10x GENOMICS

User Guide | CG000786 | Rev A

GEM-X
Flex Gene Expression Reagent Kits

For Singleplexed Samples

For use with:

GEM-X Flex Sample Preparation v2 Kit, *48 rxns PN-1000781*

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

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

GEM-X Flex Human Transcriptome Probe Kit, *4 samples PN-1000783*

GEM-X Flex Mouse Transcriptome Probe Kit, *4 samples PN-1000784*

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

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.

Notices

Document Number

CG000786 | Rev A

Legal Notices

Instrument & Licensed Software Updates Warranties

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

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Document Revision Summary

Document Number

CG000786 | Rev A

Title

GEM-X Flex Gene Expression Reagent Kits For Singleplexed Samples

Revision

N/A to Rev A

Revision Date

October 09, 2024

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Introduction

GEM-X Flex Reagent Kits

Refer to SDS for handling and disposal information

GEM-X Flex Sample Preparation v2 Kit, 48 rxns PN-1000781*

*This kit provides sufficient reagents to process:

- 48 samples when using the Demonstrated Protocol Fixation of Cells & Nuclei for GEM-X Flex Gene Expression (CG000782)
- 24 samples when using the Demonstrated Protocol Tissue Fixation & Dissociation for GEM-X Flex Gene Expression (CG000783)
- 96 samples when using the Demonstrated Protocol Sample Preparation from FFPE Tissue Sections for GEM-X Flex Gene Expression (CG000784)
- 48 samples when using the Demonstrated Protocol Blood Fixation and Cell Isolation for GEM-X Flex Gene Expression (CG000785)

GEM-X Flex Reagent Bundles for Singleplexing - Human & Mouse

Reagent Kit Bundle	Part Number (Kits)	Components	Component Part Number	Quantity
GEM-X Flex Gene Expression Human, 4 samples	1000792	GEM-X Flex GEM & Library Kit, 4 rxns	1000782	1
		GEM-X Flex Gel Bead Kit, 4 rxns	1000790	1
		GEM-X Flex Hybridization & Wash Kit, 24 rxns	1000789	1
		GEM-X Flex Human Transcriptome Probe Kit, 4 samples	1000783	1
GEM-X Flex Gene Expression Mouse, 4 samples	1000796	GEM-X Flex GEM & Library Kit, 4 rxns	1000782	1
		GEM-X Flex Gel Bead Kit, 4 rxns	1000790	1
		GEM-X Flex Hybridization & Wash Kit, 24 rxns	1000789	1
		GEM-X Flex Mouse Transcriptome Probe Kit, 4 samples	1000784	1

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

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

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

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

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

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

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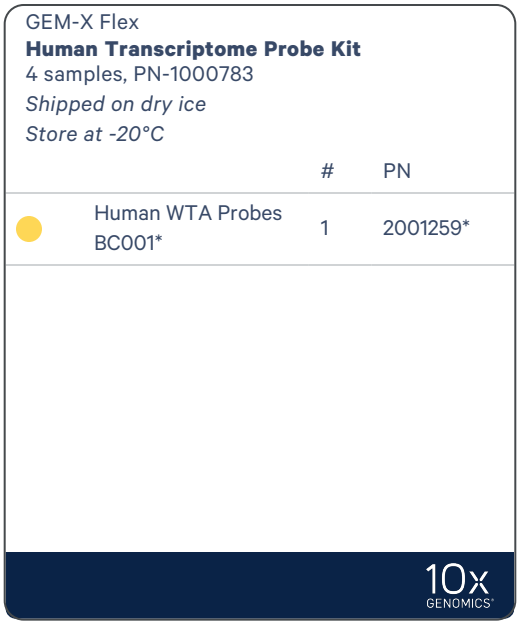
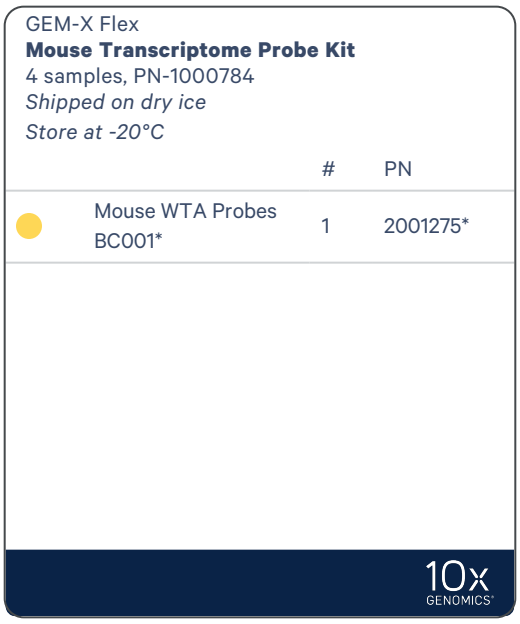
GEM-X Flex Human Transcriptome Probe Kit, 4 samples PN-1000783



*The tube name and part number may vary from BC001 to BC016 and PN-2001259 to 2001274. All probes are expected to perform equivalently.

GEM-X Flex Mouse Transcriptome Probe Kit, 4 samples PN-1000784



*The tube name and part number may vary from BC001 to BC016 and PN-2001275 to 2001290. All probes are expected to perform equivalently.

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

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

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

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

Partitioning Oil B
Shipped at ambient temperature
Store at ambient temperature

		#	PN
◯	Partitioning Oil B	4	2001213

Recovery Agent
Shipped at ambient temperature
Store at ambient temperature

		#	PN
◯	Recovery Agent	4	220016

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

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

10x GENOMICS

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

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

	#	PN
Dual Index Plate TS Set A	1	3000511

10x Genomics Accessories

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

Third-Party Items

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

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

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

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

Protocol Steps & Timing

Steps	Timing	Stop & Store
Sample Fixation	Variable*	STOP 4°C ≤1 week/-80°C ≤12 months
	*Refer to the appropriate Demonstrated Protocols for details.	
Step 1: Probe Hybridization (page 35)		
1.1 Probe Hybridization (page 37)	16-24 h	
Step 2: Post-Hybridization Washing (page 39)		
2.1 Post-Hybridization Wash (page 41)	60 min	STOP -80°C ≤12 months
Step 3: GEM Generation and Barcoding (page 45)		
3.1 Prepare GEM Master Mix + Sample Dilution (page 48)	30 min	
3.2 Load GEM-X Chip (page 52)	10 min	
3.3 Run the Chromium X/iX (page 54)	6 min	
3.4 Transfer GEMs (page 55)	5 min	
3.5 GEM Incubation (page 56)	125 min	STOP 4°C ≤1 week (GEMs) -80°C ≤12 months (washed undiluted sample from step 2.1)
Step 4: GEM Recovery and Pre-Amplification (page 57)		
4.1 Post-GEM Incubation – Recovery (page 59)	10 min	
4.2 Pre-Amplification PCR (page 60)	55 min	STOP 4°C ≤72 h/-20°C ≤1 week
4.3 DNA Cleanup – SPRIselect (page 61)	30 min	STOP 4°C ≤72 h/-20°C ≤4 weeks
Step 5: GEM-X Flex – Gene Expression Library Construction (page 62)		
5.1 Sample Index PCR (page 64)	40 min	STOP 4°C ≤72 h
5.2 Post Sample Index PCR Size Selection – SPRIselect (page 66)	30 min	STOP 4°C ≤72 h/-20°C long term
5.3 Post Library Construction QC (page 67)	60 min	

Sample Preparation

This User Guide is compatible with fixed cells, fixed nuclei, PBMCs and leukocytes derived from fixed blood, cells derived from fixed and dissociated tissue, and nuclei derived from FFPE tissue sections.

1 Sample Fixation

Choose the appropriate protocol for fixation/isolation, depending upon the sample type.

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

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.

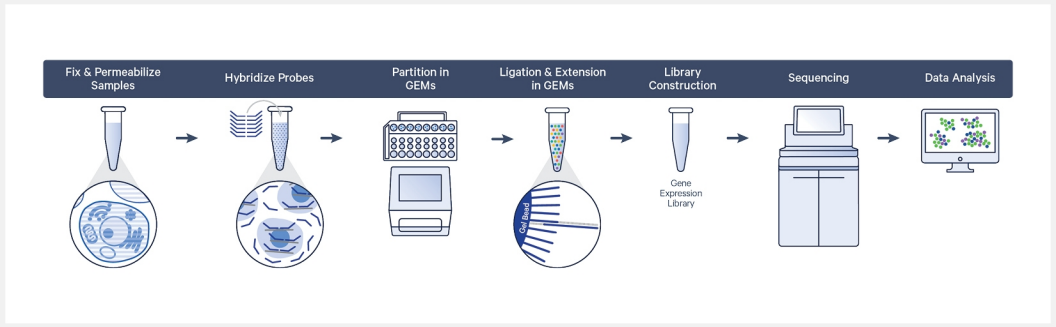
Stepwise Objectives

GEM-X Flex Gene Expression offers comprehensive, scalable solutions to measure gene expression in formaldehyde fixed samples. Gene expression is measured using probe pairs designed to hybridize to mRNA specifically.

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

Inside the GEMs, probes are ligated and the 10x GEM Barcode is added, and all ligated probes within a GEM share a common 10x GEM Barcode. Barcoded and ligated probes are then pre-amplified in bulk, after which gene expression libraries are generated and sequenced.

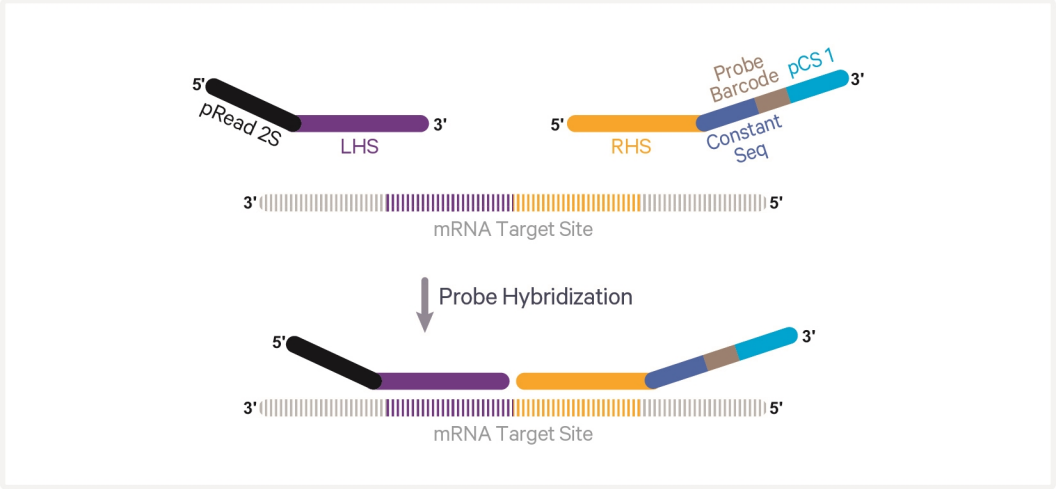
Workflow Overview



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

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.



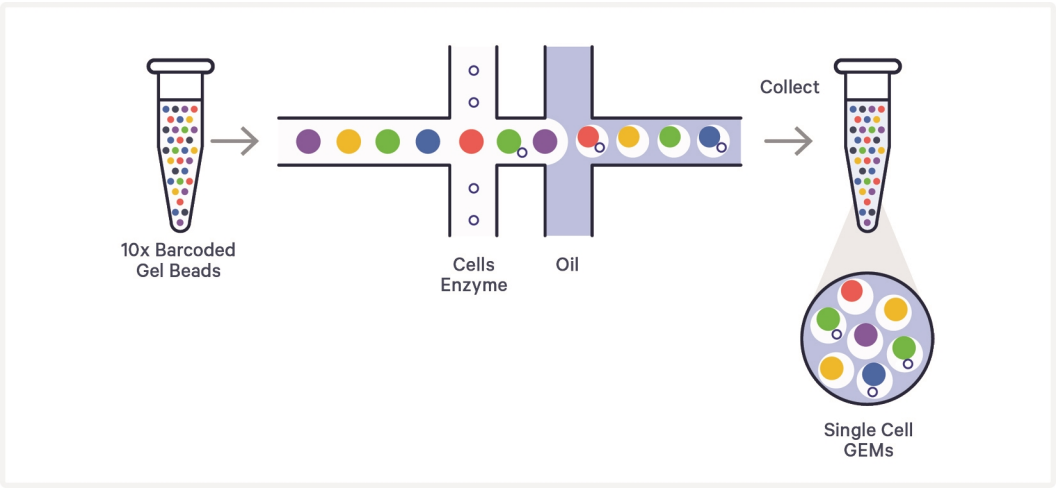
Step 2: Post-Hybridization Washing

After hybridization, the unbound probes are washed off.

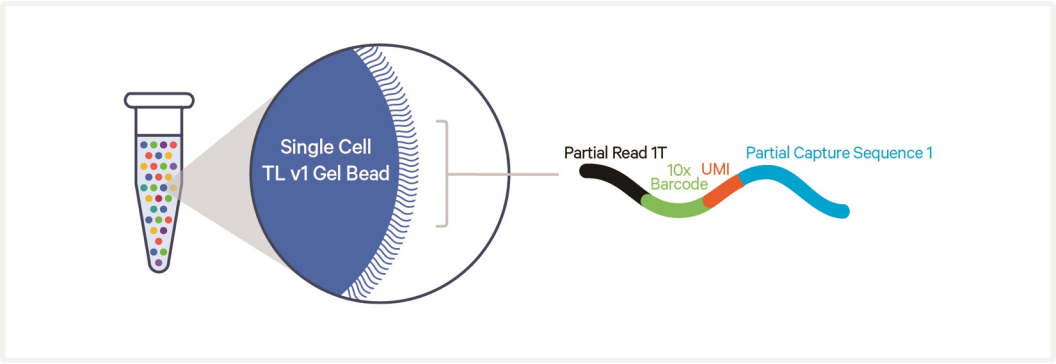
Step 3: GEM Generation & Barcoding

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

GEM Generation

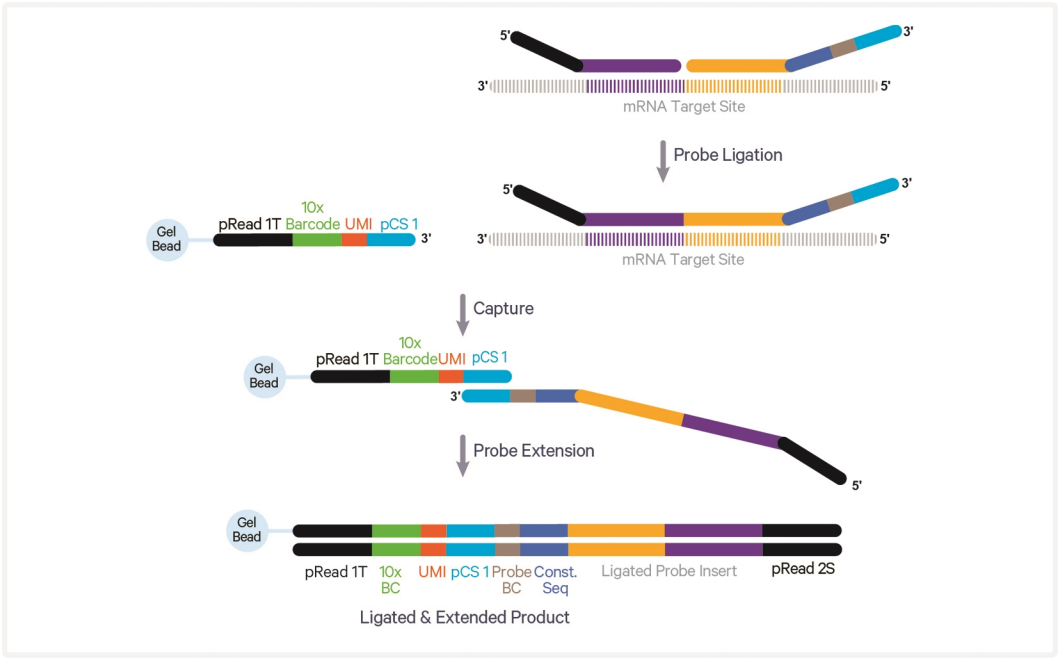


Gel Bead



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

Inside Individual GEMs

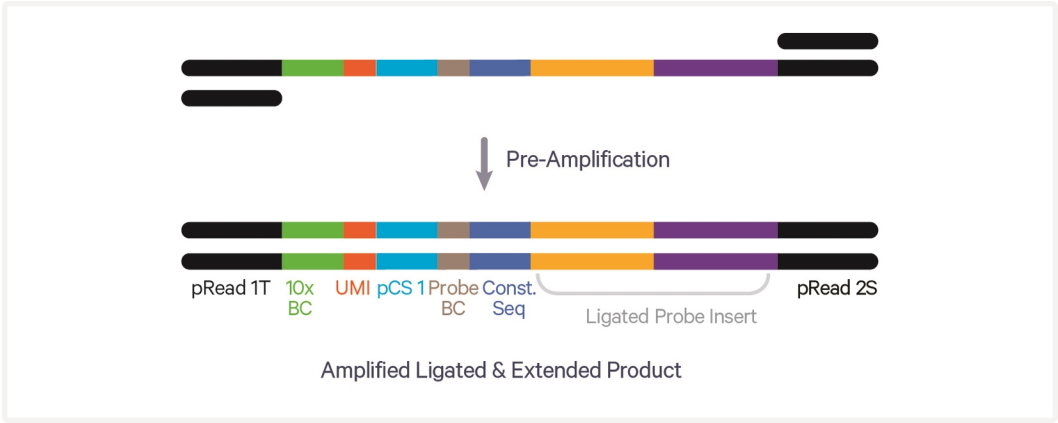


Finally, a heat denaturation step inactivates the enzymes in the GEM reaction.

Step 4: GEM Recovery & Pre-Amplification

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

DNA Pre-Amplification - Gene Expression

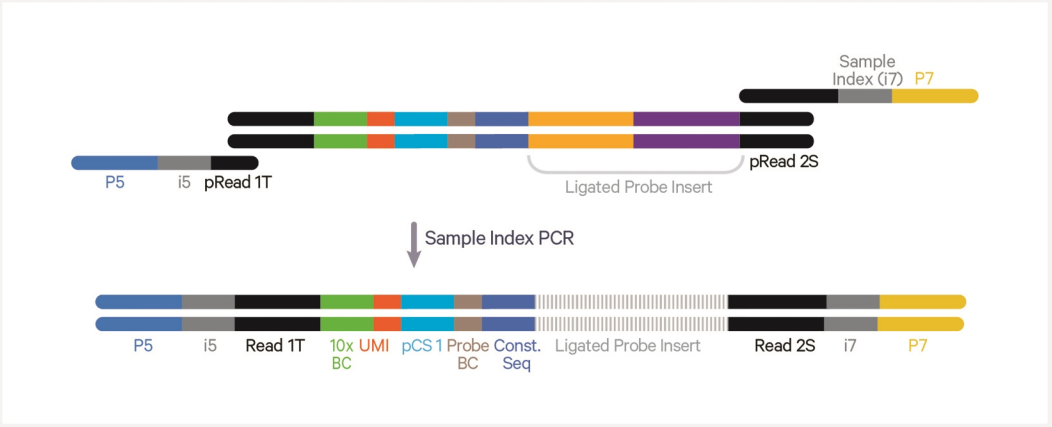


Step 5: GEM-X Flex – Gene Expression Library Construction

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

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

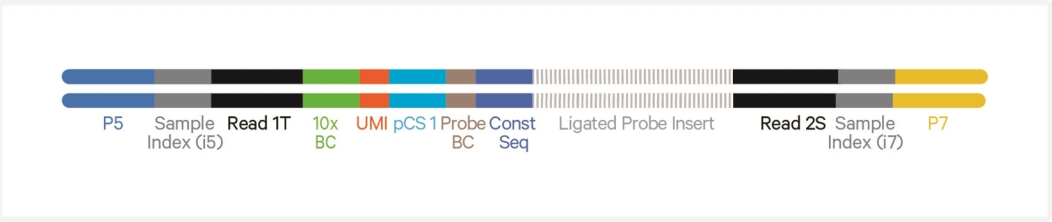
Pooled Amplified DNA Processed in Bulk



Step 6: Sequencing

A GEM-X Flex – Gene Expression library comprises standard Illumina paired-end constructs which begin and end with P5 and P7. The 16 bp 10x GEM Barcode and 12 bp UMI are encoded in Read 1T. Small RNA Read 2 (Read 2S) sequences the ligated probe insert.

GEM-X Flex – Gene Expression Library



See Appendix for Oligonucleotide Sequences on page 84

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Tips & Best Practices

Icons

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

GEM-X specific steps

Emulsion-safe Plastics

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

General Reagent Handling

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

Pipette Calibration

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

Probe Hybridization

Sample Input

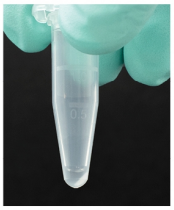
- Minimum sample input for hybridization is **25,000** fixed cells/nuclei and maximum input is **500,000** fixed cells/nuclei. If sufficient cells are available, it is recommended to default to 300,000 fixed cells/nuclei for setting up hybridization.
  However, in case of leukocytes isolated from fixed blood (CG000785), splenocytes (CG000782), and cells from fixed & dissociated spleens and pancreas (CG000783), it is recommended to use ≤100,000 cells, as higher cell loads may lead to a slight decrease in data quality.
- Using <50,000 cells/barcode may make it difficult to target maximum cell load.
- It may be possible to use <25,000 cells/nuclei, but it may lead to:
  - Loss of pellet
  - Not enough cells for storage
  - Not enough cells left after washing to target maximum cell load (20,000 cells/Probe Barcode)
  - Difficulty in counting cells, may require concentration
  - Observance of an atypical library trace and increase in wasted data due to excess supernatant left behind during post-hybridization washes
- Mitigation strategies when using lower cell input
  - Follow better sample preparation practices including use of a swinging bucket rotor.
  - During probe hybridization, up to 15 µl supernatant can be left behind to avoid losing the pellet.
  - During post-hybridization wash, up to 30 µl supernatant can be left behind to avoid losing the pellet.

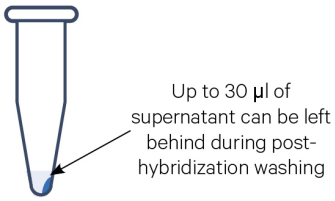
Incubation Time

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

Post-Hybridization Sample Washing & Recovery

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





Cell Counts for Chip Loading

- The GEM-X Flex Gene Expression is designed to target 500-20,000 cells per sample with a per sample undetected multiplet rate of 0.2% to 8.0%.
- Recommended starting point is to target ~4,000 cells, and a multiplet rate of ~1.6%.
- The minimum cell input concentration to get maximum cell recovery is 836 cells/µl.

Undetectable Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~0.2	725	500
~0.4	1,450	1,000
~0.8	2,900	2,000
~1.6	5,800	4,000
~2.4	8,700	6,000
~3.2	11,600	8,000
~4.0	14,500	10,000
~5.0	18,125	12,500
~6.0	21,750	15,000
~7.0	25,375	17,500
~8.0	29,000	20,000

Sample Filtration

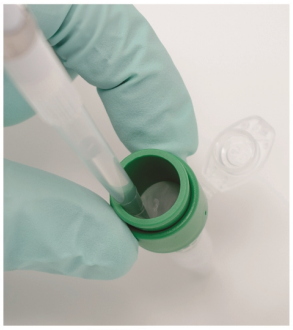
- After post-hybridization wash, pass the sample through a 30 µm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation) into a new 1.5-ml microcentrifuge tube.

  DO NOT use 40 µm Flowmi Tip Strainer for filtration.

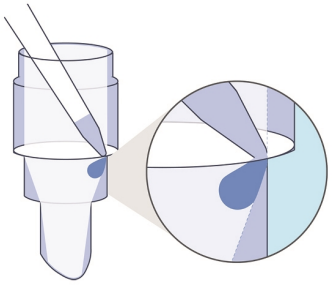
- Hold the pipette tip at an angle and touch the filter membrane where the filter meets the wall. Slowly pipette through the filter. Tap gently or centrifuge briefly if liquid remains at the end of the filter.
- To maximize recovery, residual volume can be pipetted from underneath the filter.

Filtration using 30 µm Filters

Pre-Separation Filters from Miltenyi Biotec



Celltrix Filters from Sysmex



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

Pre-Separation Filters from Miltenyi Biotec

Celltrix Filters from Sysmex

Cell Counting

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

Combination of counters and dyes tested for counting fixed cells/nuclei

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.*

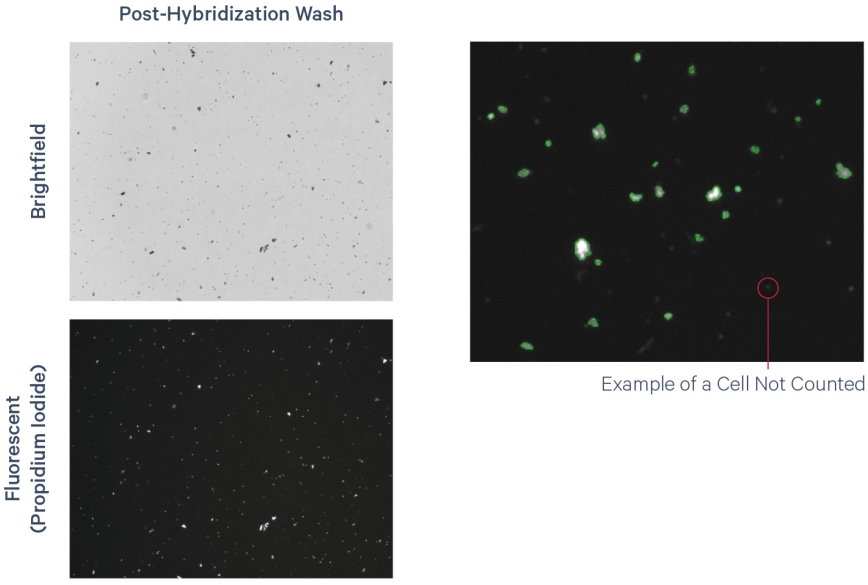
- Focus cells/nuclei under the brightfield before switching to the fluorescent channel.
- Increase exposure time to help adjust signal to noise during counting.
- Do a final visual inspection to confirm the counts are accurate. After obtaining the counts, switch between brightfield and fluorescent channel 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.

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

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

Representative chip image

GEM-X Chip Loading

- Place the assembled chip and holder flat (gasket attached) on the bench with the lid open.
- Dispense at the bottom of the wells without introducing bubbles.
- When dispensing Gel Beads into the chip, wait for the remainder to drain into the bottom of the pipette tips and dispense again to ensure complete transfer.
- See Load GEM-X Chip on page 52 for specific instructions.

Chromium X/iX Firmware

- Firmware version 2.0 or higher is required in the Chromium X/iX to run the GEM-X chips.
- The current firmware version of the instrument can be located using the system menu.

- Consult the Chromium X Series Instrument with Readiness Test User Guide (CG000396) for detailed instructions on updating the firmware.

50% Glycerol Solution for Addition to Unused Chip Wells

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

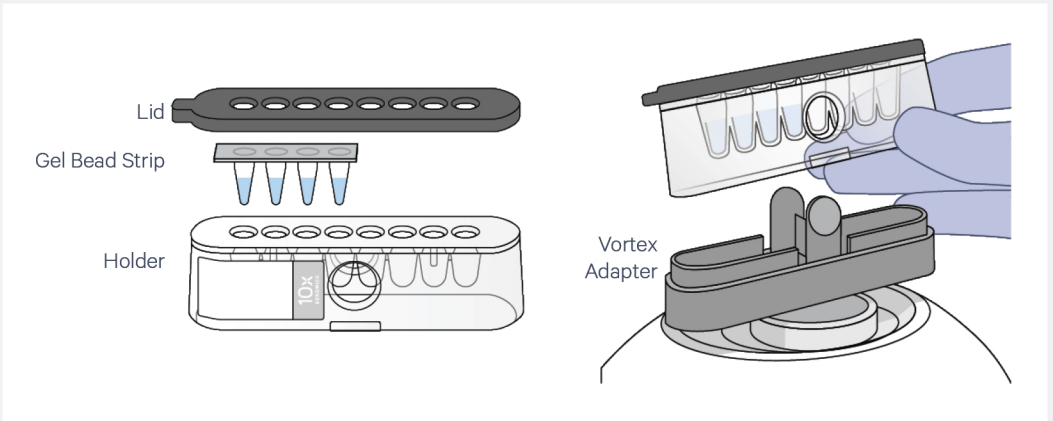
  OR

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

50% Glycerol Solution for Sample Storage

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

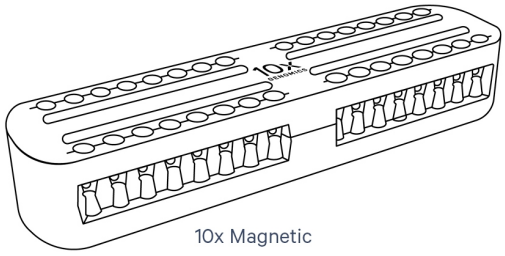
Gel Bead Handling



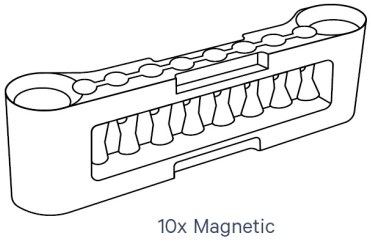
- Use one tube of Gel Beads per sample. DO NOT puncture the foil seals of tubes not used at the time.
- After removing the Gel Bead strip from the packaging, equilibrate the Gel Bead strip to **room temperature** for at least **30 min** before use.
- Store unused Gel Beads at **−80°C** and avoid more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

10x Magnetic Separator

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



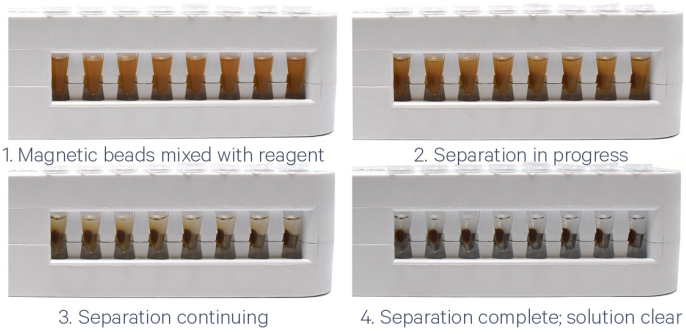
10x Magnetic Separator B



10x Magnetic Separator

Magnetic Bead Cleanup Steps

- During magnetic bead based cleanup steps that specify waiting “until the solution clears”, visually confirm clearing of solution before proceeding to the next step. See panel below for an example.
- The time needed for the solution to clear may vary based on specific step, reagents, volume of reagents etc.
- Images below are representative - actual color of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.
- **Visually Confirm Clearing of Magnetic Bead Solution**



SPRIselect Cleanup & Size Selection

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

Tutorial — SPRIselect Reagent: DNA Sample Ratios
SPRI beads selectively bind DNA according to the ratio of SPRIselect reagent (beads).

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

Sample Indices in Sample Index PCR

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

Sample Index PCR Cycles

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

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

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

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

Step 1:

Probe Hybridization

1.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Thaw & Keep Warm				
☐	Hyb Buffer B	2001312	Thaw at 42°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. DO NOT keep the thawed buffer on ice, or the solution will precipitate. Thawed Hyb Buffer B can be kept at 42°C for up to 1 h.	–20°C
☐	Enhancer	2000482	Thaw for 10 min at 65°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min. If not used within 10 min and precipitation is observed, reheat at 65°C to ensure enhancer is fully dissolved again before use.	–20°C
Place on Ice				
☐	Fixed Cell Suspension	—	Consult GEM-X Flex Gene Expression - Protocol Planner (CG000780) for details on applicable Demonstrated Protocols.	—
☐	Human WTA Probes* May vary from BC001 to BC016 OR Mouse WTA Probes* May vary from BC001 to BC016	May vary from 2001259 to 2001274 May vary from 2001275 to 2001290	Thaw on ice. Vortex and centrifuge briefly.	–20°C

*All probes are expected to perform equivalently.

1.1 Probe Hybridization

Before starting this protocol, ensure that:

- Samples have been appropriately fixed and quenched.
  Consult GEM-X Flex Gene Expression - Protocol Planner (CG000780) for details on the fixation protocols to use.
- Determine the number of cells that will be used in each hybridization reaction.

  Minimum sample input for hybridization is **25,000** fixed cells/nuclei and maximum input is **500,000** fixed cells/nuclei. If sufficient cells are available, it is recommended to default to 300,000 fixed cells/nuclei for setting up hybridization.

  DO NOT exceed 500,000 cells in one hybridization reaction.

  In case of leukocytes, splenocytes, and cells from fixed & dissociated spleen and pancreas tissues, it is recommended to use ≤100,000 cells as higher cell loads may lead to a slight decrease in data quality.

TIPS See Tips & Best Practices for Probe Hybridization on page 23.

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

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

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

Hyb Mix *Add reagents in the order listed*	PN	1X* + 20% (µl)	4X* + 20% (µl)
Hyb Buffer B *Thaw at 42°C. Add warm to the mix and if appears milky keep it back on 42°C.*	2001312	42.0	168.0
Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.*	2000482	6.0	24.0
Total	-	**48.0**	**192.0**

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

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

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

e. Remove the supernatant.

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

TIPS

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

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

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

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

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

g. Add **10 µl** Human/Mouse WTA Probes to the **40 µl** mixture of Hyb Mix and fixed sample and gently pipette mix 10x with pipette set at 40 µl.

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

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

Step 2:

Post-Hybridization Washing

2.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Thaw & Keep Warm				
☐	Enhancer	2000482	Thaw for 10 min at 65°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min. If not used within 10 min and precipitation is observed, reheat at 65°C to ensure enhancer is fully dissolved again before use.	–20°C
Place on Ice				
☐	Conc. Post-Hyb Buffer B	2001308	Thaw at room temperature and keep on ice.	–20°C
Obtain				
☐	Sample Filters Sysmex Sterile Single-pack CellTrics Filters/Miltenyi Biotec Pre-Separation Filters (30 µm)	—	Manufacturer's recommendations.	Ambient
☐	10x Vortex Adapter	330002	See Tips & Best Practices.	Ambient
☐	Glycerol for molecular biology, ≥99% *Prepare fresh 50% glycerol solution for sample storage*	—	See Tips & Best Practices.	—

2.1 Post-Hybridization Wash

During the centrifugation steps, up to 30 µl supernatant may be left behind if working with <300,000 cells. See Post-Hybridization Sample Washing & Recovery on page 24 for more details. If uncertain about the volume remaining in the tube, add 30 µl water/PBS to an empty tube of the same size and use it for a visual estimation.

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

Post-Hyb Wash Buffer B *Add reagents in the order listed*	PN	1X + 10% (ml)*	4X + 10% (ml)*
Nuclease-free Water	-	1.98	7.92
Conc. Post-Hyb Buffer B	2001308	0.11	0.44
Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate.* *Add warm to the mix.*	2000482	0.11	0.44
Total	-	**2.2**	**8.8**

**Volumes are in ml*

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

c. Add an appropriate volume of Post-Hyb Wash Buffer B to the sample.

For 1.5-ml microcentrifuge tube: Add **900 µl** Post-Hyb Wash Buffer B. Pipette mix 5x.

For 8-tube strips: Add **175 µl** Post-Hyb Wash Buffer B, gently pipette mix, and transfer to a 1.5-ml microcentrifuge tube. Wash the tube strips with additional buffer, transfer to the microcentrifuge tube, and add the remaining volume of buffer for a total of **900 µl** Post-Hyb Wash Buffer B to the sample.

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

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

f. Remove the supernatant without disturbing the pellet.

TIPS

Up to 30 µl supernatant may be left behind if working with <300,000 cells. See Post-Hybridization Sample Washing & Recovery on page 24 for more details.

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

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

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

j. Remove the supernatant without disturbing the pellet.

TIPS

Up to 30 µl supernatant may be left behind if working with <300,000 cells. See Post-Hybridization Sample Washing & Recovery on page 24 for more details.

When using nuclei derived from FFPE tissue sections, perform one extra 0.5 ml wash by repeating steps g-j one more time

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

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

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

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

The buffer prepared at this step is also sufficient for sample dilution at step 3.1. Maintain at 4°C.

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

TIPS

o. Remove the supernatant without disturbing the pellet. Up to 30 µl supernatant may be left behind if working with <300,000 cells. See Post-Hybridization Sample Washing & Recovery on page 24 for more details.

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

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

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

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

- See Cell Suspension Volume Calculator on page 49 to determine the desired cell stock concentration based on targeted cell recovery.

- Calculate the volume of resuspension buffer using the cells input into probe hybridization, an estimated ~70% cell recovery* during the washing step, and the desired cell stock concentration. See example calculation on the next page.

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

Example Calculation
Volume of Resuspension Buffer
$= \frac{\text{Cell Input into Probe Hybridization x Expected Recovery Rate}}{\text{Desired Cell Stock Concentration}}$
Cell Input: 250,000
Desired Cell Stock Concentration: 500 cells/µl
$= \frac{250{,}000 \times 0.70}{500 \text{ cells/µl}}$
=350 µl

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

DO NOT use 40 µm Flowmi Tip Strainer for filtration.

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

TIPS *See Sample Filtration on page 25 for details.*

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

For samples with ~25,000 cells, concentrate the sample as follows:

- Centrifuge a known sample volume at **850 rcf** for **5 min** at **room temperature**.
- Carefully remove supernatant leaving behind ~**50 µl**, and pipette thoroughly to resuspend the cell pellet.
- Take ~**5-12.5** µl to count and determine the concentration.

See Tips & Best Practices for Cell Counting on page 26. A serial dilution may be needed to accurately determine cell concentration.

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

- Centrifuge a known sample volume at **850 rcf** for **5 min** at **room temperature**.
- Carefully remove only a fraction of the supernatant. Pipette thoroughly to resuspend the cell pellet in the remaining volume. The amount of supernatant removed should be proportional to the desired increase in concentration.

  For example, to increase the concentration 4-fold from a starting volume of 400 µl, centrifuge, then remove 300 µl supernatant, and finally resuspend the cell pellet in the remaining 100 µl (400/100 = 4).
- Recount to confirm final concentration.

t. 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 48 for details.

Sample Storage

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

Using Stored Samples

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

10x GENOMICS

Step 3:

GEM Generation and Barcoding

3.0 Get Started

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

Item		10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐	Single Cell TL v1 Gel Beads	2000538	Equilibrate to room temperature 30 min before loading the chip.	–80°C
☐ ○	Reducing Agent B	2000087	Vortex, verify no precipitate, centrifuge briefly.	–20°C
Thaw & Keep Warm				
☐	Enhancer	2000482	Thaw for 10 min at 65°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min. If not used within 10 min and precipitation is observed, reheat at 65°C to ensure enhancer is fully dissolved again before use.	–20°C
Place on Ice				
☐	GEM Enzyme Mix B	2001302	Centrifuge briefly before adding to the mix.	–20°C
☐	GEM Reagent Mix	2000491	Thaw at room temperature. Vortex, verify no precipitate, centrifuge briefly. Keep on ice.	–20°C
☐	Post-Hyb Resuspension Buffer B *Use the Post-Hyb Resuspension Buffer B prepared at the previous step (2.1) for sample dilution.*	—	Additional buffer can be prepared using the buffer preparation table in step 2.1.	4°C
Obtain				
☐	Partitioning Oil B	2001213	—	Ambient
☐	GEM-X FX Chip	2001257	See Tips & Best Practices.	Ambient
☐	Chromium X Series Chip Holder	3000598	See Tips & Best Practices.	Ambient

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

3.1 Prepare GEM Master Mix + Sample Dilution

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

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

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

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

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

Place remaining undiluted sample on ice. These samples can be stored later after GEM incubation. Guidelines for storage of remaining samples are provided in step 3.5 *GEM Incubation on page 56*.

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.

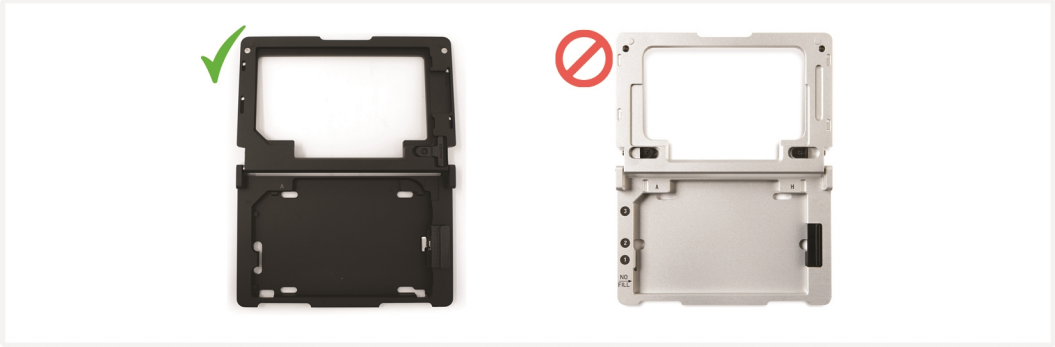
Cell Suspension Volume Calculator

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								
	500	1000	2000	4000	8000	10000	12000	15000	20000
500	1.5	2.9	5.8	11.6	23.2	29.0			
	33.2	31.8	28.9	23.1	11.5	5.7			
1000	0.7	1.5	2.9	5.8	11.6	14.5	17.4	21.8	29.0
	34.0	33.2	31.8	28.9	23.1	20.2	17.3	12.9	5.7
1500	0.5	1.0	1.9	3.9	7.7	9.7	11.6	14.5	19.3
	34.2	33.7	32.8	30.8	27.0	25.0	23.1	20.2	15.4
2000	0.4	0.7	1.5	2.9	5.8	7.3	8.7	10.9	14.5
	34.3	34.0	33.2	31.8	28.9	27.4	26.0	23.8	20.2
2500	0.3	0.6	1.2	2.3	4.6	5.8	7.0	8.7	11.6
	34.4	34.1	33.5	32.4	30.1	28.9	27.7	26.0	23.1
3000	0.2	0.5	1.0	1.9	3.9	4.8	5.8	7.3	9.7
	34.5	34.2	33.7	32.8	30.8	29.9	28.9	27.4	25.0
3500	0.2	0.4	0.8	1.7	3.3	4.1	5.0	6.2	8.3
	34.5	34.3	33.9	33.0	31.4	30.6	29.7	28.5	26.4
4000	0.2	0.4	0.7	1.5	2.9	3.6	4.4	5.4	7.3
	34.5	34.3	34.0	33.2	31.8	31.1	30.3	29.3	27.4
4500	0.2	0.3	0.6	1.3	2.6	3.2	3.9	4.8	6.4
	34.5	34.4	34.1	33.4	32.1	31.5	30.8	29.9	28.3
5000	0.1	0.3	0.6	1.2	2.3	2.9	3.5	4.4	5.8
	34.6	34.4	34.1	33.5	32.4	31.8	31.2	30.3	28.9
5500	0.1	0.3	0.5	1.1	2.1	2.6	3.2	4.0	5.3
	34.6	34.4	34.2	33.6	32.6	32.1	31.5	30.7	29.4
6000	0.1	0.2	0.5	1.0	1.9	2.4	2.9	3.6	4.8
	34.6	34.5	34.2	33.7	32.8	32.3	31.8	31.1	29.9
6500	0.1	0.2	0.4	0.9	1.8	2.2	2.7	3.3	4.5
	34.6	34.5	34.3	33.8	32.9	32.5	32.0	31.4	30.2
7000	0.1	0.2	0.4	0.8	1.7	2.1	2.5	3.1	4.1
	34.6	34.5	34.3	33.9	33.0	32.6	32.2	31.6	30.6
7500	0.1	0.2	0.4	0.8	1.5	1.9	2.3	2.9	3.9
	34.6	34.5	34.3	33.9	33.2	32.8	32.4	31.8	30.8
8000	0.1	0.2	0.4	0.7	1.5	1.8	2.2	2.7	3.6
	34.6	34.5	34.3	34.0	33.2	32.9	32.5	32.0	31.1
8500	0.1	0.2	0.3	0.7	1.4	1.7	2.0	2.6	3.4
	34.6	34.5	34.4	34.0	33.3	33.0	32.7	32.1	31.3
Yellow boxes	Indicate a low transfer volume that may result in higher cell load variability								

Assemble GEM-X FX Chip

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



See Tips & Best Practices on page 21 for chip handling instructions.

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

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

c. Open the chip holder.

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

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

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

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

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

i. The assembled chip is ready for loading the indicated reagents. See Load GEM-X Chip on page 52 for reagent volumes and loading order.

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

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

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

Representative chip image

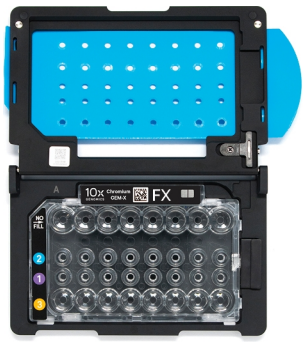
3.2 Load GEM-X Chip

TIPS

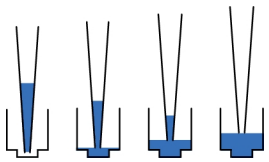
- After removing chip from the sealed bag, use in **≤24 h**.
- Open the lid (gasket attached) of the assembled chip and lay flat for loading.
- Ensure that the Gel Beads are properly thawed and ready to use.
- When loading the chip, raising and depressing the pipette plunger should each take **~5 sec**.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

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

GEM-X FX Chip, gasket attached
Representative chip image



Pipette technique

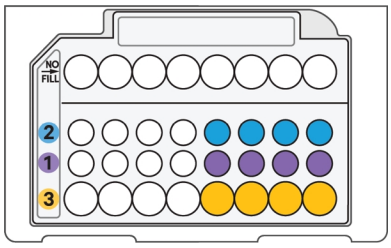


The Chromium X Series Chip Holder, Chip Gasket X/iX, and GEM-X chip images shown below are representative and do not show the specific color & label. Chip holder and gasket should be black and blue in color, respectively. Refer to Assemble GEM-X FX Chip on page 50 for details.

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

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

Glycerol
in GEM-X FX Chip

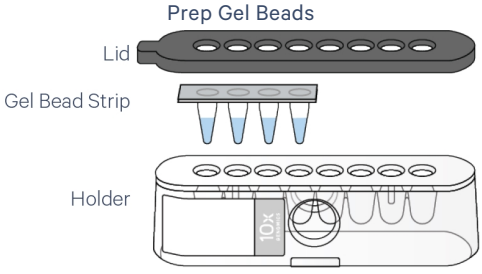


DO NOT add 50% glycerol solution to the wells in top row labeled NO FILL.
DO NOT use any substitute for 50% glycerol solution.

b. Prepare Gel Beads

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

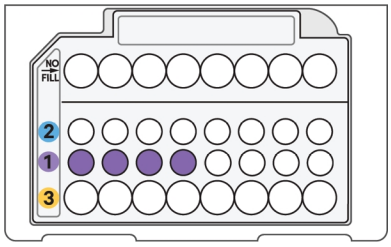
Prep Gel Beads



c. Load Row Labeled 1

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

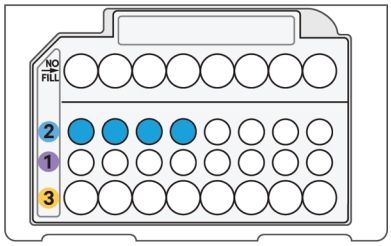
60 µl Master Mix + Cell Suspension in GEM-X FX Chip



d. Load Row Labeled 2

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

60 µl Gel Beads in GEM-X FX Chip

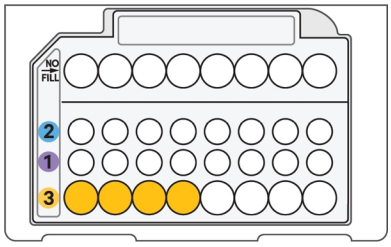


e. Load Row Labeled 3

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

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

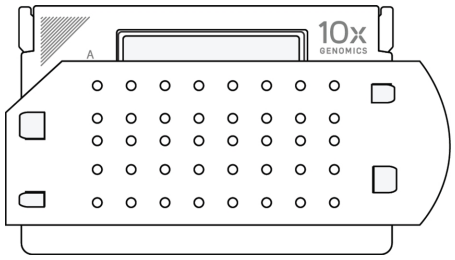
250 µl Partitioning Oil B in GEM-X FX Chip



f. Prepare for Run

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

GEM-X FX Chip, closed



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

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

3.3 Run the Chromium X/iX

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

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

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

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

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

Run Chromium X/iX

3.4 Transfer GEMs

a. Place a tube strip on ice.

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

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

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

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

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

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

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

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

Representative chip image

3.5 GEM Incubation

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

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

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

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

DO NOT store the GEMs at −20°C.

c. Undiluted sample placed on ice at step 3.1 Prepare GEM Master Mix + Sample Dilution on page 48 can either be discarded or stored at **−80°C** for up to **12 months**. See Sample Storage below:

Sample Storage of Undiluted Sample

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

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

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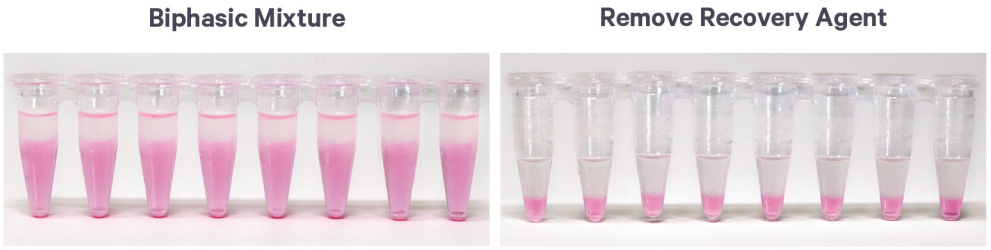
Step 4:

GEM Recovery and Pre-Amplification

4.0 Get Started

Item			10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
☐	◯	Reducing Agent B	2000087	Thaw, vortex, verify no precipitate, centrifuge briefly.	−20°C
☐	●	Pre-Amp Primers 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.	—

4.1 Post-GEM Incubation – Recovery



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

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

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

DO NOT invert without firmly securing the caps.

c. Wait **2 min**.

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

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

d. Centrifuge briefly.

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

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

4.2 Pre-Amplification PCR

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

Pre-Amplification Mix *Add reagents in the order listed*		PN	1X (µl)	4X + 10% (µl)
○	**Amp Mix C**	2001311	25.0	110.0
●	**Pre-Amp Primers B** Verify name & PN ⚠	2000529	10.0	44.0
	Total		**35.0**	**154.0**

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

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

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

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

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

4.3 DNA Cleanup – SPRIselect

a. Prepare Elution Solution. Vortex and centrifuge briefly.

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

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

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

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

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

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

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

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

h. Remove the ethanol.

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

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

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

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

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

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

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

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

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Step 5:

GEM-X Flex – Gene Expression Library Construction

5.0 Get Started

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

5.1 Sample Index PCR

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

b. Determine sample index PCR cycle numbers using one of the following methods (See Sample Index PCR Cycles on page 34 for guidance on which method to use):

Using qPCR: See Optional: Gene Expression Library Cycle Number Determination Using qPCR on page 79

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

Total Cycles*

Targeted Cell Recovery	Cell Lines	PBMCs & Nuclei	Cells from Fixed & Dissociated Tissues**	Leukocytes & Nuclei from FFPE Tissue
500-2,000	11	15	14-15	16
2,000-4,000	10	14	13-14	15
4,000-7,000	9	13	12-13	14
7,000-12,000	8	12	11-12	13
12,000-20,000	7	11	10-11	12

**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.*

***For cells derived from the fixed and dissociated tissue samples, the cycle number will depend on the RNA expression level of the tissue and on overall quality of the tissue prior to fixation. Additional optimization may be required.*

c. Prepare Sample Index PCR Mix on ice.

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

d. Transfer **ONLY 20 µl** sample from the step DNA Cleanup – SPRIselect on page 61 to a new tube strip. The remaining sample can be stored at −20°C for up to 4 weeks, for generating additional libraries.

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

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

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

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

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

5.2 Post Sample Index PCR Size Selection – SPRIselect

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

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

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

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

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

f. Remove the ethanol.

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

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

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

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

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

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

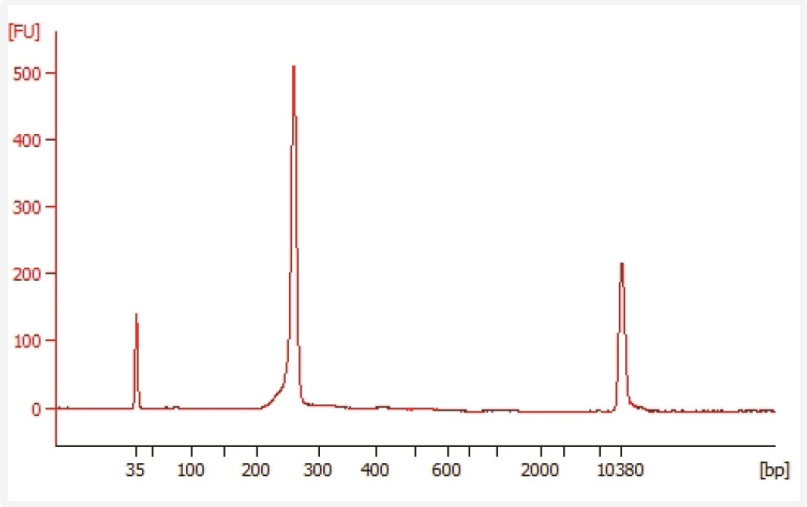
m. Transfer **40 µl** to a new tube strip.

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

5.3 Post Library Construction QC

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

Representative Trace



Alternate QC Method

Agilent TapeStation

LabChip

Fragment Analyzer

See Appendix for:

- Post Library Construction Quantification on page 81
- Agilent TapeStation Traces on page 82
- LabChip Traces on page 82
- Fragment Analyzer Traces on page 83

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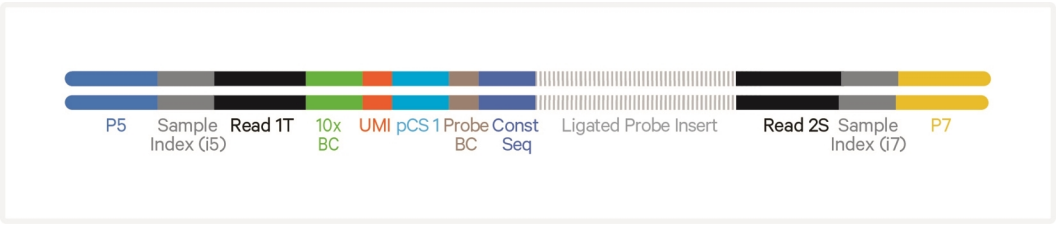
Step 6:

Sequencing

Sequencing Libraries

GEM-X Flex – 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 – 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.

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

Illumina Sequencer Compatibility

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

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

Sample Indices

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

GEM-X Flex – Library Sequencing Parameters

Parameter	Description
Sequencing Depth	Minimum 10,000 read pairs per cell
Sequencing Type	Paired-end, dual indexing
Sequencing Read	**Recommended Number of Cycles**
Read 1 i7 Index i5 Index Read 2	28 cycles 10 cycles 10 cycles 90 cycles*
	**Minimum required Read 2 length is 50 bp*

Library Loading

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

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

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

Library Loading

Instrument	Loading Concentration (pM)	PhiX (%)
MiSeq	12	1
NextSeq 500/550	2.5	1
NextSeq 1000/2000	650	1
NovaSeq 6000 standard*	100-150	1
NovaSeq 6000 Xp workflow*	150-200	1
NovaSeq X series	150-200	1

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

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

Library Pooling

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

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

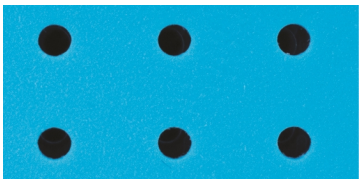
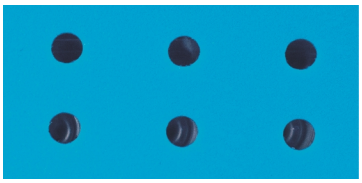
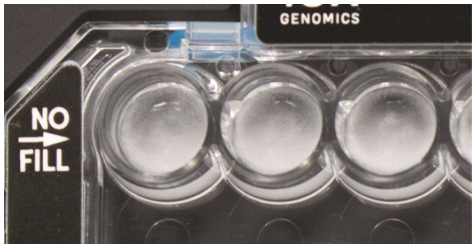
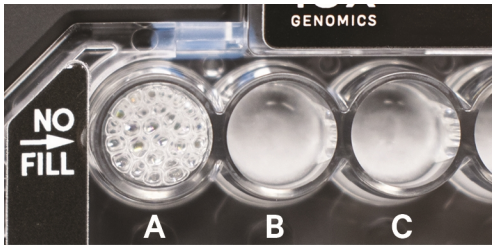
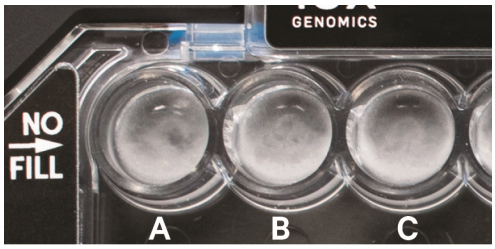
Loupe Browser

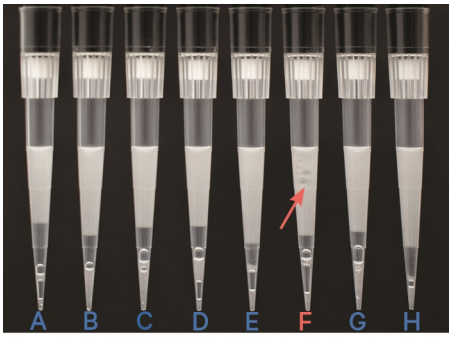
Loupe Browser is an interactive data visualization tool that requires no prior programming knowledge.

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

Troubleshooting

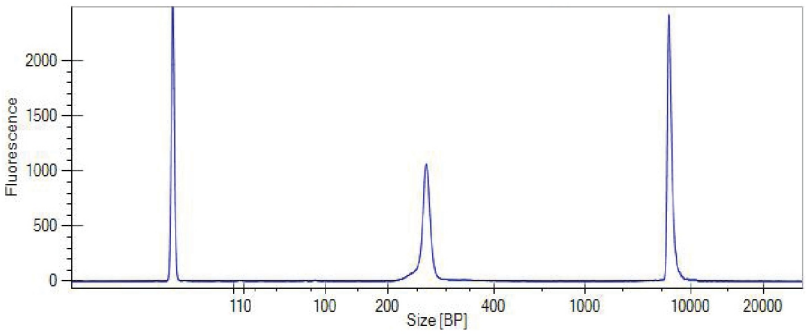
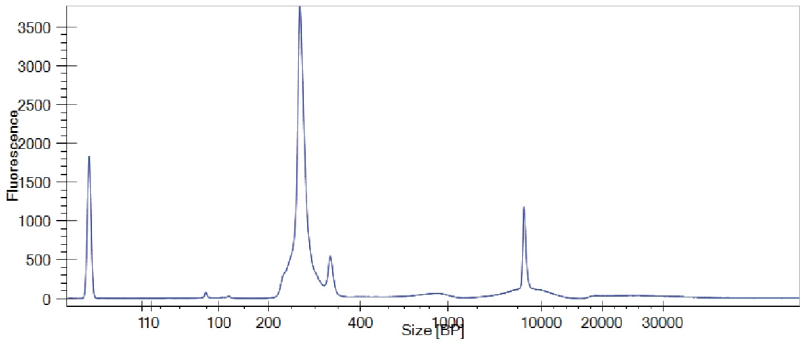
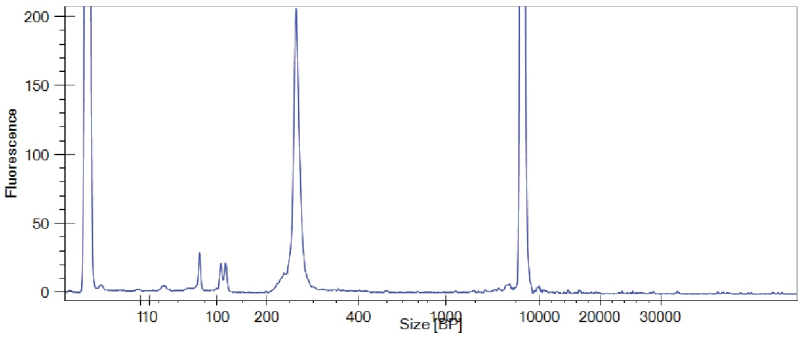
GEM Generation & Barcoding

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

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

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

Post Library Construction QC

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

Chromium X Series Errors

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

There are two types of errors:

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

a. System Error

b. Pressure Error

c. Chip Error

d. Run Error

e. Temperature Error

f. Software Error

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

a. Gasket Error

b. Tray Error

c. Chip Error

d. Unsupported Chip Error

e. Network Error

f. Update Error

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

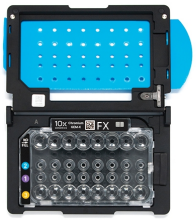
Appendix

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 52 for details.

Steps

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



Color Legend

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

a. Add 50% glycerol solution to each unused well

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

b. Prepare Gel Beads

- Vortex for 30 sec
- Centrifuge for 5 sec

c. Load Row Labeled 1

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

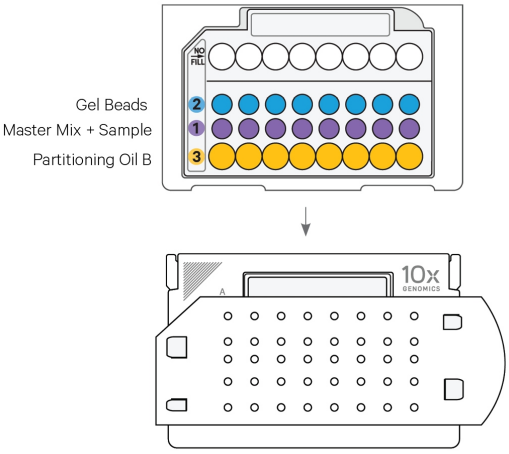
d. Load Row Labeled 2

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

e. Load Row Labeled 3

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

f. Close the lid and prepare for run.



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

Optional: Gene Expression Library Cycle Number Determination Using qPCR

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

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

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

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

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

*Includes 1 negative control

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

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

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.

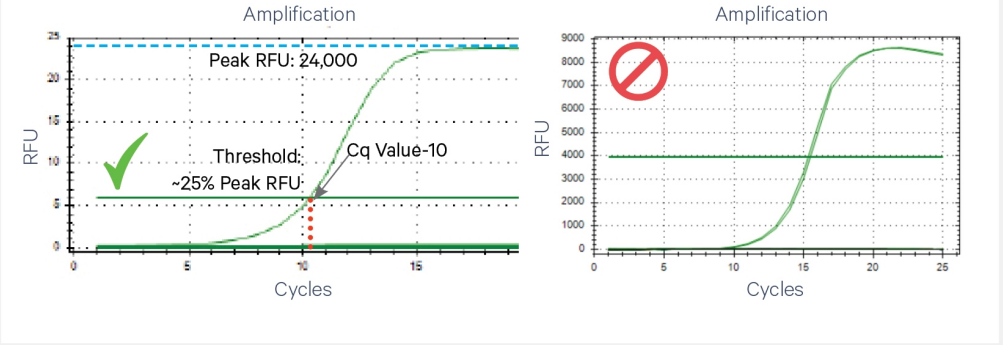
g. Prepare a qPCR system with the following protocol, place the plate in the qPCR system, and start the program.

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

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.

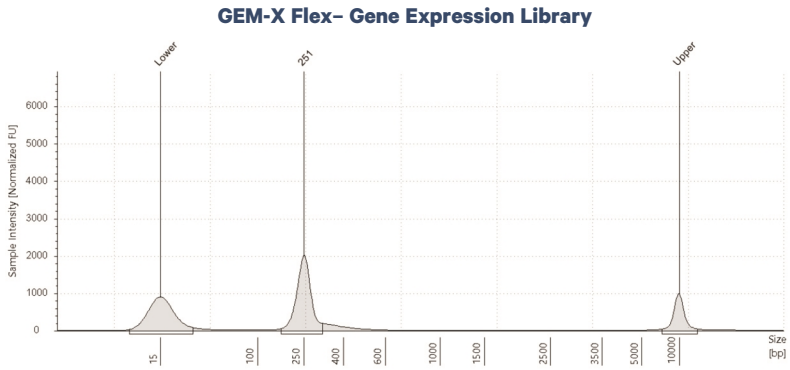
f. Incubate in a thermal cycler with the following protocol.

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

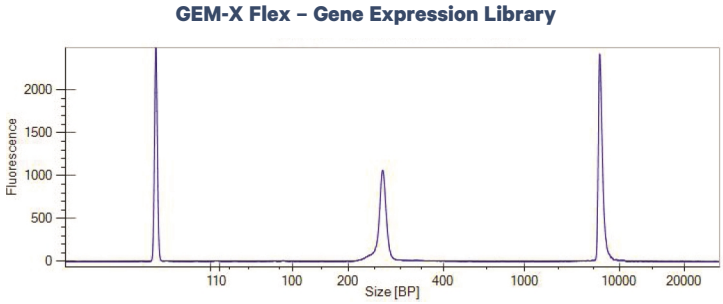
Agilent TapeStation High Sensitivity D5000 ScreenTape was used.



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

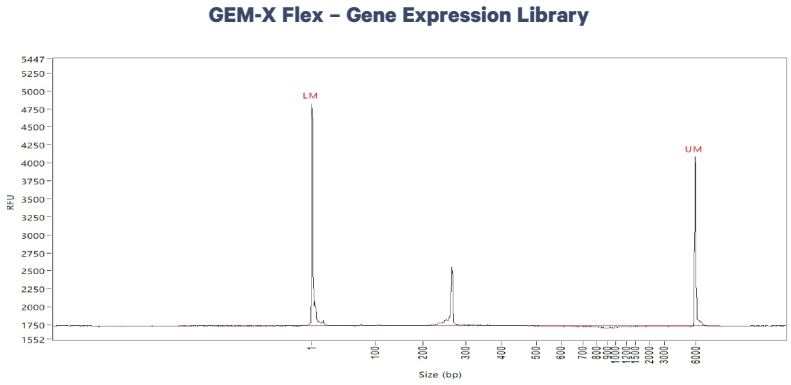
LabChip Traces

DNA High Sensitivity Reagent Kit was used.



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

Fragment Analyzer Traces



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

Oligonucleotide Sequences

Gel Bead Primer

Gel Bead Primers

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

Pre-Amp Primers B

Forward Primers

5’-CTACACGACGCTCTTCCGATCT-3’

Reverse Primer

5'-CCTTGGCACCCGAGAATTCCA-3'

Dual Index Kit TS Set A

Forward Primers

5’-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTC-3'

Reverse Primers

5'-CAAGCAGAAGACGGCATACGAGAT-N10-GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA-3'

GEM-X Flex– Gene Expression Library

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

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