automatically. System requires a few seconds before the tray can be ejected again.

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



4.4 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

4.5 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 78 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 3.1A-q (for Pooled Wash workflow) or 3.1B-p (for Individual Wash workflow) *of Post-Hybridization Wash step, completing all subsequent steps before proceeding to step 4.1. Samples may undergo a color change during storage (e.g. black, light gray, or green), however this will not impact assay performance.*

Step 5:

GEM Recovery and Pre-Amplification

5.0 Get Started 89

5.1 Post-GEM Incubation – Recovery 90

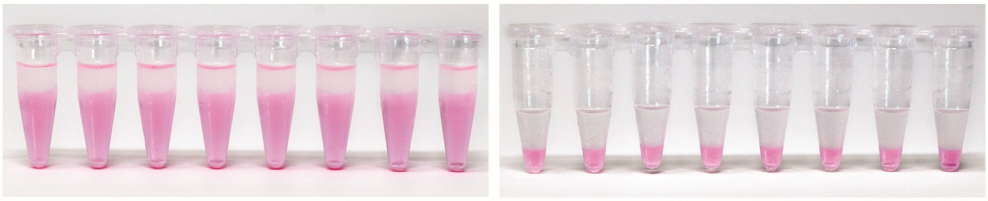
5.2 Pre-Amplification PCR 91

5.3 DNA Cleanup – SPRIselect 92

5.0 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 B *Verify name & PN*	2000529	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.	—

5.1 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.

5.2 Pre-Amplification PCR

a. Prepare Pre-Amplification Mix on ice. Add reagents in the order listed. Vortex and centrifuge briefly.

	Pre-Amplification Mix	PN	1X (µl)	1X + 10% (µl)	4X + 10% (µl)
○	**Amp Mix C**	2001311	25.0	27.5	110.0
●	**Pre-Amp Primers B** Verify name & PN	2000529	10.0	11.0	44.0
	Total		**35.0**	**38.5**	**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.

5.3 DNA Cleanup – SPRIselect

a. Prepare Elution Solution. Add reagents in the order listed. Vortex and centrifuge briefly.

	Elution Solution	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.

Step 6:

GEM-X Flex v2 – Gene Expression Library Construction

6.0 Get Started 94

6.1 Sample Index PCR 95

6.2 Post Sample Index PCR Size Selection – SPRIselect 98

6.3 Post Library Construction QC 99

6.0 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.	—
☐		EvaGreen Plus Dye, 20X in Water *Needed for optional Cycle Number Determination using qPCR*	—	Manufacturer's recommendations.	Ambient
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

- 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.
- Determine sample index PCR cycle numbers using one of the following methods (See Sample Index PCR Cycles on page 46 for guidance on which method to use):

  Using qPCR: See Optional: Gene Expression Library Cycle Number Determination Using qPCR on page 115

  Using Targeted Cell Recovery: Use the table below for guidance:

Targeted Cell Recovery	Cell Lines/Dissociated Tumor Cells	PBMCs/ Nuclei/Cells from Fixed & Dissociated Tissue	Leukocytes/Nuclei from FFPE Tissue
500-2,000	11	15	16
2,000-4,000	10	14	15
4,000-7,000	9	13	14
7,000-12,000	8	12	13
12,000-25,000	7	11	12
25,000-50,000	6	10	11
50,000-128,000	5	9	10
128,000-250,00	5	8	9
250,000-320,000	5	7	8
For >320,000 cells, set up 4 separate SI-PCR reactions for each sample using the cycle number provided below:			
320,000-500,000	5	9	10
500,000-1 x 10^6	5	8	9

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.
For dissociated tumor cells, cycle numbers for cell lines can be used as a starting point.
For dissociated primary cells, cycle numbers for PBMCs can be used as a starting point.

- If targeting ≤320,000 cells, perform sample index PCR in a single tube and add 20 µl sample. One library will be generated per pool if targeting up to 320,000.

- If targeting between 320,000 and 1 x 10^6 cells, prepare four separate SI-PCR Mixes. Add 5 µl of the sample to each tube, effectively splitting the sample into four PCR reactions. Ensure that unique sample indexes are used for each PCR reaction. Four libraries will be generated per pool if targeting over 320,000 cells.

a. Prepare appropriate Sample Index PCR Mix on ice. Add reagents in the order listed.

Amp Mix C volume provided in the GEM-X Flex GEM & Library Kit v2 (4 rxns, PN-1000918) is sufficient for four pools, with 4-split SI-PCR for each pool.

For Targeting up to 320,000 Cells

	Sample Index PCR Mix *For up to 320,000 Cells*	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**

For Targeting 320,000–1 x 10^6 cells

Version Specific

For >320,000 cells, set up 4 separate SI-PCR reactions for each sample.

	Sample Index PCR Mix For 320,000 - 1 x 10^6 cells	PN	1X (µl)	1X + 10% (µl)	4X + 10% (µl)
◯	**Amp Mix C**	2001311	50.0	55.0	220.0
	Nuclease-free Water	—	25.0	27.5	110.0
	Total		**75.0**	**82.5**	**330.0**

b. Transfer **appropriate volume** of sample from the step DNA Cleanup – SPRIselect on page 92 to a new tube strip.

The remaining sample can be stored at −20°C for up to 4 weeks, for generating additional libraries.

i. For up to 320,000 cells: Transfer **ONLY 20 µl** sample per reaction

ii. For 320,000 - 1 x 10^6 cells: Transfer **ONLY 5 µl** sample per reaction

c. Add **appropriate volume** of Sample Index PCR Mix to sample.

i. For up to 320,000 cells: Add **60 µl** PCR mix per reaction

ii. For 320,000 - 1 x 10^6 cells: Add **75 µl** PCR mix per reaction

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

e. 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 previously	
6	72°C	00:01:00
7	4°C	Hold

STOP

f. 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.

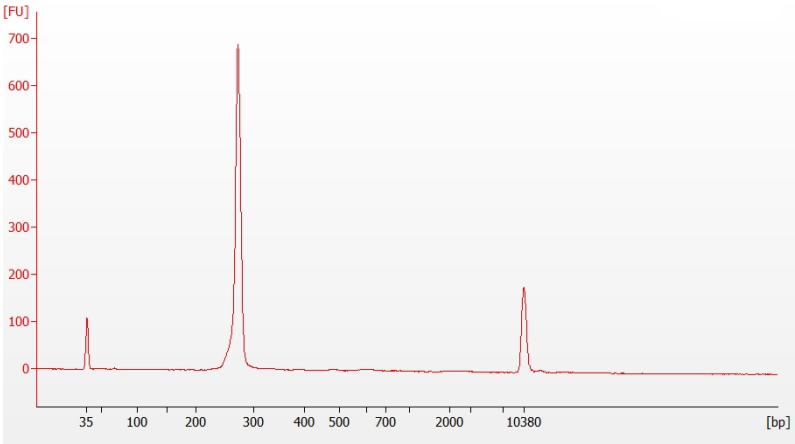
STOP

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 150-350 bp to determine average size of the library.

Representative Trace



If additional peaks (40-180 bp) exceed 5% of the total, repeat step 6.2 Post Sample Index PCR Double Sided Size Selection - SPRIselect. Dilute the sample with EB to bring the library volume to 100 µl before performing step 6.2a. See Troubleshooting on page 104 for guidance on how to determine the percentage of additional peaks.

Alternate QC Method

Agilent TapeStation

LabChip

See Appendix for:

- Post Library Construction Quantification on page 118
- Agilent TapeStation Traces on page 119
- LabChip Traces on page 120

Step 7:

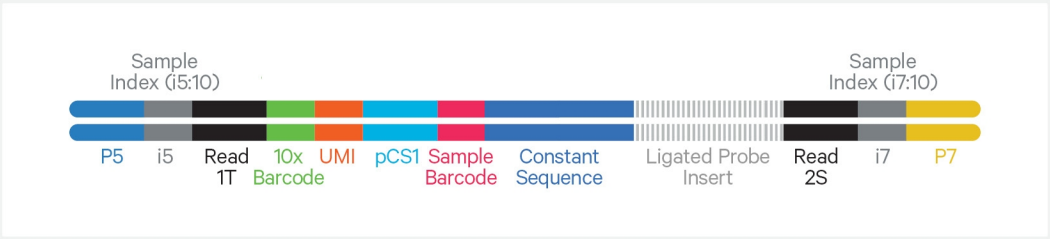
Sequencing

Sequencing Libraries	101
Sequencer Compatibility	101
Sample Indices	102
Library Loading	102
Data Analysis and Visualization	103

Sequencing Libraries

GEM-X Flex v2 – Gene 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 v2 – 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 sequences, and the Sample Barcode that identifies the sample.

Sample Barcode can be sequenced in both Read 1 and Read 2 sequencing configurations. See 10x Genomics support website for library sequencing parameters.

A single library contains reads derived from up to 384 samples, with cell barcodes composed of a Sample Barcode and a 10x GEM Barcode (10x Barcode).

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

Sequencer Compatibility

10x Genomics libraries contain P5 and P7 adaptors, which can be used for Illumina sequencing. These libraries can also be modified to enable sequencing on various long and short-read sequencing platforms, with some platforms requiring third-party analysis tools. For a list of tested sequencers, consult the Sequencer Compatibility for GEM-X Flex Gene Expression page on the 10x Genomics support site.

Some variation in assay performance is expected based on sequencer choice. For more information on sequencing platform compatibility, refer to the 10x Genomics support website.

Sample Indices

Each sample index in the Dual Index Kit TS Set A (PN-1000251) 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.

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.

For a list of sequencers and loading concentrations, consult the 10x Genomics support website.

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, 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

Troubleshooting

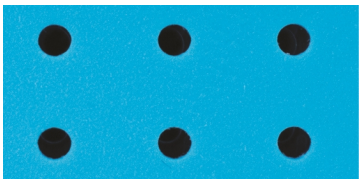
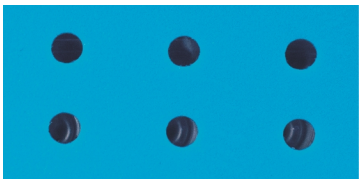
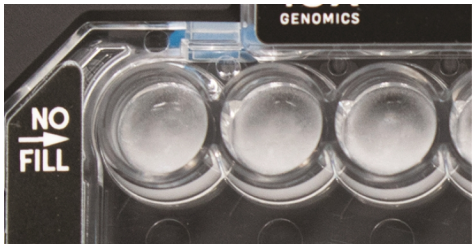
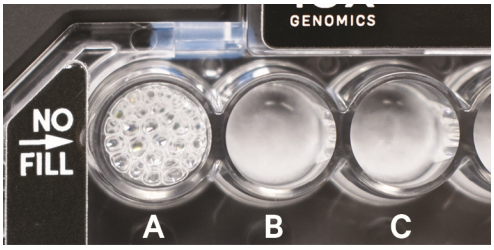
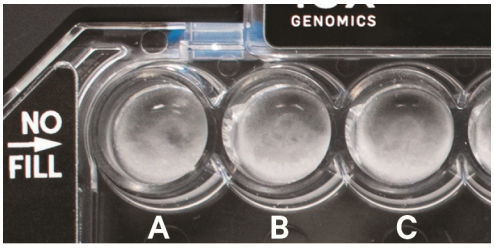
GEM Generation & Barcoding 105

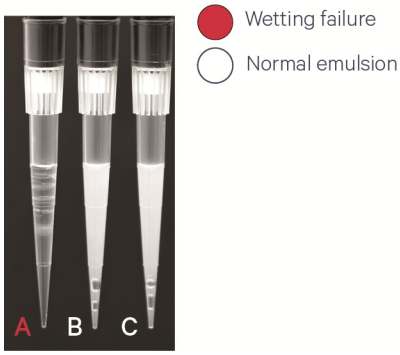
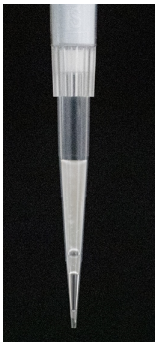
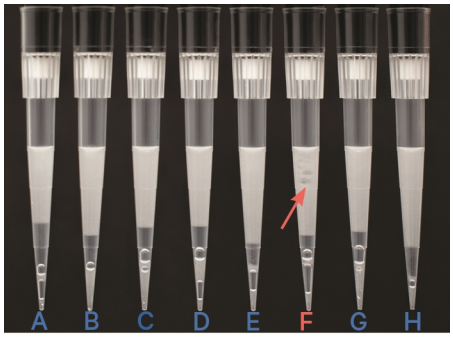
Post Library Construction QC 107

Chromium X Series Errors 109

Post-Storage Gel Formation in Fixed Samples 110

GEM Generation & Barcoding

STEP	Normal	Impacted
4.2 **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.*
4.4 **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.**

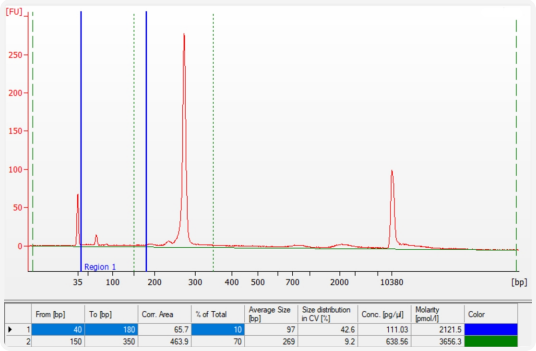
STEP	Normal	Impacted
4.4 **Transfer GEMs from chip**		
	All liquid levels are similar in volume and opacity without air trapped in the pipette tips.	Pipette tip A shows wetting failure. Pipette tips B-C show uniform emulsions & slightly low volumes. (~95 µl) Most wetting failures will not impact emulsion 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).
		
	The image above shows recovery of slightly lower emulsion volume (90-95 µl). This is not a clog, but a pipetting error during chip loading or emulsion recovery. It is acceptable to proceed to the next steps in this case, but the cell recovery may be slightly lower. To prevent this, use a calibrated pipette and follow the loading and recovery instructions closely.	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

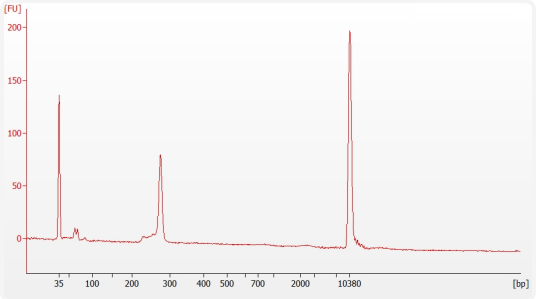
6.3 Post Library Construction QC

Incomplete Clean up



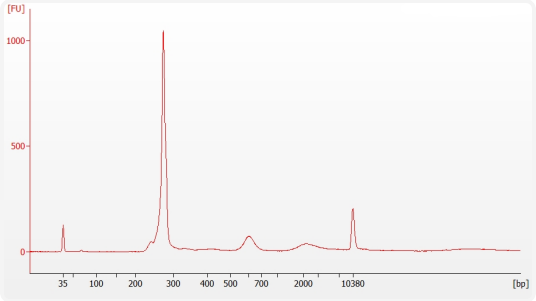
The image above shows incomplete cleanup with additional peaks (40-180 bp) at 10% (4th column) of the total. It is recommended to repeat step 6.2 Post Sample Index PCR Size Selection - SPRIselect.

Under Cycling



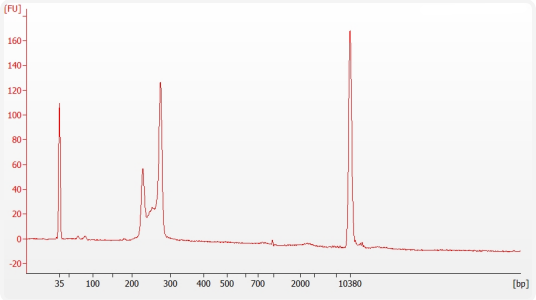
A higher proportion of low molecular weight species in the library trace and a library concentration of <50 nM indicates under cycling.

Over Cycling



Additional higher molecular weight peaks indicate over cycling.

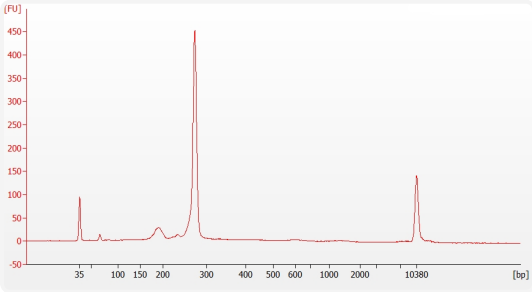
Incomplete Sample Indexing



6.3 Post Library Construction QC

A high abundance of partially indexed material (peak at 230 bp) indicates incomplete indexing. When targeting >320,000 cells or when cycle number determination using qPCR indicates 5 cycles or less for any number of cells targeted, prepare four SI-PCR reactions using 5 µl sample per reaction and a minimum of 5 cycles.

Presence of singlet unligated probes



An additional peak (~190 bp) might appear from singlet unligated probes, especially in samples with low RNA content. This peak can also occasionally be seen if the sample has a lot of debris or if the post-hybridization washes were not performed optimally. Repeating the SPRIselect cleanup is unlikely to remove this additional peak. However, the data generated should still be suitable for subsequent analysis.

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.

Post-Storage Gel Formation in Fixed Samples

Occasionally, minor gel formation may be observed during post-storage processing of fixed sample stored at -80°C. This observation is not related to specific sample types or storage duration. Appearance of gel is significantly larger than expected size of the cell pellet. Proceeding with the downstream workflow without removing the gel may impact assay sensitivity and increase sample loss. If gel is observed after first centrifugation step during post-storage processing, removing the gel using the following guidance is highly recommended.

a. Using a pipette tip, remove as much of the supernatant as possible without removing the gel.

b. Resuspend the sample along with gel in **500 µl** 0.5X PBS (no BSA, no RNase Inhibitor).

c. Incubate for **10 min** at **42°C**.

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

e. Remove the supernatant without touching the pellet.

If gel is still present, repeat steps b-e.

f. Resuspend the final cell pellet in Quenching Buffer B and proceed with workflow.

Based on our limited testing, data from these samples will not be negatively impacted when performing the above steps to remove gel.

Appendix

Probe & Barcode Oligo Hybridization for Subpooling 112

Expected Cell Concentration at Different PBS Dilution 113

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Optional: Gene Expression Library Cycle Number Determination Using qPCR 115

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Probe & Barcode Oligo Hybridization for Subpooling

Subpooling one sample across multiple Sample Barcodes will require minor modifications during Probe and Barcode Oligo Hybridizations. This section provides an overview of those modifications and key considerations when planning subpooling. Detailed buffer volumes are not included in this section.

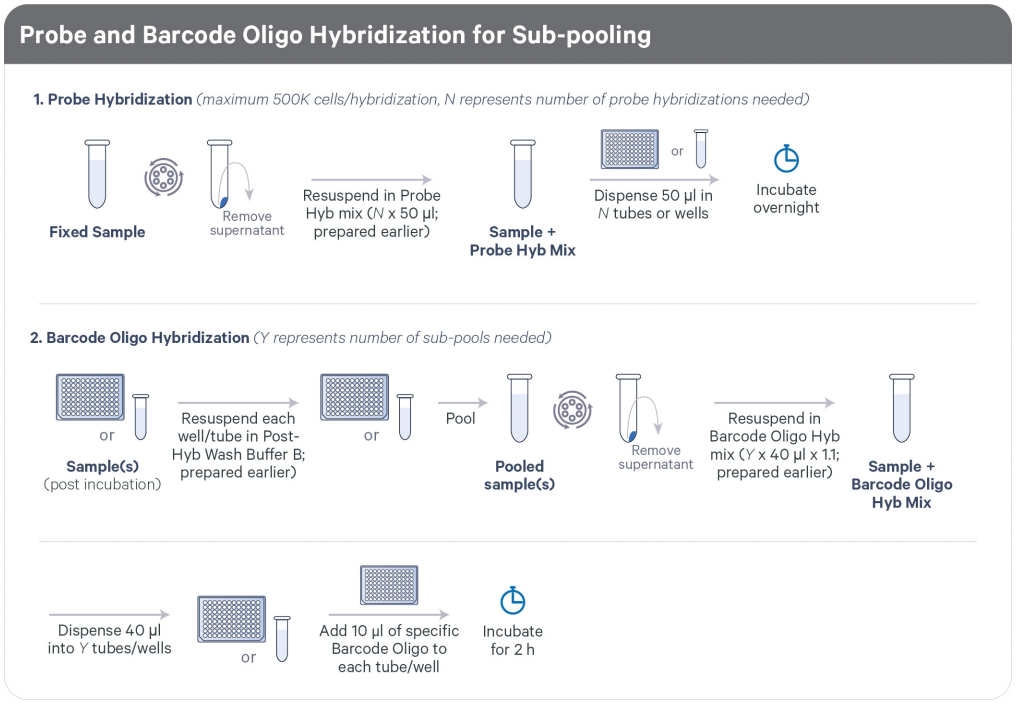
- Determine the number of probe hybridizations needed so that the cell number does not exceed the maximum recommended number for that sample type per hybridization. This can be done by dividing the total number with the maximum recommended number per hybridization.

Example Calculation # of Probe Hybs
Number of Probe Hybridizations
$= \frac{\text{Total \# of Cells}}{\text{Maximum \# in Probe Hybridization}}$
$= \frac{1{,}000{,}000}{500{,}000} = \mathbf{\sim 2}$

- Prepare appropriate Probe Hyb Mix and Barcode Oligo Hyb Mix for sample subpooling as shown in the illustrated overview. Keep the number of cells above 10,000 in the Barcode Oligo Hybridization and follow the Tips listed below.

TIPS

  - *Ensure that the sample cell pellet is completely resuspended in Probe Hyb Mix or Barcode Oligo Hyb Mix. Incomplete resuspension can lead to variability in number of cells per hybridization.*
  - *If resuspending sample in Probe Hyb Mix or Barcode Oligo Hyb Mix for more than 16 hybridizations, start by adding <1 ml Hyb mix to the pelleted sample and pipette mix to resuspend completely. Ensure no clumps are present and then add the remaining Hyb mix for the final volume and pipette mix.*
  - *If dispensing sample + Barcode Oligo Hyb mix to a large number of tubes/wells, mix the suspension periodically to prevent cell settling.*



See Probe Hybridization on page 47 and Barcode Oligo Hybridization on page 53 for details.

Expected Cell Concentration at Different PBS Dilution

This table provides expected approximated cell concentration after diluting in PBS at step 3.1A-d. 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.

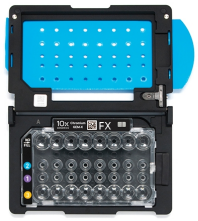
PBS Dilution	~ Concentration at Different Starting Cell # in Barcode Oligo Hybridization			
	50,000	**100,000**	**300,000**	**500,000**
No Dilution	180 cells/µl	360 cells/µl	1,080 cells/µl	1,800 cells/µl
1:2 Dilution (10 µl diluted sample + 10 µl PBS)	90 cells/µl	180 cells/µl	540 cells/µl	900 cells/µl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

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 83 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

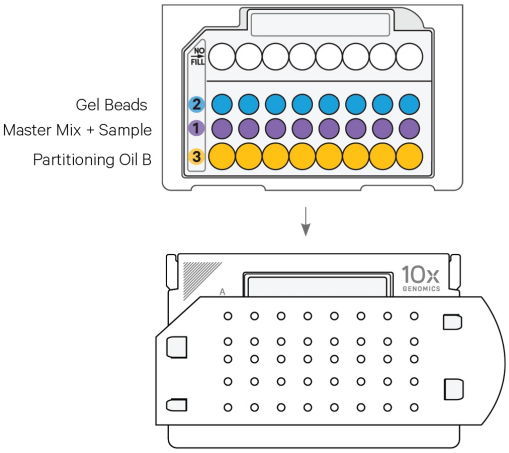
b. **Prepare Gel Beads**
   - Vortex for 30 sec
   - Centrifuge for 5 sec

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 v2 - 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
320,000-1 x 10^6	2	318	320

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.

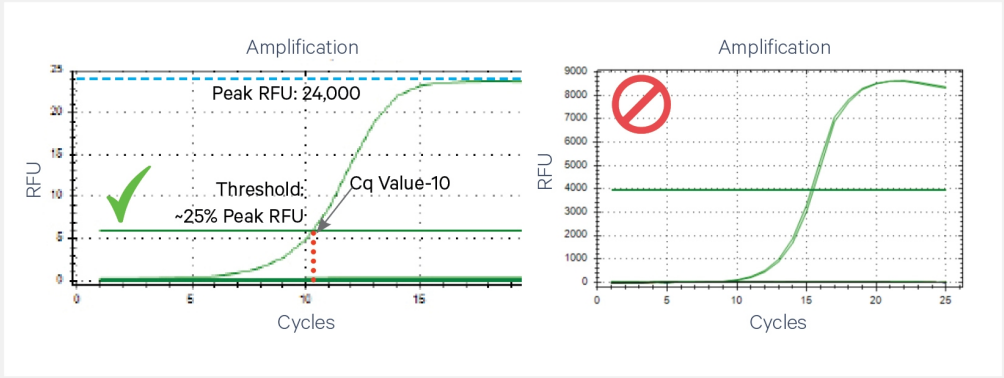
g. Prepare a qPCR system with the following protocol, place the plate in the qPCR system, and start the program.

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)
For >320,000 cells, set up 4 separate SI PCR reactions for each sample using the cycle number provided below:		
320,000-1 x 10^6	Cq value-5	Cq value = 12.6 Rounded Cq value = 13 Cycle no. = 8 (13-5)

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.

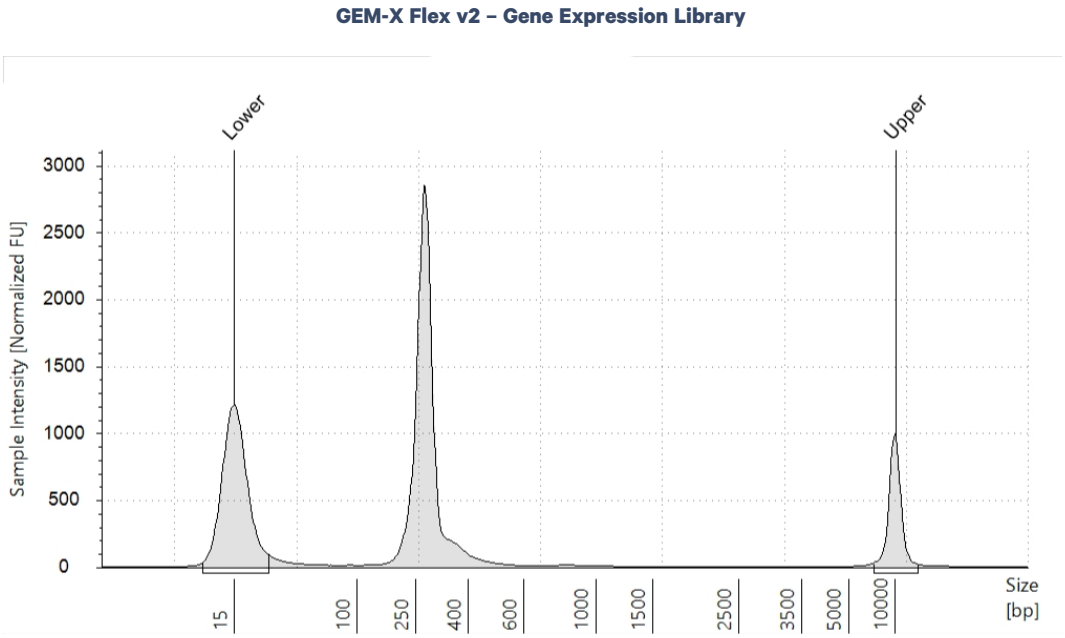
f. Incubate in a thermal cycler with the following protocol.

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.

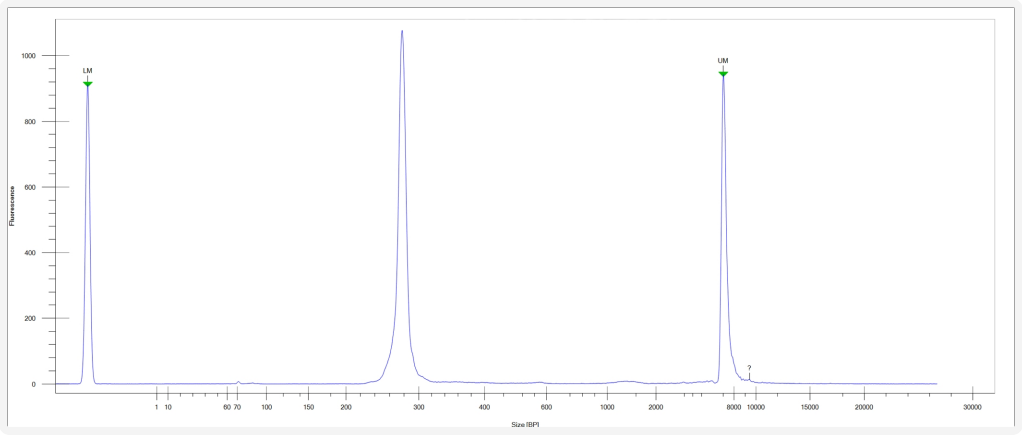


All traces are representative. Samples were run at 1:80 dilution.

LabChip Traces

DNA High Sensitivity Reagent Kit was used.

GEM-X Flex v2 – Gene Expression Library



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

Oligonucleotide Sequences

Gel Bead Primer

Gel Bead Primers

5'-CTACACGACGCTCTTCCGATCT-N16-N12-N(0,3)-TTGCTAGGACCG-3'

Pre-Amp Primers B

Forward Primers

5'-CTACACGACGCTCTTCCGATCT-3'

Reverse Primer

5'-CCTTGGCACCCGAGAATTCCA-3'

Dual Index Kit TS Set A

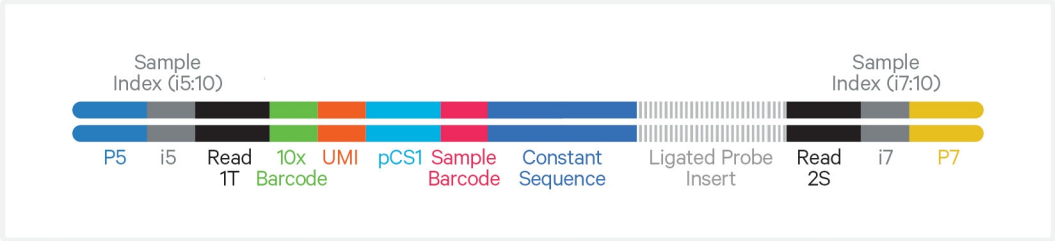
Forward Primers

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTC-3'

Reverse Primers

5'-CAAGCAGAAGACGGCATACGAGAT-N10-GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA-3'

GEM-X Flex v2 – Gene Expression Library

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N12-N(0,3)-TTGCTAGGACCG-BC10-AACCGCGTATTCAGGTTTTCTTATATGGG-Ligated_Probe_insert-TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'

3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12--N(0,3)-AACGATCCTGGC-BC10-TTGGCGCATAAGTCCAAAAGAATATACCC-Ligated_Probe_insert-ACCTTAAGAGCCCACGGTTCCTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'