



User Guide | CG000769 | Rev A

GEM-X Universal

3' Gene Expression v4 4-plex

On-chip Multiplexing (OCM) with Feature Barcode technology for Cell Surface Protein

For use with:

GEM-X Universal 3' Gene Expression v4 4-plex

16 samples PN-1000779

GEM-X OCM 3' Chip Kit v4 4-plex

2 chips PN-1000747

Chromium GEM-X Single Cell 3' Feature Barcode Kit v4

16 rxns PN-1000702

Dual Index Kit TT Set A

96 rxns PN-1000215

Dual Index Kit NT Set A

96 rxns PN-1000242

Take 1 minute to evaluate this protocol. Scan this code or [click here](#).



Notices

Document Number

CG000769 | Rev A

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Document Revision Summary

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GEM-X Universal 3' Gene Expression v4 4-plex On-chip Multiplexing (OCM) with Feature Barcode technology for Cell Surface Protein

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November 14, 2024

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Introduction






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GEM-X Universal 3' Reagent Kits v4 4-plex

Refer to SDS for handling and disposal information

GEM-X Universal 3' Kit v4 4-plex, 16 samples PN-1000779

Chromium GEM-X Single Cell 3' GEM Kit v4 4 rxns, PN-1000688 <i>Shipped on dry ice</i> <i>Store at -20°C</i>			
		#	PN
	RT Reagent E	1	2001106
	RT Enzyme E	1	2001146
	Template Switch Oligo B	1	2001027
	Reducing Agent B	1	2000087
	Cleanup Buffer	1	2000088
	cDNA Primers	1	2000089
	Amp Mix	1	2000103
			

Library Construction Kit C 4 rxns, PN-1000689 <i>Shipped on dry ice</i> <i>Store at -20°C</i>			
		#	PN
	Fragmentation Enzyme	1	2000090
	Fragmentation Buffer	1	2000091
	Ligation Mix	1	2001109
	DNA Ligase	1	220110
			


GEM-X OCM 3' Gel Bead Kit v4 4-plex 16 samples, PN-1000749 <i>Shipped on dry ice</i> <i>Store at -80°C</i>			
		#	PN
	GEM-X OCM 3' Gel Bead v4	2	2001126
			

Dynabeads™ MyOne™ SILANE PN-2000048 <i>Shipped at ambient temperature</i> <i>Store at 4°C</i>			
		#	PN
	Dynabeads MyOne SILANE	1	2000048

GEM-X OCM 3' Chip Kit v4 4-plex, 2 chips PN-1000747


Chromium

Partitioning Oil B*Shipped at ambient temperature**Store at ambient temperature*

		#	PN
	Partitioning Oil B	4	2001213

Chromium

Recovery Agent*Shipped at ambient temperature**Store at ambient temperature*

		#	PN
	Recovery Agent	4	220016



GEM-X

3' OCM Chip & Gaskets*Shipped at ambient temperature**Store at ambient temperature*

	#	PN
GEM-X 3' OCM Chip	2	2001099
X/iX Chip Gasket, 2-pack	1	3000656

10x
GENOMICS

Chromium GEM-X Single Cell 3' Feature Barcode Kit v4, 16 rxns PN-1000702

Chromium GEM-X Single Cell 3' Feature Barcode Kit v4 16 rxns, PN-1000702 <i>Shipped on dry ice</i> <i>Store at -20°C</i>			
		#	PN
	Feature cDNA Primers 2	1	2000097
	Amp Mix	1	2000047
			

Dual Index Kit TT Set A, 96 rxns PN-1000215

Dual Index Kit TT Set A <i>Shipped on dry ice</i> <i>Store at -20°C</i>		
	#	PN
Dual Index Plate TT Set A	1	3000431

Dual Index Kit NT Set A, 96 rxns PN-1000242

Dual Index Kit NT Set A <i>Shipped on dry ice</i> <i>Store at -20°C</i>		
	#	PN
Dual Index Plate NT Set A	1	3000483

10x Genomics Accessories

Product	Part Number (Kit)	Part Number (Item)
10x Vortex Adapter	120251	330002
10x Magnetic Separator B*	1000709 (Chromium X/iX Series Accessory Kit)/	2001212
Chromium X/iX Chip Holder (also referred to as Chromium X Series Chip Holder)	1000821 (Chromium X Series Accessory Kit)/ 1000707 (GEM-X Transition Kit)	3000598

**10x Magnetic Separator (PN-230003) is untested but interchangeable with the 10x Magnetic Separator B (PN-2001212).*

Third-Party Items

Successful execution of Chromium Gene Expression Solutions 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 Gene Expression v4 & Immune Profiling v3 - Protocol Planner (CG000748) for a detailed list of the following third-party items:

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



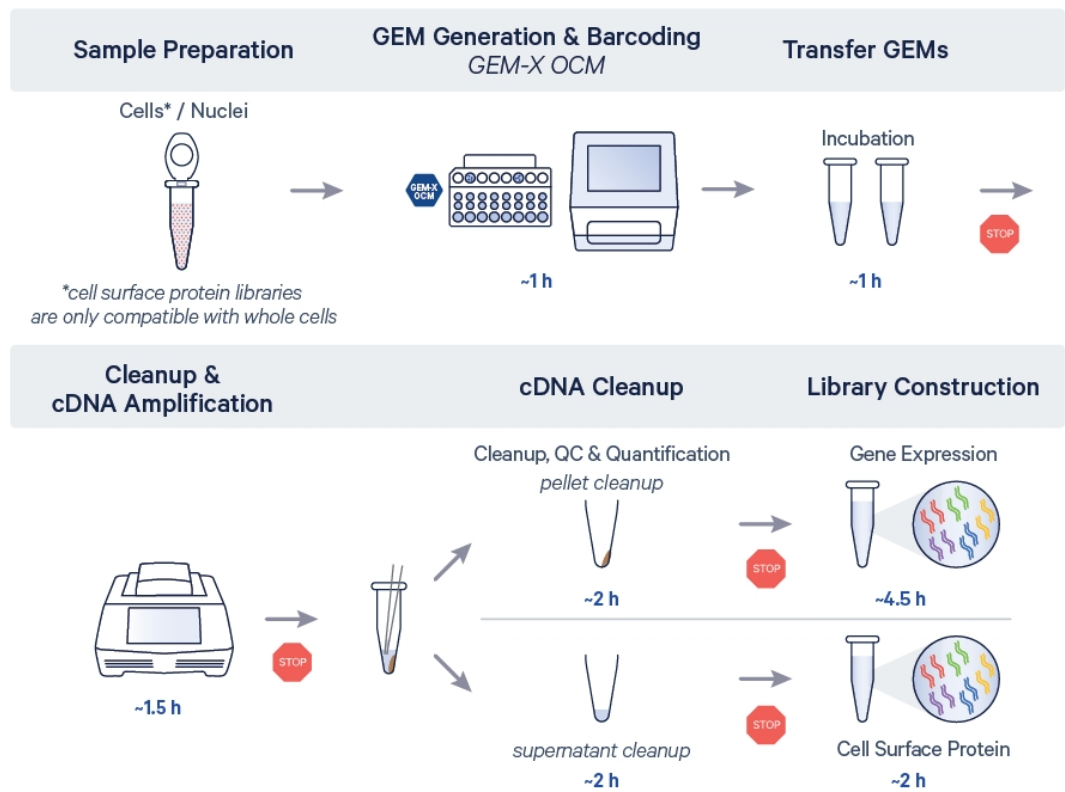
10x Genomics has tested all items listed in the Protocol Planner. These items perform optimally with the assay. Substituting materials may adversely affect assay performance.

Protocol Steps & Timing

Steps	Timing	Stop & Store	
Sample Preparation	variable*		
	*Refer to the appropriate Demonstrated Protocols for details.		
Step 1: GEM Generation and Barcoding (page 34)			
1.1 Prepare Master Mix (page 36)	20 min		
1.2 Load GEM-X OCM Chip (page 42)	10 min		
1.3 Run the Chromium X Series Instrument (page 45)	4 min		
1.4 Transfer GEMs (page 46)	3 min		
1.5 GEM-RT Incubation (page 48)	55 min		4°C ≤72 h/-20°C ≤1 week
Step 2: Post GEM-RT Cleanup & cDNA Amplification (page 49)			
2.1 Post GEM-RT Cleanup – Dynabeads (page 52)	45 min		
2.2 cDNA Amplification (page 55)	40 min		4°C ≤72 h/-20°C ≤1 week
2.3 cDNA Cleanup – SPRIselect (page 57)			
2.3A Pellet Cleanup	30 min		4°C ≤72 h/-20°C ≤4 weeks
2.3B Supernatant Cleanup	20 min		
2.4 Post cDNA Amplification QC & Quantification (page 60)	50 min		
Step 3: 3' Gene Expression Library Construction (page 62)			
3.1 GEX Fragmentation, End Repair & A-tailing (page 65)	50 min		
3.2 GEX Post Fragmentation, End Repair & A-tailing Double Sided – SPRIselect (page 66)	30 min		
3.3 GEX Adaptor Ligation (page 67)	25 min		
3.4 GEX Post Ligation Cleanup – SPRIselect (page 68)	30 min		
3.5 GEX Sample Index PCR (page 69)	40 min		4°C ≤72 h
3.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect (page 70)	30 min		4°C ≤72 h/-20°C long term
3.7 Post Library Construction QC (page 71)	50 min		
Step 4: Cell Surface Protein Library Construction (page 72)			
4.1 Sample Index PCR (page 74)	40 min		
4.2 Post Sample Index PCR Size Selection – SPRIselect (page 76)	20 min		4°C ≤72 h/-20°C long term
4.3 Post Library Construction QC (page 77)	50 min		

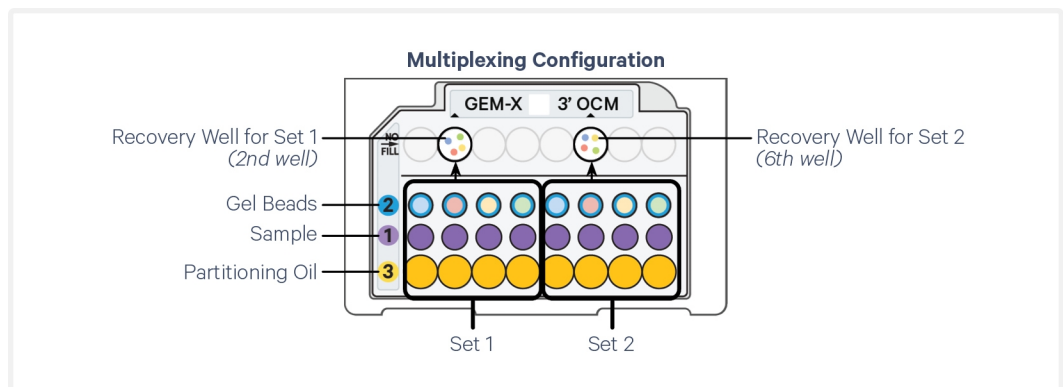
Stepwise Objectives

Workflow Overview



The GEM-X Universal 3' Gene Expression v4 4-plex assay provides a scalable microfluidic platform for on-chip multiplexing (OCM) of up to 8 samples (two sets of up to 4 samples each) to assess cell surface protein expression combined with single cell 3' gene expression by profiling 500-5,000 individual cells per sample.

A pool of ~900,000 barcodes are sampled separately to index each cell's transcriptome and cell surface protein in a given sample. This is done by partitioning up to 4 samples from each multiplexing set with corresponding gel beads (each with a unique list of barcodes). A pool of nanoliter-scale Gel Beads-in-emulsion (GEMs) are generated in a single recovery well for each set (wells 2 and 6 in top row). The GEM pool in each recovery well is used to generate cDNA from poly-adenylated mRNAs and DNA from cell surface proteins, where DNA derived from a cell share a common barcode. From each pool, a dual indexed library is generated and sequenced, and 10x Barcodes are used to associate the individual reads back to the individual partitions and samples.



This document outlines the protocol for generating Single Cell 3' Gene Expression and Cell Surface Protein libraries from the same cells.

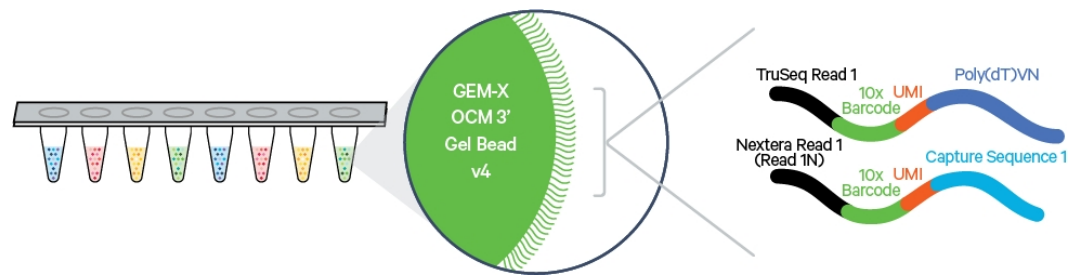
GEM-X OCM 3' Gel Beads v4 4-Plex

For each multiplexing set, the 4 color coded gel beads, each with a unique list of ~900,000 barcodes, are loaded in consecutive wells of the chip and are associated with a specific sample.

In addition to the poly(dT) primer that enables the production of barcoded, full-length cDNA from poly-adenylated mRNA, the GEM-X OCM 3' Gel Beads v4 also include an additional primer sequence (Capture Sequence 1) that enables the capture and priming of Feature Barcode technology compatible targets or analytes of interest.

The poly(dT) primers along with the capture sequence primers are used in this protocol for generating Single Cell 3' Gene Expression and Cell Surface Protein libraries.

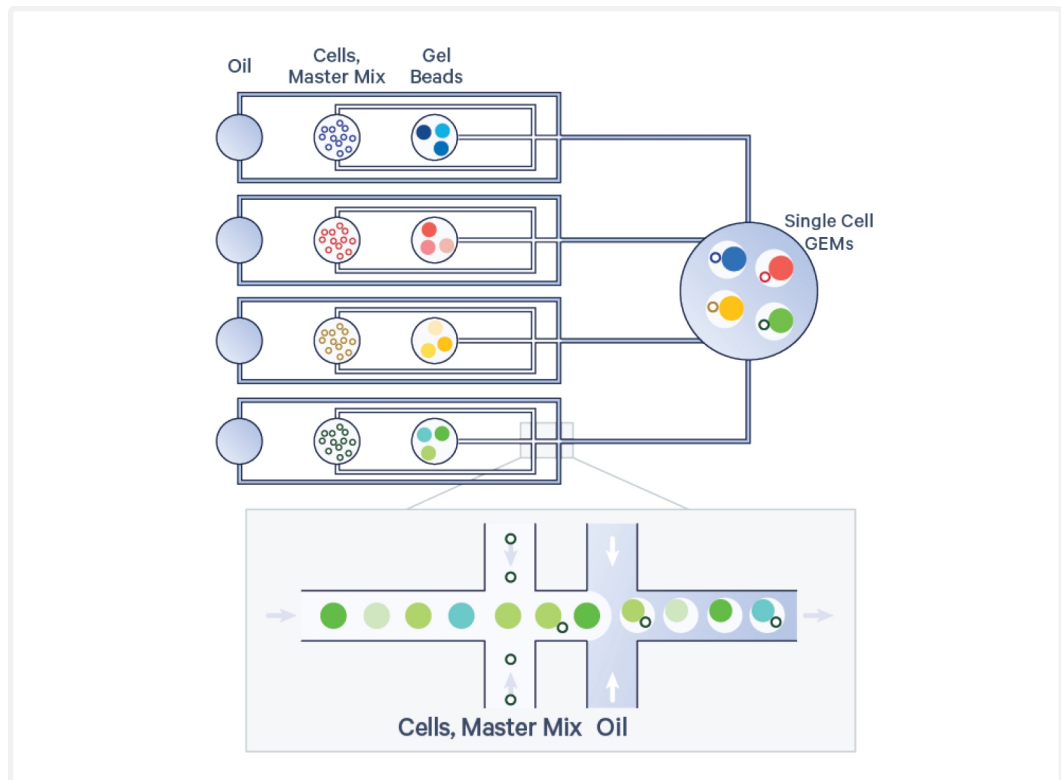
Gel Bead



Step 1: GEM Generation & Barcoding

For each multiplexing set, GEMs are generated from 4 samples by combining each sample (cells + Master Mix) with uniquely barcoded Gel Beads, and Partitioning Oil B into a single recovery well on the top row of the chip (well 2 for the first set and well 6 for the second set). To achieve single cell resolution, cells are delivered at a limiting dilution, such that the majority (~85-99%) of generated GEMs contain no cell, while the remainder largely contain a single cell.

GEM-X OCM Chip Workflow



Immediately following GEM generation, GEMs (containing ~3,600,000 unique barcodes from the 4 pooled samples) are transferred from the two recovery wells to two separate consecutive tubes of a tube strip. The Gel Bead is dissolved, primers are released, and any copartitioned cell is lysed. The poly (dT) and the capture sequence primer in the gel bead are engaged simultaneously in two different reactions inside individual GEMs.

A. Primers containing:

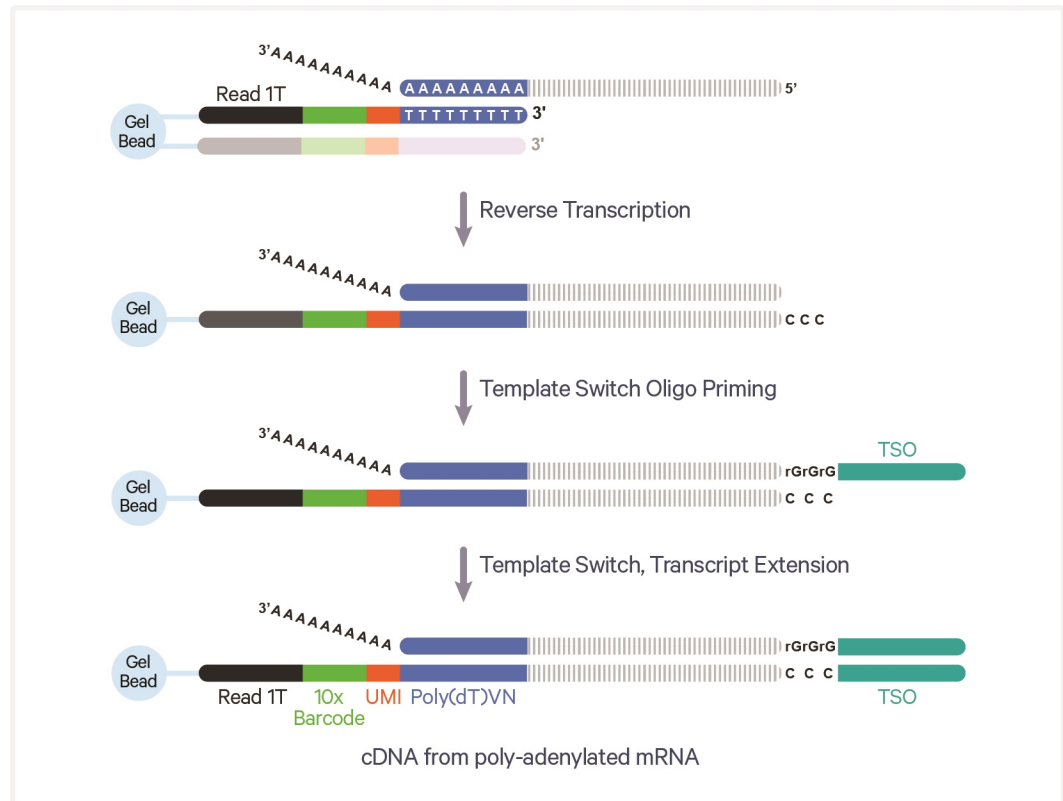
- an Illumina TruSeq Read 1 (Read 1T: read 1 sequencing primer)
- 16 nt 10x Barcode
- 12 nt unique molecular identifier (UMI)
- 30 nt poly(dT) sequence

B. Primers containing:

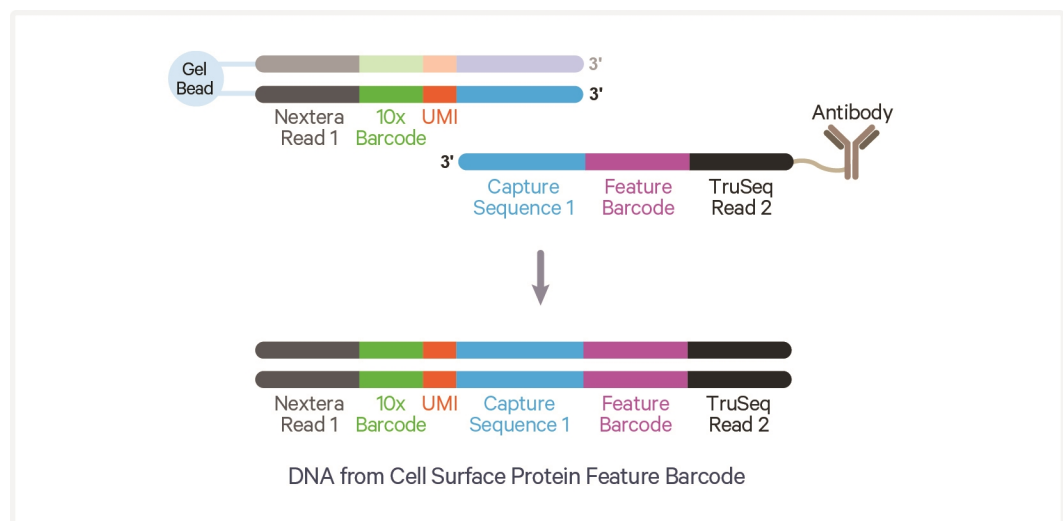
- an Illumina Nextera Read 1 (Read 1N; read 1 sequencing primer)
- 16 nt 10x Barcode
- 12 nt unique molecular identifier (UMI)
- Capture Sequence 1

Both primers are mixed with both the cell lysate and a Master Mix containing reverse transcription (RT) reagents. Incubation of the GEMs produces barcoded, full-length cDNA from poly-adenylated mRNA from reagents in (A) and barcoded DNA from the cell surface protein Feature Barcode oligonucleotides from reagents in (B).

Inside Individual GEMs - A. Gene Expression Primer



B. Feature Barcode Primer



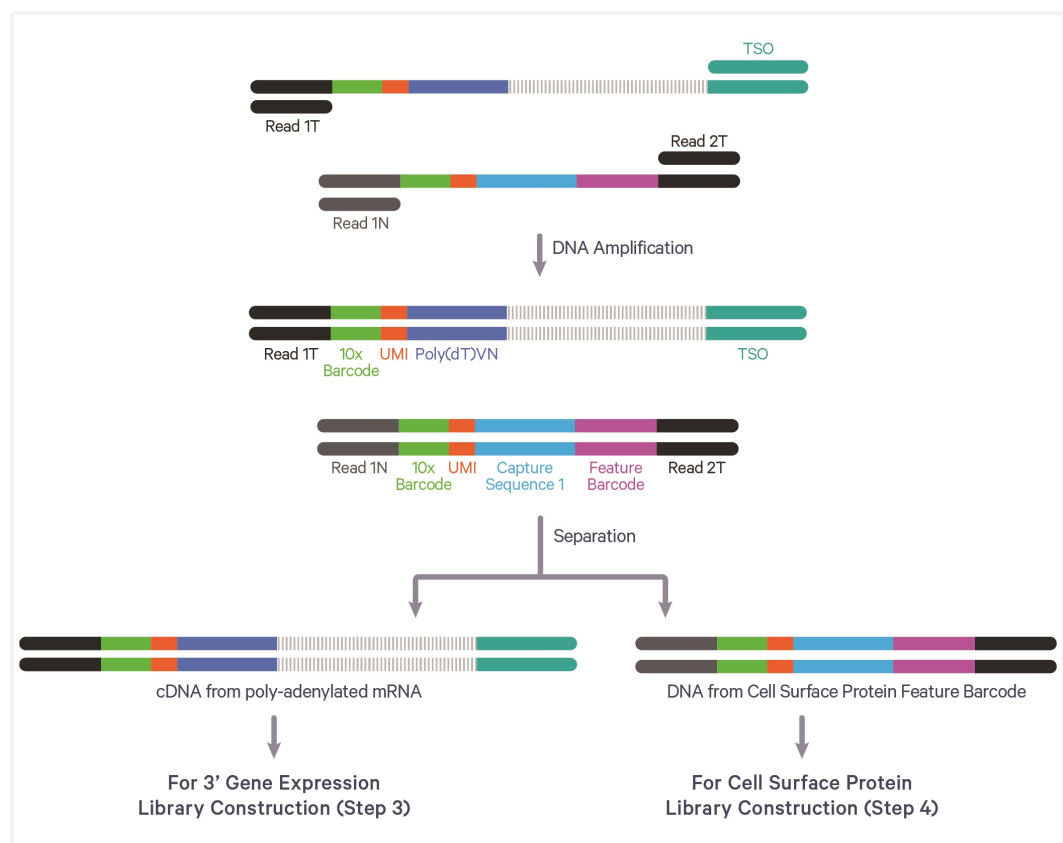
cDNA from poly-adenylated mRNA and DNA from cell surface protein Feature

Barcode are generated simultaneously from the same single cell inside the GEM.

Step 2: Post GEM-RT Cleanup & cDNA Amplification

After incubation, GEMs are broken and pooled fractions are recovered. Silane magnetic beads are used to purify the cell barcoded products from the post GEM-RT reaction mixture, which includes leftover biochemical reagents and primers. The cell barcoded cDNA molecules are amplified via PCR to generate sufficient mass for library constructions. Size selection is used to separate the amplified cDNA molecules for 3' Gene Expression and Cell Surface Protein library construction.

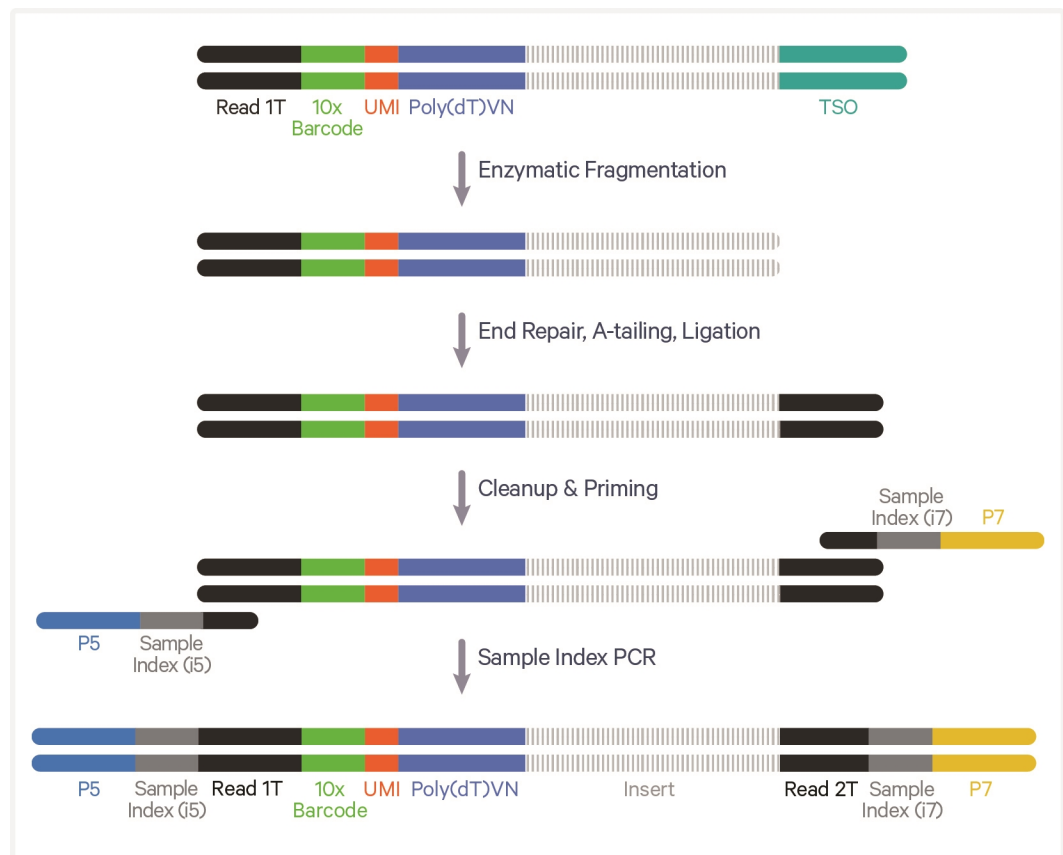
Pooled cDNA Amplification



Step 3: 3' Gene Expression Library Construction

Enzymatic fragmentation and size selection are used to optimize the cDNA amplicon size. P5 and P7 adaptors, i7 and i5 sample indexes, and TruSeq Read 2 (read 2 primer sequence) are added via End Repair, A-tailing, Adaptor Ligation, and PCR. The final libraries contain the P5 and P7 primers used in Illumina amplification.

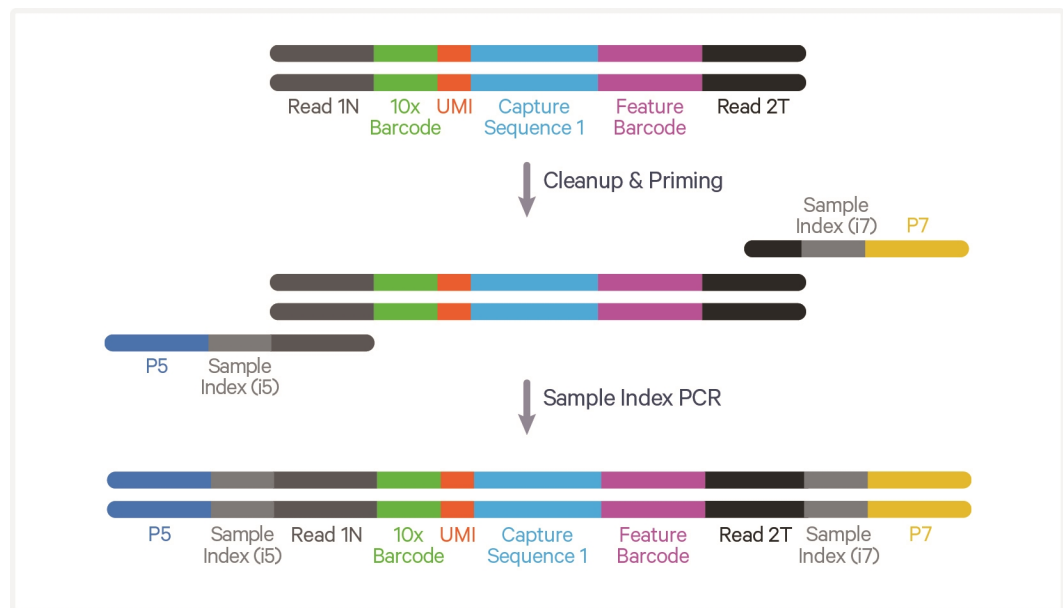
Amplified cDNA Processing (dual index)



Step 4: Cell Surface Protein Library Construction

Amplified DNA from cell surface protein Feature Barcode oligonucleotides is used for library construction. P5 and P7 adaptors, i7 and i5 sample indexes, and TruSeq Read 2 (read 2 primer sequence) are added via PCR. The final libraries contain the P5 and P7 primers used in Illumina amplification.

Amplified DNA Processing (dual index)

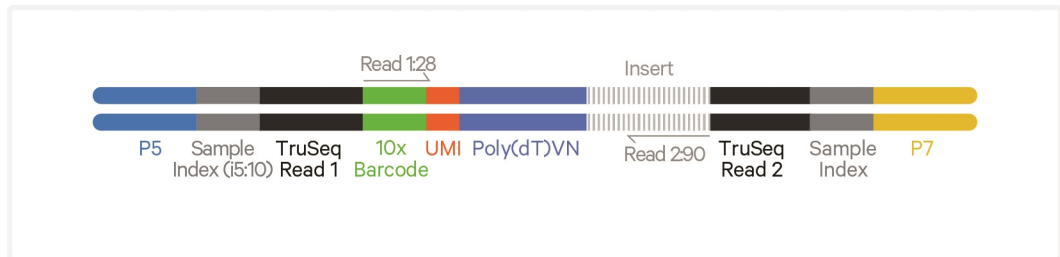


Sequencing

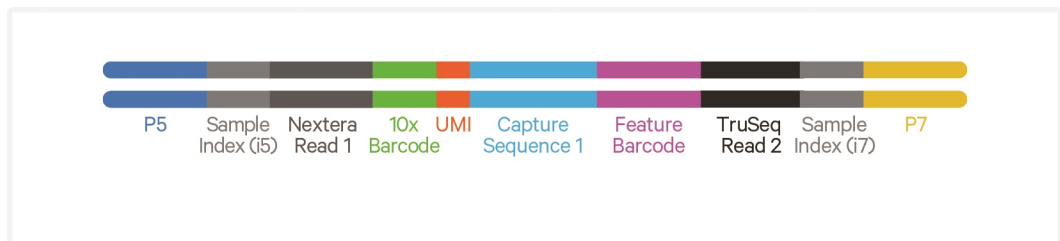
The configuration of sequencing-ready libraries generated using the GEM-X Universal 3' assay is described below:

The Single Cell 3' libraries comprise standard Illumina paired-end constructs which begin and end with P5 and P7. The 16 bp 10x Barcode and 12 bp UMI are encoded in Read 1, while Read 2 is used to sequence the cDNA fragment in 3' Gene Expression libraries and the Feature Barcode in the Cell Surface Protein libraries. i7 and i5 sample index sequences are incorporated as the sample index reads. Standard Illumina sequencing primer sites TruSeq Read 1 and TruSeq Read 2 in the 3' Gene Expression libraries and Nextera Read 1 and TruSeq Read 2 in the Cell Surface Protein libraries are used in paired-end sequencing. Sequencer compatibility, sample indices, library loading and pooling, recommended read depths and run parameters are summarized in the sequencing chapter.

Chromium Single Cell 3' Gene Expression Library



Chromium Single Cell 3' Cell Surface Protein Library

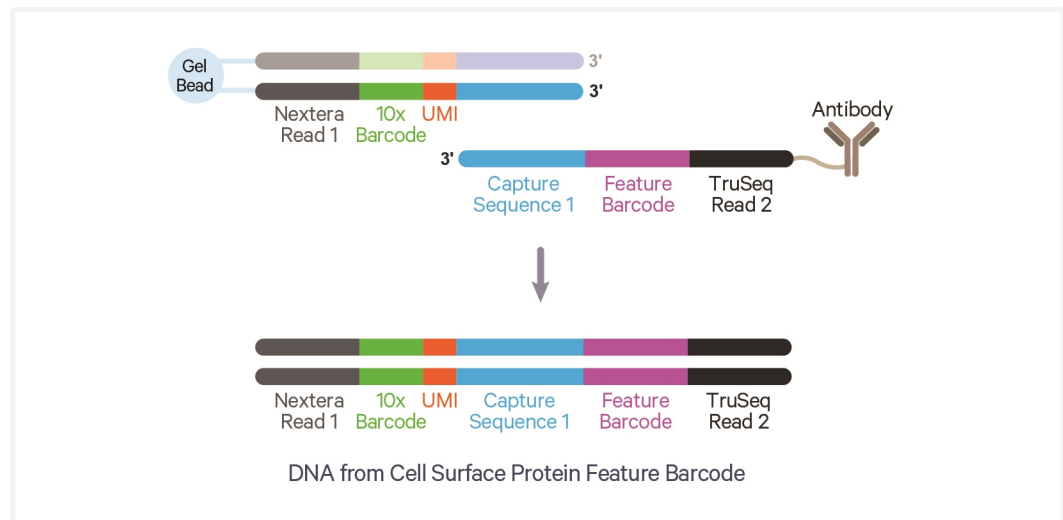


See [Oligonucleotide Sequences](#) on page 95

Cell Surface Protein Labeling Guidelines

Overview

Cell surface proteins can be labeled using a Feature Barcode oligonucleotide conjugated to a specific protein binding molecule, such as an antibody. The Feature Barcode conjugated molecule bound to the cell surface protein can be directly captured by the Gel Bead inside a GEM during GEM generation and amplified (see [Stepwise Objectives](#) for assay scheme specifics). The amplified DNA generated from the Feature Barcode can be used for Cell Surface Protein Library Construction.



Demonstrated Protocols for cell surface protein labeling

- For antibody-oligonucleotide conjugation guidance and cell surface protein labeling protocol, consult [Demonstrated Protocol Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology](#) (Document CG000149).



Failure to label cell surface proteins with a Feature Barcode oligonucleotide conjugated to a specific protein binding molecule prior to using the cells for GEM Generation & Barcoding will preclude generation of Cell Surface Protein library.

Tips & Best Practices



Icons

					
Tips & Best Practices section for additional guidance	Signifies critical step requiring accurate execution	Troubleshooting section for additional guidance	Version specific protocol updates	GEM-X OCM specific protocol updates	GEM-X specific protocol updates

Emulsion-safe Plastics

- Use validated emulsion-safe plastic consumables when handling GEMs as some plastics can destabilize GEMs.
- Consult GEM-X Gene Expression v4 & Immune Profiling v3 - Protocol Planner (CG000748) for a detailed list of plastics and other consumables.

Cell Concentration

- The optimal input cell concentration depends upon the desired cell recovery target:

Optimal Input Cell Concentration	Cell Recovery Target
700-1,200 cells/ μ l	500-5,000 cells

- The presence of dead cells and debris in the suspension may reduce the recovery rate. Consult the following documents for more information on preparing cells:
 - 10x Genomics Single Cell Protocols Cell Preparation Handbook (Documents CG00053)
 - Best Practices to Minimize Chromium Next GEM Chip Clogs and Wetting Failure (Document CG000479)

Multiplet Rate (%) per Sample*	# of Cells Loaded	# of Cells Recovered per sample
~1.5%	~1,650	~1,000
~3.1%	~3,300	~2,000
~4.6%	~4,950	~3,000
~6.1%	~6,600	~4,000
~7.6%	~8,250	~5,000

*Refers to the per sample multiplet rate in one fourth of the total GEMs

General Reagent Handling

- Fully thaw and thoroughly mix reagents before use.
- Keep all enzymes and reagent mixes on ice during setup and use. Promptly move reagents back to the recommended storage.
- Calculate reagent volumes with 10% excess of 1 reaction values.
- Limit Partitioning Oil exposure to air to minimize evaporation.
- If using multiple chips, use separate reagent reservoirs for each chip during loading.
- Thoroughly mix samples with the beads during bead-based cleanup steps.

50% Glycerol Solution for Addition to Unused Chip Wells

- If loading only one set (each set = 4 samples), add 50% glycerol solution to each unused well in row 1, 2, and 3 of the second set.
- 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.

Pipette Calibration

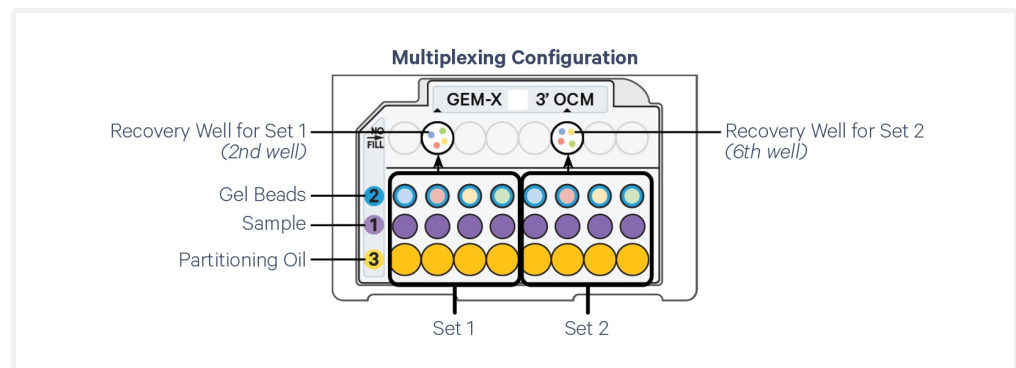
- Follow manufacturer's calibration and maintenance schedules.
- Pipette accuracy is particularly important when using SPRiSelect reagents.

GEM-X OCM Chip

- GEM-X 3' OCM Chip facilitates on-chip multiplexing by enabling GEM generation from up to 4 samples that can be recovered from a single recovery well.
- The GEM-X OCM chip is subdivided into two on-chip multiplexing sets.

- Each set is configured to multiplex up to 4 independent samples by co-partitioning them with corresponding gel beads (each with unique barcodes) to generate a pool of GEMs in a single recovery well (wells 2 and 6 in top row).
- The GEMs from each recovery well are used to generate a single library that is sequenced and computationally demultiplexed during data analysis.
- It is recommended to run samples in a set of 4. If fewer than 4 samples are run in a set, the same volume of sample master mix, gel bead and partitioning oil should be loaded to the corresponding wells as would be added for the set of 4 samples.
- If <4 samples are available, consider running replicates to maximally utilize the reagents and chip.
- All 4 samples loaded in a single multiplexing set should have similar RNA content and cell/nuclei number. If the RNA content is not known or varies significantly between the samples in one multiplexing set, on chip multiplexing is not recommended.

Refer to the [Sample Multiplexing Guidelines on page 40](#) before loading the chip.



Chromium X Series Chip Holder

- Chromium X/iX or X Series Chip Holders encase GEM-X 3' OCM 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 OCM 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.
- When running 4 samples (loading only one multiplexing set), fill the unused wells of the other set in rows labeled 1, 2, and 3 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. See [Sample Multiplexing Guidelines on page 40](#) for details.
- 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 compatible Chromium X series instrument.
- Keep the chip horizontal to prevent wetting the gasket with oil, which depletes the input volume and may adversely affect the quality of the resulting emulsion. DO NOT pick up the assembled chip holder by the holder lid as this could accidentally lead to spillage or dropping of the chip holder.

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 3' OCM Chip is shown below. The Chromium X/iX Chip Holder is also referred to as Chromium X Series Chip holder.



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.
- Refer to [Load GEM-X OCM Chip on page 42](#) for specific instructions.

Chromium X Series Instrument Firmware

- To run the GEM-X 3' OCM chips in the Chromium X instrument, firmware version 2.0.0 or higher is required; however the most updated version is recommended for best performance.
- To run the GEM-X 3' OCM chips in the Chromium Xo instrument, use the most updated firmware version.

- The current firmware version of the instrument will be displayed on the instrument homescreen.
- Consult the Chromium X Series Instrument User Guide with Readiness Test (CG000396) for detailed instructions on updating the firmware.
- After an instrument run, to troubleshoot any potential clogs and failures, it is recommended to photograph the GEMs in the chip recovery wells and the pipette tips during GEM retrieval (or the tubes after GEM transfer).

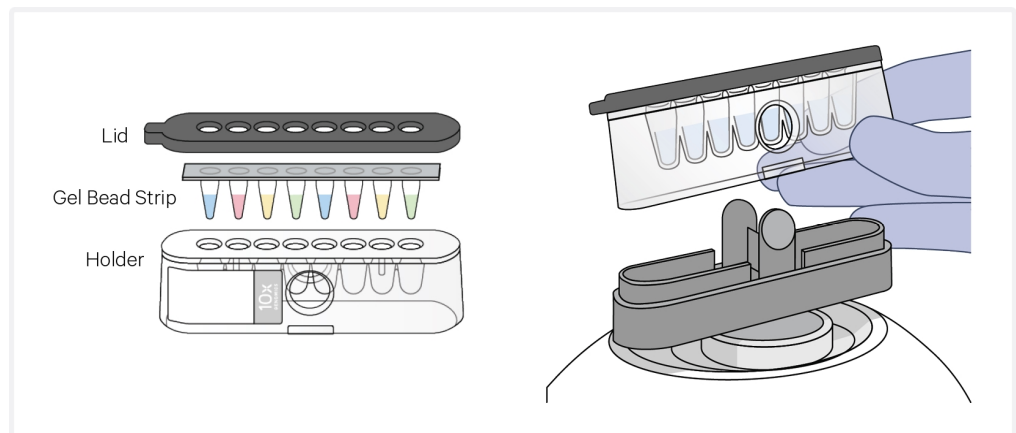
Gel Bead Handling

- 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.



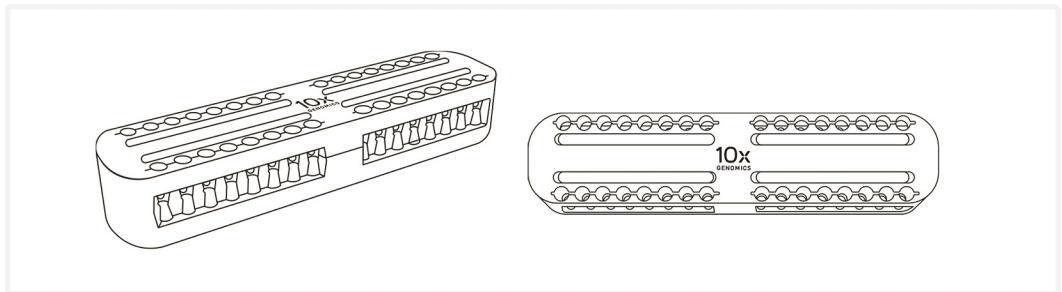
DO NOT store Gel Beads at **-20°C**.

- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**.
- Note the color coded Gel Beads in the tube strip, starting with blue in the first tube followed by red, yellow, and green (see illustration below). When loading the chip, follow the guidance for loading the correct sequence of colored beads to the chip wells.
- 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. DO NOT reuse pipette tips and do not combine aliquots to avoid cross contamination.



10x Magnetic Separator B

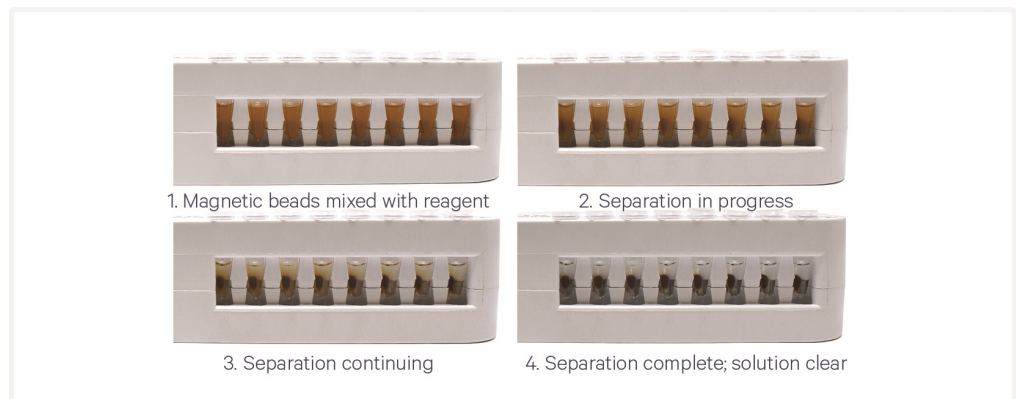
- Images below are illustrative - actual appearance of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.
- Offers two positions of the magnets (high and low) relative to a tube, depending on its orientation. Flip the magnetic separator over to switch between high (magnet•**High**)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



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

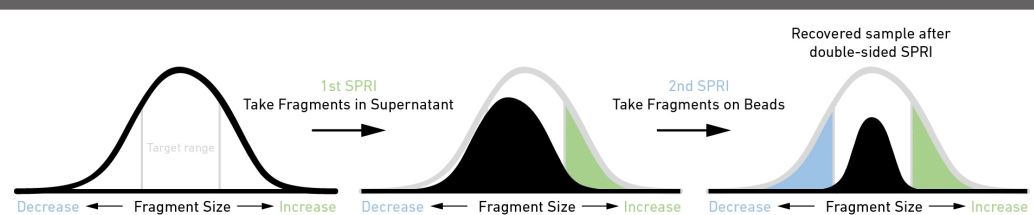
- After aspirating the desired volume of SPRIselect reagent, examine the pipette tips before dispensing to ensure the correct volume is transferred.
- Pipette mix thoroughly as insufficient mixing of sample and SPRIselect reagent will lead to inconsistent results.
- Use fresh preparations of 80% ethanol.

Tutorial — SPRIselect Reagent:DNA Sample Ratios

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

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

Schematic of Double Sided Size Selection



After the first SPRI, supernatant is transferred for a second SPRI while larger fragments are discarded (green). After the second SPRI, fragments on beads are eluted and kept while smaller fragments are discarded (blue). Final sample has a tight fragment size distribution with reduced overall amount (black).

Tutorial — Double Sided Size Selection

a. First SPRIselect: Add **50 μl** SPRIselect reagent to **100 μl** sample (**0.5X**).

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

b. Second SPRIselect: Add **30 μl** SPRIselect reagent to supernatant from step a (**0.8X**).

$$\text{Ratio: } \frac{\text{Total Volume of reagent added to the sample (step a + b)}}{\text{Original Volume of DNA sample}} = \frac{50 \mu\text{l} + 30 \mu\text{l}}{100 \mu\text{l}} = \mathbf{0.8X}$$

Enzymatic Fragmentation

Ensure enzymatic fragmentation reactions are prepared on ice and then loaded into a thermal cycler pre-cooled to **4°C** prior to initiating the Fragmentation, End Repair, and A-tailing incubation steps.

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.

Index Hopping Mitigation

Index hopping can impact pooled samples sequenced on Illumina sequencing platforms that utilize patterned flow cells and exclusion amplification chemistry. To minimize index hopping, follow the guidelines listed below.

- Remove adaptors during cleanup steps.
- Ensure no leftover primers and/or adaptors are present when performing Post-Library Construction QC.
- Store each library individually at **4°C** for up to **72 h** or at **-20°C** for **long-term** storage. DO NOT pool libraries during storage.
- Pool libraries prior to sequencing. An additional 1.0X SPRI may be performed for the pooled libraries to remove any free adaptors before sequencing.
- Hopped indices can be computationally removed from the data generated from single cell dual index libraries.

Step 1:




GEM Generation and Barcoding

1.0 Get Started	35
1.1 Prepare Master Mix	36
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1.2 Load GEM-X OCM Chip	42
1.3 Run the Chromium X Series Instrument	45
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1.5 GEM-RT Incubation	48

1.0 Get Started



Firmware version 2.0.0 or higher is required for Chromium X instrument used for this protocol, however the most updated firmware version is recommended for best performance. For Chromium Xo instrument, use the most updated firmware version. Chromium iX is not compatible without a licensing upgrade.

Item			10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
<input type="checkbox"/>		GEM-X OCM 3' Gel Bead v4	2001126	Equilibrate to room temperature for 30 min before loading the chip.	−80°C
<input type="checkbox"/>		RT Reagent E	2001106	Vortex, verify no precipitate, centrifuge briefly. If a precipitate is observed, warm the tube with hands until the precipitate dissolves. Vortex and centrifuge briefly.	−20°C
<input type="checkbox"/>		Template Switch Oligo B	2001027	Centrifuge briefly, resuspend in 65 µl Low TE Buffer. Vortex 15 sec at maximum speed, centrifuge briefly. Resuspended solution can be used immediately. After resuspension, store at −80°C . Thaw at room temperature for ≥ 30 minutes in subsequent uses.	−20°C
<input type="checkbox"/>		Reducing Agent B	2000087	Vortex, verify no precipitate, centrifuge briefly.	−20°C
Place on Ice					
<input type="checkbox"/>		Labeled Cell Suspension	—	Consult Demonstrated Protocol Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (CG000149)	—
<input type="checkbox"/>		RT Enzyme E	2001146	Centrifuge briefly before adding to the mix.	−20°C
<input type="checkbox"/>		1X PBS	—		—
Obtain					
<input type="checkbox"/>		Partitioning Oil B	2001213	—	Ambient
<input type="checkbox"/>		GEM-X 3' OCM Chip <i>Verify name and PN</i>	2001099	See Tips & Best Practices.	Ambient
<input type="checkbox"/>		X/iX Chip Gasket	3000656	See Tips & Best Practices.	Ambient
<input type="checkbox"/>		Chromium X/iX or Chromium X Series Chip Holder	3000598	See Tips & Best Practices.	Ambient

Item		10x PN	Preparation & Handling	Storage
<input type="checkbox"/>	10x Vortex Adapter	330002	See Tips & Best Practices.	Ambient
<input type="checkbox"/>	50% glycerol solution <i>If loading only one set (each set = 4 samples)</i>	—	See Tips & Best Practices.	—



1.1 Prepare Master Mix

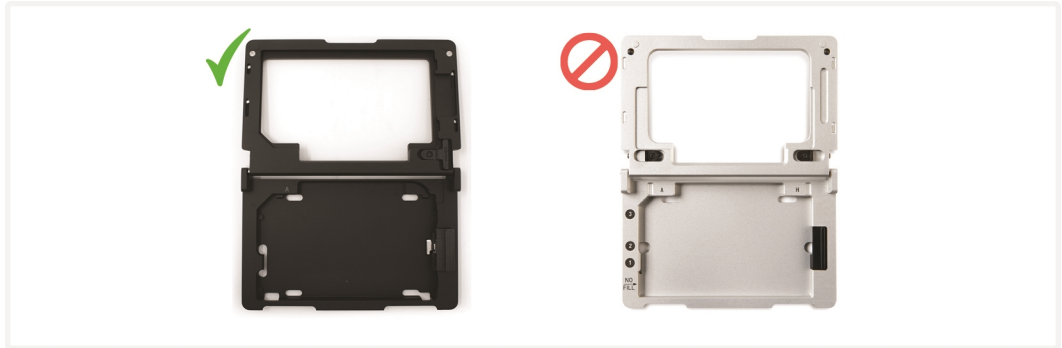
- a. Prepare Master Mix on ice. Pipette mix 15x and centrifuge briefly.

Master Mix <i>Add reagents in the order listed</i>		PN	4X + 10% (μ l)	8X + 10% (μ l)
	RT Reagent E	2001106	18.9	37.8
	Template Switch Oligo B	2001027	3.5	7.0
	Reducing Agent B	2000087	2.2	4.4
	RT Enzyme E	2001146	7.9	15.8
Total		—	32.5	65.0

- b. Add **7.4 μ l** Master Mix into each tube of a PCR 8-tube strip on ice.

Assemble GEM-X OCM Chip

The GEM-X chip is only compatible with Chromium X/iX Chip Holder (also referred to as Chromium X Series Chip Holder; PN-3000598). DO NOT use any other holder.



See [Tips & Best Practices on page 22](#) for chip handling instructions.

- 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.



- Remove the chip from the sealed bag. Use the chip within **≤ 24 h**.
- Align notch on the chip (upper left corner) and the open holder with the gasket attached.
- Slide the chip to the left until the guide on the holder is inserted into the chip. Depress the right hand side of the chip until the spring-loaded clip engages.

A generic image representative of the GEM-X 3' OCM Chip is shown below.

- Keep the assembled unit with the attached gasket open until ready for and while dispensing reagents into the wells.
- 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 and 3. DO NOT load reagents in the top row labeled NO FILL. See step 1.2 for details.



Cell Suspension Volume Calculator Table

For step 1.2 of this protocol.



DO NOT add 1X PBS directly to single cell suspension. Add PBS to the Master Mix. See step 1.2c.

Cell Recovery Target – 500-5,000

Volume of Cell Suspension Stock per reaction (µl) Volume of PBS per reaction (µl)						
Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery					
	500	1000	2000	3000	4000	5000
100	8.3					
	1.3					
200	4.1	8.3				
	5.5	1.3				
300	2.8	5.5				
	6.8	4.1				
400	2.1	4.1	8.3			
	7.5	5.5	1.3			
500	1.7	3.3	6.6			
	7.9	6.3	3.0			
600	1.4	2.8	5.5	8.3		
	8.2	6.8	4.1	1.3		
700	1.2	2.4	4.7	7.1	9.4	
	8.4	7.2	4.9	2.5	0.2	
800	1.0	2.1	4.1	6.2	8.3	
	8.6	7.5	5.5	3.4	1.3	
900	0.9	1.8	3.7	5.5	7.3	9.2
	8.7	7.8	5.9	4.1	2.3	0.4
1000	0.8	1.7	3.3	5.0	6.6	8.3
	8.8	7.9	6.3	4.6	3.0	1.3
1100	0.8	1.5	3.0	4.5	6.0	7.5
	8.8	8.1	6.6	5.1	3.6	2.1
1200	0.7	1.4	2.8	4.1	5.5	6.9
	8.9	8.2	6.8	5.5	4.1	2.7
1300	0.6	1.3	2.5	3.8	5.1	6.3
	9.0	8.3	7.1	5.8	4.5	3.3
1400	0.6	1.2	2.4	3.5	4.7	5.9
	9.0	8.4	7.2	6.1	4.9	3.7
1500	0.6	1.1	2.2	3.3	4.4	5.5
	9.0	8.5	7.4	6.3	5.2	4.1
1600	0.5	1.0	2.1	3.1	4.1	5.2
	9.1	8.6	7.5	6.5	5.5	4.4
1700	0.5	1.0	1.9	2.9	3.9	4.9
	9.1	8.6	7.7	6.7	5.7	4.7
1800	0.5	0.9	1.8	2.8	3.7	4.6
	9.1	8.7	7.8	6.8	5.9	5.0
1900	0.4	0.9	1.7	2.6	3.5	4.3
	9.2	8.7	7.9	7.0	6.1	5.3
2000	0.4	0.8	1.7	2.5	3.3	4.1
	9.2	8.8	7.9	7.1	6.3	5.5
Volumes that would exceed the allowable PBS volume in each reaction						
Low transfer volume that may result in higher cell load variability						
Optimal range of cell stock concentration to maximize the likelihood of achieving the desired cell recovery target (500-5,000 cells)						

Sample Multiplexing Guidelines

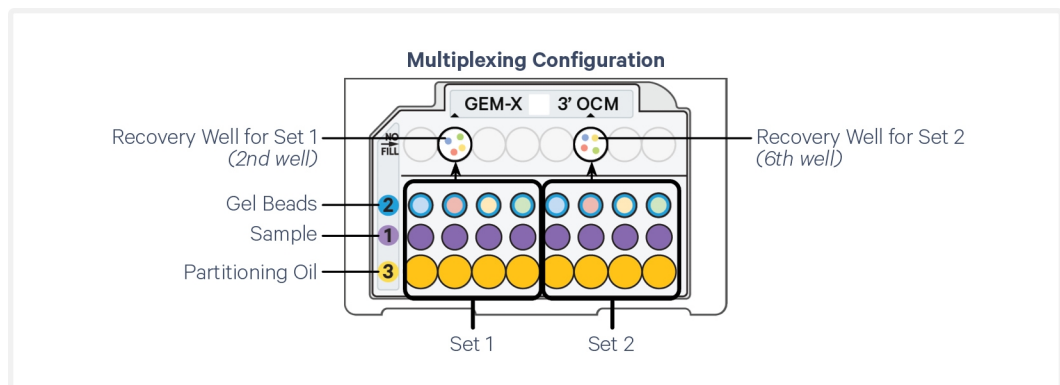
Read these guidelines before loading the GEM-X OCM Chip

GEM-X OCM chip configuration

The GEM-X OCM chip is subdivided into two multiplexing sets, each set configured to multiplex up to 4 samples by co-partitioning them with corresponding gel beads (each with unique barcodes) to generate a pool of GEMs in a single recovery well (wells 2 and 6 in top row). The GEMs from each recovery well are used to generate a single library that is sequenced and computationally demultiplexed by using 10x Barcodes during data analysis.



All 4 samples loaded in a single multiplexing set should be from the same species and have similar RNA content. If loading nuclei suspension, all 4 samples in the set should be nuclei. If the RNA content is not known or varies significantly between the samples in one multiplexing set, on-chip multiplexing is not recommended. Depending on experimental goals, if dissimilar samples are multiplexed, additional sequencing may be required to get sufficient saturation/data across all multiplexed samples.



Chip loading overview

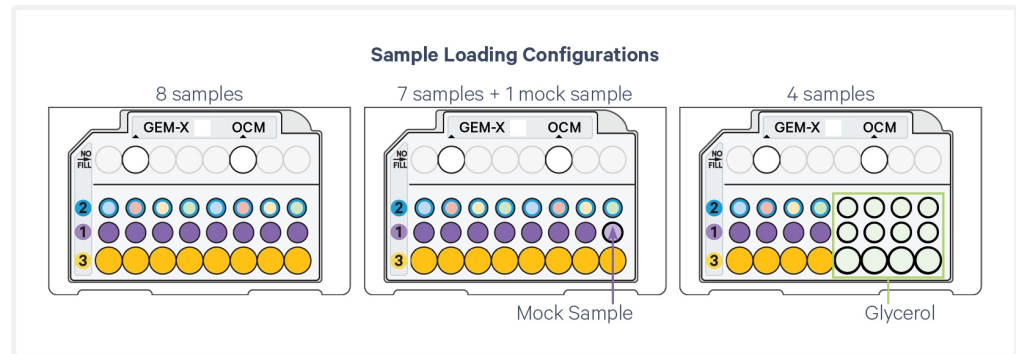
- **Row labeled 1:** 4 samples can be loaded in 4 consecutive wells of each set, with the option of loading just one set (4 samples) or both sets (8 samples). If running fewer than 4 samples in a set, load mock samples (PBS + Master Mix) to ensure that all 4 wells of the set are loaded. If only one multiplexing set is being used, the wells in the second set should be loaded with 50% glycerol.
- **Row labeled 2:** For the 4 samples loaded in a set, the 4 corresponding consecutive wells are loaded with gel beads, where the blue gel bead is loaded in the first well of the set followed by red, yellow, and green. If a mock sample is loaded, the corresponding gel bead should still be loaded.



Note the position of the sample and the corresponding gel bead color loaded on the chip. This information will be required during data analysis.

- **Row labeled 3:** Partitioning Oil B is loaded in the corresponding wells of the set. If a mock sample was loaded, the oil should still be loaded in the corresponding well.

Examples of chip loading configurations



Follow the step-by-step chip loading instructions provided in step 1.2.



1.2 Load GEM-X OCM 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**.

Color Legend

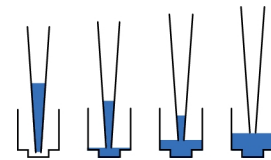
- 2: Gel beads (4 colors - blue, red, yellow, & green)
- 1: Sample
- 3: Oil

The Chromium X/iX or X Series Chip Holder, X/iX Chip Gasket, 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 OCM Chip on page 37](#) for details.

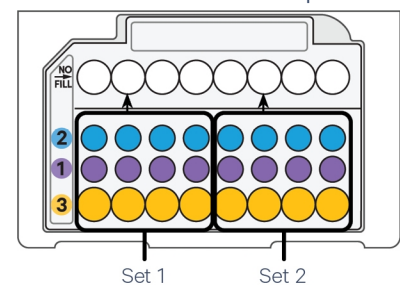
GEM-X 3' OCM Chip, gasket attached
Representative chip image



Pipette technique



Glycerol
in GEM-X 3' OCM Chip



a. If loading only one set (each set = 4 samples), add 50% glycerol solution to each unused well in row 1, 2, and 3 of the second set

If running fewer than 4 samples in a set, DO NOT load glycerol to the unused wells in that set. Refer to [Sample Multiplexing Guidelines on page 40](#) for guidance on mock samples.

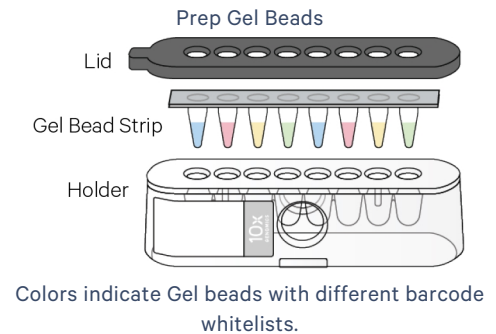
- **15 µl** in each unused well in row labeled **1**
- **18 µl** in each unused well in row labeled **2**
- **70 µl** in each unused well in row labeled **3**

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

DO NOT use any substitute for 50% glycerol solution.

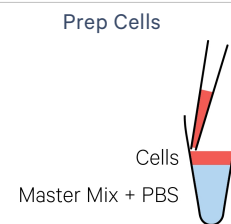
b. Prepare Gel Beads

- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**. Gel Beads are color coded to indicate two sets of 4 Gel Beads each, where each color represents a unique set of barcodes.
- 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. Blue tube should be loaded on the left. Secure the holder lid.



c. Prepare Master Mix + Cell Suspension

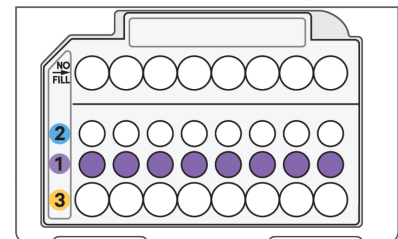
- Refer to the [Cell Suspension Volume Calculator Table](#).
- Add the appropriate volume of **1X PBS** to Master Mix. Pipette mix 5x. Centrifuge briefly.
- Add corresponding volume of **single cell suspension** into the Master Mix + PBS (total 17 μ l in each tube).
- Gently pipette mix the cell suspension before adding to the Master Mix.
- If running less than 4 samples in a multiplexing set, prepare 17 μ l mock sample (add 1X PBS 9.6 μ l to Master Mix 7.4 μ l).



d. Load Row Labeled 1

- Using a multichannel pipette (P20), gently mix the Master Mix + Cell Suspension.
- Using the same pipette tip, dispense **15 μ l** Master Mix + Cell Suspension into the bottom center of each well in **row labeled 1** without introducing bubbles.
- Wait **30 sec**, and then proceed immediately to the next step.
- If running less than 4 samples in a multiplexing set, load **15 μ l** mock sample (**1X PBS** + Master Mix) in the designated well.

15 μ l Master Mix + Cell Suspension
in GEM-X 3' OCM Chip

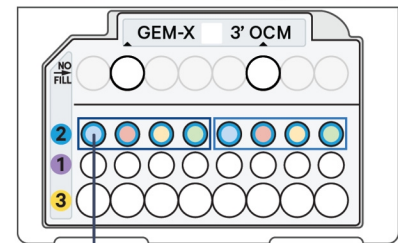


e. Load Row Labeled 2

- Puncture the foil seal of the Gel Bead tubes. Check orientation to ensure blue is loaded in the first well of the set.
- Using a multichannel pipette (P20), slowly aspirate **18 µl** Gel Beads.
- Dispense into the bottom center of each well in **row labeled 2** without introducing bubbles. Load the gel beads such that the blue gel beads are loaded in the first well of the set.
- Wait **30 sec.**
- If a mock sample is loaded, the corresponding gel bead should still be loaded.

18 µl Gel Beads
in GEM-X 3' OCM Chip

Gel Bead Loading



Blue gel beads in first well

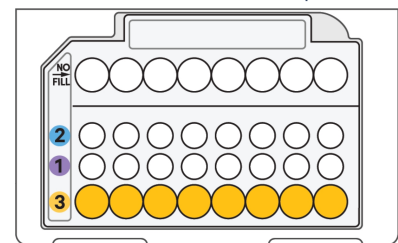
f. Load Row Labeled 3

- Using a multichannel pipette (P100/200), dispense **70 µl** Partitioning Oil B into the wells in **row labeled 3**.
- If a mock sample is loaded, the oil should still be loaded in the corresponding well.



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

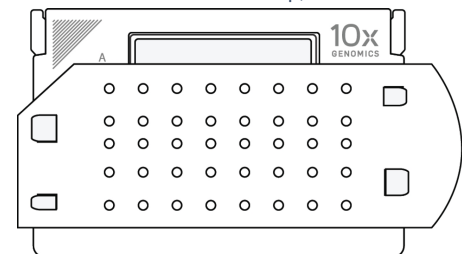
70 µl Partitioning Oil B
in GEM-X 3' OCM Chip

**g. 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 horizontal to avoid wetting the gasket.

*Run the chip on compatible Chromium X Series instrument **immediately** after loading the Partitioning Oil B.*

GEM-X 3' OCM Chip, closed

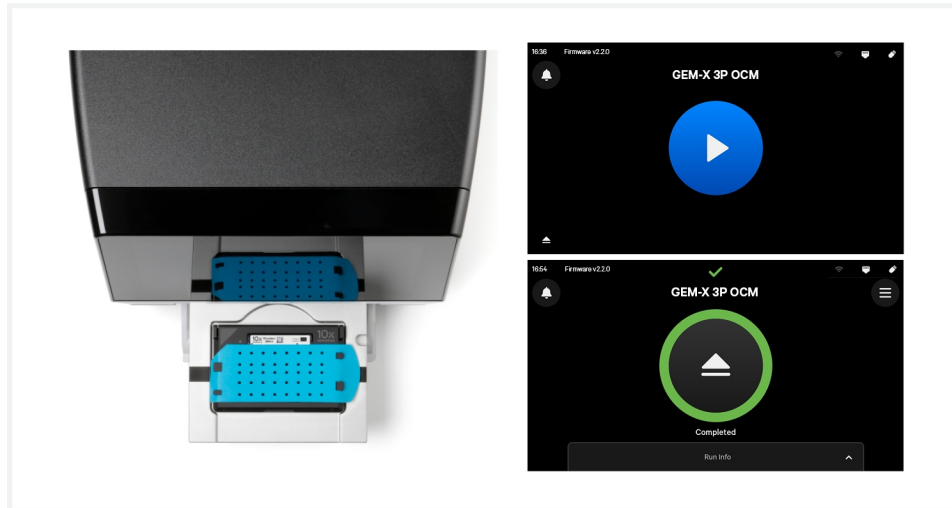



1.3 Run the Chromium X Series Instrument



Firmware version 2.0.0 or higher is required for Chromium X instrument used for this protocol, however the most updated firmware version is recommended for best performance. For Chromium Xo instrument, use the most updated firmware version.

Consult the Chromium X Series User Guide (CG000396) for detailed instrument operation instructions and follow the Chromium X series touchscreen prompts for execution.




- a. Press the eject button on the instrument 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 3P OCM program on the screen. Press the play button.
-  d. At completion of the run (**~4 min**), the instrument will chime.
Immediately proceed to the next step.

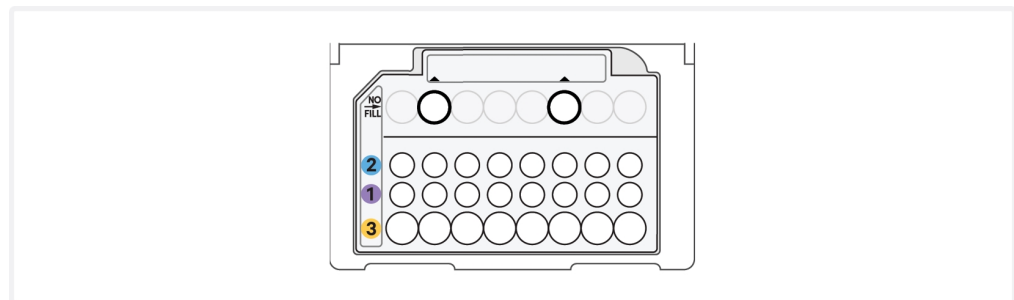


1.4 Transfer GEMs

- a. Place a tube strip on ice.
- b. Press the eject button of the instrument and remove the chip.
- c. Discard the gasket. Open the chip holder. Inspect and photograph GEMs while the chip is still horizontal (see [Troubleshooting on page 84](#)).



- d. Fold the lid back until it clicks to expose the wells at 45 degrees.
- e.  Visually compare the remaining volume in rows labeled 1. An abnormally high volume in one well relative to other wells may indicate a clog. Photograph the GEM(s) in the chip.
- f. Using a single channel pipette, slowly aspirate **100 µl** GEMs separately from the lowest points of the **second** and **sixth** recovery wells in the top NO FILL row without creating a seal between the tips and the bottom of the wells.



- g. Withdraw the pipette tip from the well. GEMs should appear opaque and uniform in both the recovery wells. Excess Partitioning Oil (clear) in the pipette tip indicates a potential clog. Photograph GEMs in the pipette tip against a dark background.



See [Troubleshooting on page 84](#) for additional guidance for clogs/emulsion failures.



- h. Over the course of **~20 sec**, dispense GEMs retrieved from recovery wells 2


and 6 into two separate consecutive tubes of a tube strip on ice with the pipette tip against the sidewall of the tube.

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.

1.5 GEM-RT 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 125 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
	48°C	125 µl	~55 min
	Step	Temperature	Time hh:mm:ss
	1	48°C	00:45:00
	2	85°C	00:05:00
	3	4°C	Hold










- b. Store at **4°C** for up to **72 h** or at **-20°C** for up to **a week**, or proceed to the next step.

Step 2:

Post GEM-RT Cleanup & cDNA Amplification

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2.2 cDNA Amplification	55
Step Overview (steps 2.2 & 2.3)	56
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2.3A Pellet Cleanup for 3' Gene Expression Library	58
2.3B Transferred Supernatant Cleanup for Cell Surface Protein	59
2.4 Post cDNA Amplification QC & Quantification	60

2.0 Get Started

Item			10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
<input type="checkbox"/>		Reducing Agent B	2000087	Thaw, vortex, verify no precipitate, centrifuge briefly.	-20°C
<input type="checkbox"/>	 	Feature cDNA Primers 2 <i>Verify name & PN</i>	2000097	Thaw, vortex, centrifuge briefly.	-20°C
<input type="checkbox"/>		Dynabeads MyOne SILANE	2000048	Vortex thoroughly (≥30 sec) immediately before adding to the mix. If still clumpy, pipette mix to resuspend completely. DO NOT centrifuge before use.	4°C
<input type="checkbox"/>		Beckman Coulter SPRIselect Reagent	—	Manufacturer's recommendations.	—
<input type="checkbox"/>		Agilent Bioanalyzer High Sensitivity Kit <i>If used for QC & quantification</i>	—	Manufacturer's recommendations.	—
<input type="checkbox"/>		DNA High Sensitivity Reagent Kit <i>If LabChip used for QC & quantification</i>	—	Manufacturer's recommendations.	—
<input type="checkbox"/>		Agilent TapeStation ScreenTape & Reagents <i>If used for QC & quantification</i>	—	Manufacturer's recommendations.	—
Place on Ice					
<input type="checkbox"/>	 	Amp Mix <i>DO NOT use the Library Amp Mix PN-2000531 (if provided) at this step to avoid a significant decrease in assay performance</i>	2000047 /2000103	Vortex, centrifuge briefly.	-20°C
Thaw at 65°C					
<input type="checkbox"/>		Cleanup Buffer	2000088	Thaw for 10 min at 65°C at max speed on a thermomixer. Verify no visible crystals. Cool to room temperature.	-20°C
Obtain					
<input type="checkbox"/>		Recovery Agent	220016	—	Ambient
<input type="checkbox"/>		10x Magnetic Separator B	2001212	—	Ambient
<input type="checkbox"/>		Qiagen Buffer EB	—	Manufacturer's recommendations.	—
<input type="checkbox"/>		Bio-Rad 10%	—	Manufacturer's	—

Item		10x PN	Preparation & Handling	Storage
	Tween 20		recommendations.	
<input type="checkbox"/>	Prepare 80% Ethanol <i>Prepare 15 ml for 8 reactions.</i>	—	Prepare fresh.	Ambient

2.1 Post GEM-RT Cleanup – Dynabeads

- a. Add **125 µl** Recovery Agent to each sample (Post GEM-RT) at room temperature. *If using sample stored at -20°C, equilibrate to room temperature before adding the Recovery Agent.*



DO NOT pipette mix or vortex the biphasic mixture. Wait **2 min.**

The resulting biphasic mixture contains Recovery Agent/Partitioning Oil (pink) and aqueous phase (clear), with no persisting emulsion (opaque).

If biphasic separation is incomplete:

- 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, centrifuge briefly, and proceed to step b. DO NOT invert without firmly securing the caps.



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

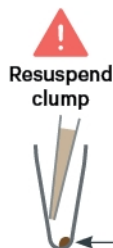


- b. Slowly remove and discard **125 µl** Recovery Agent/Partitioning Oil (pink) from the bottom of the tube. DO NOT aspirate any aqueous sample.



- c. Prepare Dynabeads Cleanup Mix.

Before using Dynabeads MyOne SILANE to prepare the Dynabeads Cleanup Mix:



Vortex the Dynabeads thoroughly (**≥30 sec**) **immediately** before adding to the mix.

- Aspirate full liquid volume in the Dynabead tube with a pipette tip to verify that beads have not settled in the bottom of the tube. If clumps are present, pipette mix to resuspend completely. DO NOT centrifuge before adding to the mix.

Dynabeads Cleanup Mix <i>Add reagents in the order listed</i>		PN	1X (μ l)	2X + 10% (μ l)	4X + 10% (μ l)
●	Cleanup Buffer	2000088	182	400.5	801
	Dynabeads MyOne SILANE	2000048	8	17.5	35
○	Reducing Agent B	2000087	5	11	22
	Nuclease-free Water	—	5	11	22
Total			200	440	880

- d. Vortex and add **200 μ l** to each tube. Pipette mix 10x (pipette set to 200 μ l). Before adding ensure that there are no clumps.



- e. Incubate **10 min** at **room temperature** (keep caps open). Pipette mix again at ~5 min after start of incubation to resuspend settled beads.
- f. Prepare Elution Solution I. Vortex and centrifuge briefly.

Elution Solution I <i>Add reagents in the order listed</i>		PN	1X (μ l)	10X (μ l)
	Buffer EB	—	98	980
	10% Tween 20	—	1	10
○	Reducing Agent B	2000087	1	10
	Total		100	1000

TIPS

- g. At the end of **10 min** incubation, place on a 10x Magnetic Separator **High** position (magnet **High**) until the solution clears.

A white interface between the aqueous phase and Recovery Agent is normal.

- h. Remove the supernatant.
- i. Add **300 μ l** 80% ethanol to the pellet while on the magnet. Wait **30 sec**.
- j. Remove the ethanol.
- k. Add **200 μ l** 80% ethanol to pellet. Wait **30 sec**.
- l. Remove the ethanol.

- m.** Centrifuge briefly. Place on the 10x Magnetic Separator **Low** position (magnet **Low**).
- n.** Remove remaining ethanol. Air dry for **1 min**.
- o.** Remove from the magnet. Immediately add **35.5 µl** Elution Solution I.
- p.** Pipette mix (pipette set to 30 µl) without introducing bubbles. Pipette mix 15x. If beads still appear clumpy, continue pipette mixing until fully resuspended.
- q.** Incubate **1 min** at **room temperature**.
- r.** Place on the magnet **Low** until the solution clears.
- s.** Transfer **35 µl** sample to a new tube strip.

2.2 cDNA Amplification

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

cDNA Amplification Mix <i>Add reagents in the order listed</i>		PN	1X (μ l)	2X + 10% (μ l)	4X + 10% (μ l)
	Amp Mix DO NOT use the Library Amp Mix PN-2000531 (if provided)	2000047/ 2000103	50	110	220
 	Feature cDNA Primers 2 Verify name & PN	2000097	15	33	66
Total			65	143	286

- b. Add **65 μ l** cDNA Amplification Reaction Mix to **35 μ l** sample (Post-GEM-RT Cleanup, step 2.1s).
- c. Pipette mix 15x (pipette set to 90 μ l). Centrifuge briefly.
- d. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 μ l	~25-50 min

Step	Temperature	Time hh:mm:ss
1	98°C	00:00:45
2	98°C	00:00:20
3	63°C	00:00:30
4	72°C	00:01:00
5	Go to Step 2, see table below for total # of cycles	
6	72°C	00:01:00
7	4°C	Hold

TIPS

Recommended starting point for cycle number optimization. The optimal cycle number is a trade-off between generating sufficient final mass for libraries & minimizing PCR amplification artifacts.

Targeted Cell Recovery (from a set with up to 4 samples)	Total Cycles
<500	13
501-6,000	12
>6,000	11



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

Step Overview (steps 2.2 & 2.3)

Amplification Products Generated in Step 2.2 – cDNA Amplification

Post GEM-RT Cleanup Products

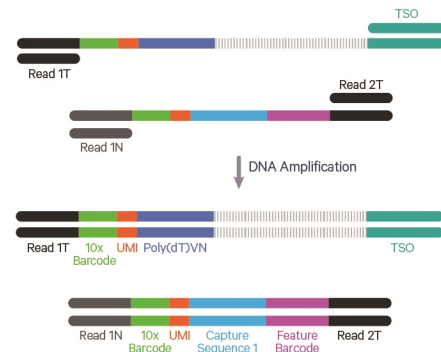
+

Feature cDNA Primers 2
PN-2000097

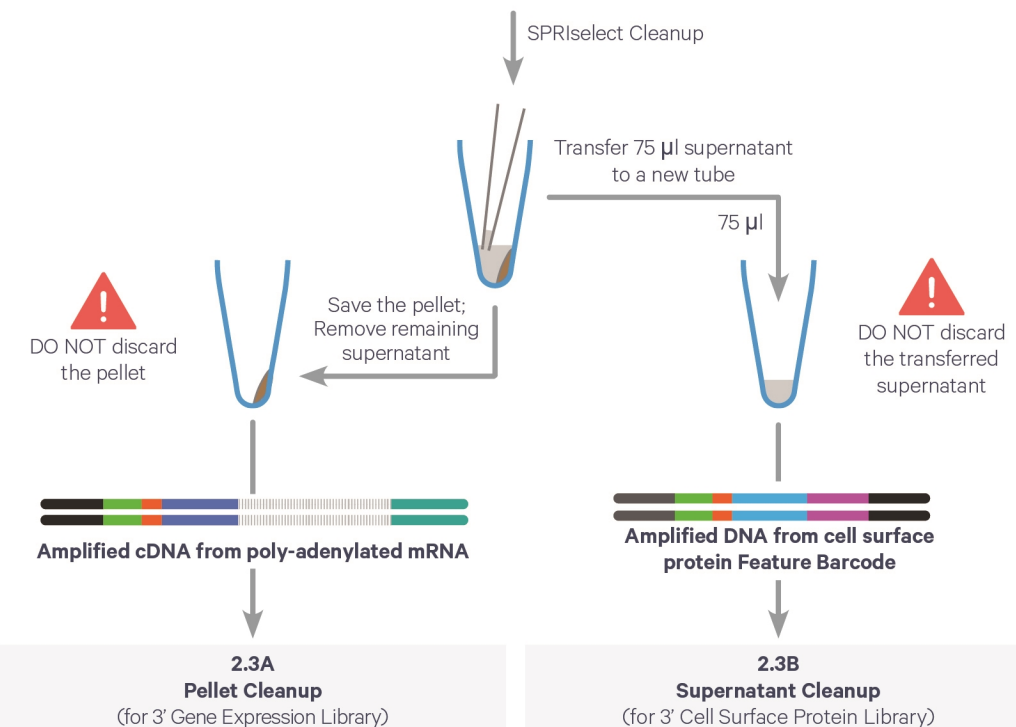
Amplification Products

cDNA from poly-adenylated mRNA


DNA from cell surface protein Feature Barcode



Step 2.3 – cDNA Cleanup – SPRIselect Overview



2.3 cDNA Cleanup – SPRIselect


- a. Vortex to resuspend the SPRIselect reagent. Add **60 µl** SPRIselect reagent (**0.6X**) to each sample and pipette mix 15x (pipette set to 150 µl).
- b. Incubate **5 min** at **room temperature**.
- c. Place on the magnet **High** until the solution clears.
-  d. Transfer and save **75 µl** supernatant in a new tube strip without disturbing the pellet. Maintain at **room temperature**. DO NOT discard the transferred supernatant (cleanup for Cell Surface Protein library construction step 2.3B).
- e. Remove the remaining supernatant from the pellet without disturbing the pellet. DO NOT discard the pellet (cleanup for 3' Gene Expression library construction). **Immediately** proceed to Pellet Cleanup (step 2.3A).

2.3A Pellet Cleanup for 3' Gene Expression Library

- a. Add **200 µl** 80% ethanol to the pellet while still on the magnet•**High**. Wait **30 sec**.
- b. Remove the ethanol.
- c. **Repeat** steps a and b for a total of 2 washes.
- d. Centrifuge briefly and place on the magnet•**Low**.
- e. Remove any remaining ethanol. Air dry for **2 min**. DO NOT exceed **2 min** as this will decrease elution efficiency.
- f. Remove from the magnet. Add **40.5 µl** Buffer EB. Pipette mix 15x.
- g. Incubate **2 min** at **room temperature**.
- h. Place the tube strip on the magnet•**High** until the solution clears.
- i. Transfer **40 µl** sample to a new tube strip.
- j. Store at **4°C** for up to **72 h** or at **-20°C** for up to **4 weeks**, or proceed to step 2.4 followed by step 3 for 3' Gene Expression Library Construction.

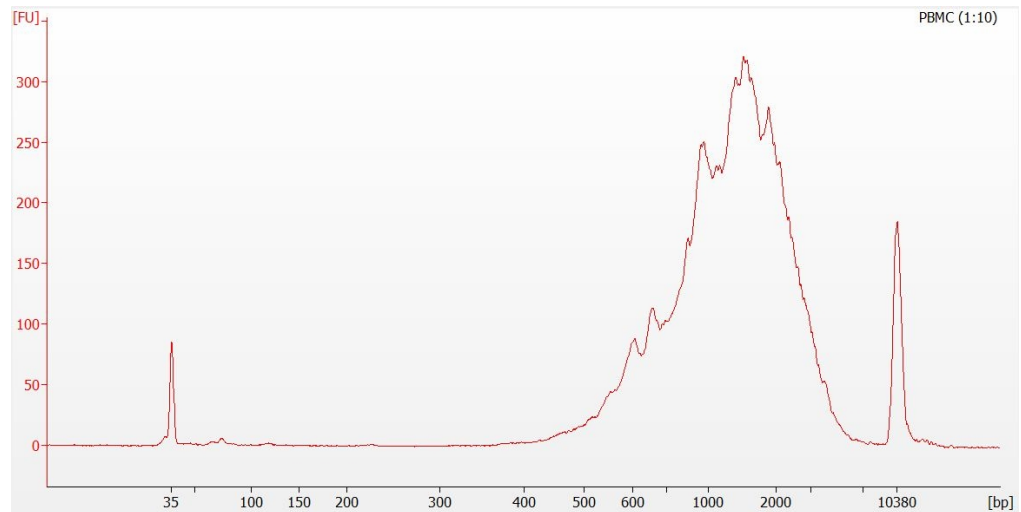


2.3B Transferred Supernatant Cleanup for Cell Surface Protein

- a. Vortex to resuspend the SPRIselect reagent. Add **70 µl** SPRIselect reagent (**2.0X**) to 75 µl of the transferred supernatant and pipette mix 15x (pipette set to 140 µl).
- b. Incubate for **5 min** at **room temperature**.
- c. Place on the magnet•**High** until the solution clears.
- d. Remove supernatant.
- e. 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. Air dry for **2 min**. DO NOT exceed **2 min** as this will decrease elution efficiency.
- j. Remove from the magnet. Add **40.5 µl** Buffer EB. Pipette mix 15x.
- k. Incubate **2 min** at **room temperature**.
- l. Place the tube strip on the magnet•**High** until the solution clears.
- m. Transfer **40 µl** sample to a new tube strip.
-  n. Store at **4°C** for up to **72 h** or at **-20°C** for up to **4 weeks**, or proceed directly to step 4 for Cell Surface Protein Library Construction.

2.4 Post cDNA Amplification QC & Quantification

- a. Run 1 μ l (see dilutions below) product from step 2.3A on an Agilent Bioanalyzer High Sensitivity chip.
- Low RNA content cells (<1 pg total RNA/cell) should be run undiluted
 - High RNA content cells should be run at 1:5 or 1:10 dilution
- DO NOT run sample from 2.3B Transferred Supernatant Cleanup step.



- b. See example calculation in the following page.

Alternate Quantification Methods

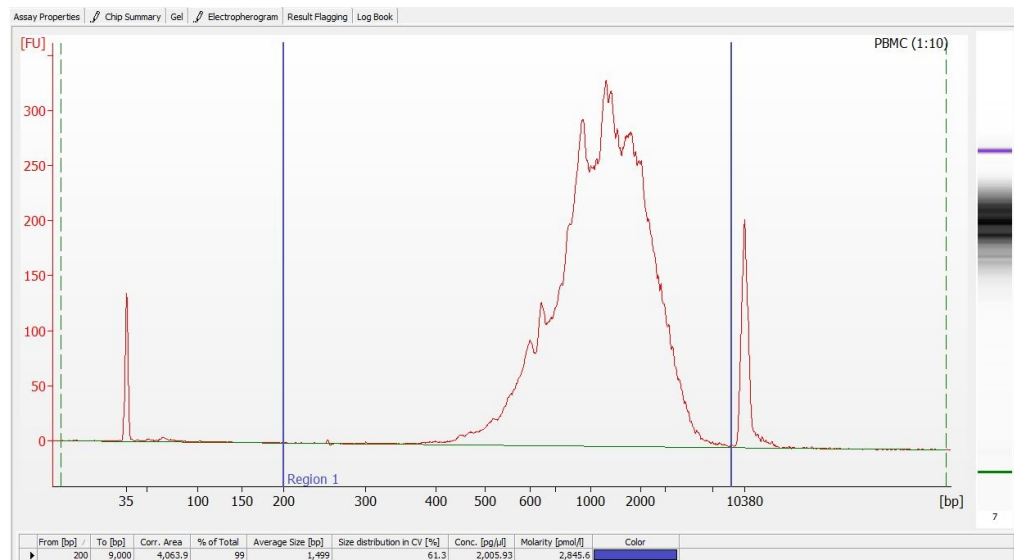
Agilent TapeStation

LabChip

See [Appendix on page 89](#) for representative traces.

Example Calculation

- Select Region: Under the “Electropherogram” view, choose the “Region Table.” Manually select the region of ~200 – ~9000 bp.
- Note Concentration [pg/ μ l]



- iii. Calculate: Multiply the diluted sample concentration [pg/μl] reported via Agilent 2100 Expert Software by the dilution factor and divide by 1000 to obtain the total cDNA yield in ng/μl.

Carry forward ONLY 25% of total cDNA yield into Gene Expression Library Construction.

Example Calculation of cDNA Yield

Concentration: **2005.93 pg/μl** Elution volume: 40 μl; Dilution Factor: 10

Total Yield

$$\begin{aligned}
 &= \frac{\text{Conc'n (pg/μl)} \times \text{Elution Vol. (μl)} \times \text{Dilution Factor}}{1000 \text{ (pg/ng)}} \\
 &= \frac{2005.93 \times 40 \times 10}{1000 \text{ (pg/ng)}} \\
 &= 802.37 \text{ ng}
 \end{aligned}$$

Carrying Forward ONLY 25% of total cDNA yield for GEX Library

$$= \text{Total cDNA} \times 0.25 = 802.37 \text{ ng} \times 0.25 = \mathbf{200.59 \text{ ng}}$$



Refer to step 3.5 for appropriate number of Sample Index PCR cycles based on carry forward cDNA product yield.

Step 3:

3' Gene Expression Library Construction

3.0 Get Started	63
Step Overview (Step 3.1d)	64
3.1 GEX Fragmentation, End Repair & A-tailing	65
3.2 GEX Post Fragmentation, End Repair & A-tailing Double Sided – SPRIselect	66
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3.5 GEX Sample Index PCR	69
3.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect	70
3.7 Post Library Construction QC	71

3.0 Get Started

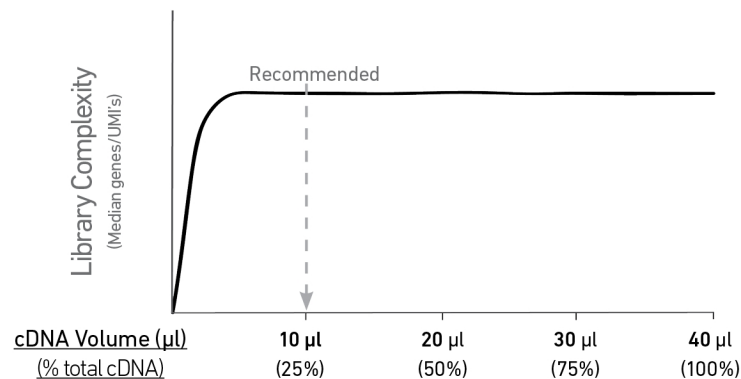
Item			10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
<input type="checkbox"/>		Fragmentation Buffer	2000091	Thaw, vortex, verify no precipitate, centrifuge.	-20°C
<input type="checkbox"/>		Ligation Mix	2001109	Thaw, vortex, verify no precipitate, centrifuge.	-20°C
<input type="checkbox"/>		Dual Index Plate TT Set A <i>Verify name & PN. Use indicated plate only</i>	3000431	—	-20°C
<input type="checkbox"/>		Beckman Coulter SPRIselect Reagent	—	Manufacturer's recommendations.	—
<input type="checkbox"/>		Agilent Bioanalyzer High Sensitivity Kit <i>If used for QC</i>	—	Manufacturer's recommendations.	—
<input type="checkbox"/>		DNA High Sensitivity Reagent Kit <i>If LabChip used for QC</i>	—	Manufacturer's recommendations.	—
<input type="checkbox"/>		Agilent TapeStation ScreenTape & Reagents <i>If used for QC</i>	—	Manufacturer's recommendations.	—
Place on Ice					
<input type="checkbox"/>		Fragmentation Enzyme <i>Ensure that Fragmentation Buffer and Fragmentation Enzyme from the same kit are used together. Lots are matched for optimal performance</i>	2000090 /2000104	Centrifuge briefly.	-20°C
<input type="checkbox"/>		DNA Ligase	220110/220131	Centrifuge briefly.	-20°C
<input type="checkbox"/>		Amp Mix or Library Amp Mix	2000047/2000103 or 2000531	Vortex, centrifuge briefly.	-20°C
<input type="checkbox"/>		KAPA Library Quantification Kit for Illumina Platforms	—	Manufacturer's recommendations.	—
Obtain					
<input type="checkbox"/>		10x Magnetic Separator B	2001212	See Tips & Best Practices.	Ambient
<input type="checkbox"/>		Qiagen Buffer EB	—	Manufacturer's recommendations.	Ambient
<input type="checkbox"/>		Prepare 80% Ethanol <i>Prepare 15 ml for 8 reactions.</i>	—	Prepare fresh.	Ambient

Step Overview (Step 3.1d)





Correlation between input & library complexity

A Single Cell Gene Expression library is generated using a fixed proportion (10 μ l, 25%) of the total cDNA (40 μ l) obtained at step 2.3A. The complexity of this library will be comparable to one generated using a higher proportion (>25%) of the cDNA. The remaining proportion (30 μ l, 75%) of the cDNA may be stored at 4°C for up to 72 h or at -20°C for long-term storage (up to 4 weeks).

Correlation: cDNA input & Library Complexity



Note that irrespective of the total cDNA yield (ng), which may vary based on cell type, targeted cell recovery etc., this protocol has been optimized for a broad range of input mass (ng), as shown in the example below. The total number of SI PCR cycles (step 3.5d) should be optimized based on carrying forward a fixed proportion (10 μ l, 25%) of the total cDNA yield calculated during Post cDNA Amplification QC & Quantification (step 2.4).



Cell	Targeted Cell Recovery	Total cDNA Yield (ng)	cDNA Input into Fragmentation		SI PCR Cycle Number
			Volume (μ l)	Mass (ng)	
High RNA Content	Low 	500 ng	10 μ l	125 ng	13
	High 	3800 ng	10 μ l	950 ng	10
Low RNA Content	Low 	2 ng	10 μ l	0.5 ng	16
	High 	400 ng	10 μ l	100 ng	12

3.1 GEX Fragmentation, End Repair & A-tailing

- a. Prepare a thermal cycler with the following incubation protocol.

Lid Temperature		Reaction Volume	Run Time
65°C		50 µl	~35 min
Step		Temperature	Time hh:mm:ss
	Pre-cool block		
	<i>Pre-cool block prior to preparing the Fragmentation Mix</i>	4°C	Hold
	Fragmentation	32°C	00:05:00
	End Repair & A-Tailing	65°C	00:30:00
	Hold	4°C	Hold

- b. Vortex Fragmentation Buffer. Verify there is no precipitate.
- c. Prepare Fragmentation Mix on ice. Add reagents in the order listed. Pipette mix and centrifuge briefly.

Fragmentation Mix <i>Add reagents in the order listed</i>		PN	1X (µl)	2X + 10% (µl)	4X + 10% (µl)
	Buffer EB	-	25	55	110
	Fragmentation Buffer	2000091	5	11	22
	Fragmentation Enzyme	2000090/ 2000104	10	22	44
Total			40	88	176



- d. Transfer **ONLY 10 µl** purified cDNA sample from Cleanup (step 2.3A) to a tube strip. Note that only 10 µl (25%) cDNA sample is sufficient for generating Gene Expression library. The remaining cDNA sample can be stored at **4°C** for up to **72 h** or at **-20°C** for up to **4 weeks** for generating additional Gene Expression libraries.
- e. Add **40 µl** Fragmentation Mix to each **10 µl** sample.
- f. Pipette mix 15x (pipette set to 30 µl) on ice. Centrifuge briefly.
- g. Transfer into the pre-cooled thermal cycler (**4°C**) and press “SKIP” to initiate the protocol.

3.2 GEX Post Fragmentation, End Repair & A-tailing Double Sided – SPRiselect

- a. Vortex to resuspend SPRiselect reagent. Add **30 µl** SPRiselect reagent(**0.6X**) to each sample. Pipette mix 15x (pipette set to 75 µl).
- b. Incubate **5 min** at **room temperature**.
- c. Place on the magnet•**High** until the solution clears. DO NOT discard supernatant.
- d. Transfer **75 µl** supernatant to a new tube strip.
- e. Vortex to resuspend SPRiselect reagent. Add **10 µl** SPRiselect reagent (**0.8X**) to each sample. Pipette mix 15x (pipette set to 80 µl).
- f. Incubate **5 min** at **room temperature**.
- g. Place on the magnet•**High** until the solution clears.
- h. Remove **80 µl** supernatant. DO NOT discard any beads.
- i. With the tube strip still on the magnet, add **125 µl** 80% ethanol to the pellet. Wait **30 sec**.
- j. Remove the ethanol.
- k. **Repeat** steps i and j for a total of 2 washes.
- l. Centrifuge briefly. Place on the magnet•**Low** until the solution clears.
- m. Remove remaining ethanol. DO NOT over dry to ensure maximum elution efficiency.
- n. Remove from the magnet. Add **50.5 µl** Buffer EB to each sample. Pipette mix 15x (pipette set to 45 µl).
- o. Incubate **2 min** at **room temperature**.
- p. Place on the magnet•**High** until the solution clears.
- q. Transfer **50 µl** sample to a new tube strip.

3.3 GEX Adaptor Ligation

- a. Prepare Adaptor Ligation Mix. Pipette mix and centrifuge briefly.

Adaptor Ligation Mix <i>Add reagents in the order listed</i>	PN	1X (μ l)	2X + 10% (μ l)	4X + 10% (μ l)
 Ligation Mix	2001109	40	88	176
 DNA Ligase	220110/220131	10	22	44
Total		50	110	220

- b. Add **50 μ l** Adaptor Ligation Mix to **50 μ l** sample. Pipette mix 15x (pipette set to 90 μ l). Centrifuge briefly.
- c. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
30°C (lid may be turned off if the instrument does not enable 30°C)	100 μ l	15 min

Step	Temperature	Time hh:mm:ss
1	20°C	00:15:00
2	4°C	Hold

3.4 GEX Post Ligation Cleanup – SPRIselect

- a. Vortex to resuspend SPRIselect Reagent. Add **80 µl** SPRIselect Reagent (**0.8X**) to each sample. Pipette mix 15x (pipette set to 150 µl).
- b. Incubate **5 min** at **room temperature**.
- c. Place on the magnet•**High** until the solution clears.
- d. Remove the supernatant.
- e. 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. Place on the magnet•**Low**.
- i. Remove any remaining ethanol. Air dry for **2 min**. DO NOT exceed 2 min as this will decrease elution efficiency.
- j. Remove from the magnet. Add **30.5 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 **30 µl** sample to a new tube strip.

3.5 GEX 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-3000431 Dual Index Plate TT Set A well ID) used.
- b. Add **50 µl** Amp Mix (PN-2000047/2000103) or Library Amp Mix (PN-2000531) to **30 µl** sample.
- c. Add **20 µl** of an individual Dual Index TT Set A to each sample and record the well ID used. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.
- d. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~30 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, see below for # of cycles	
6	72°C	00:01:00
7	4°C	Hold



The table recommends a starting point for optimization. The total cycles should be optimized based on 25% carry forward cDNA yield/input calculated during cDNA QC & Quantification (step 2.4).

Recommended Cycle Numbers

cDNA Input	Total Cycles
0.25-50 ng	14-16
50-250 ng	12-14
250-600 ng	10-12
600-1,100 ng	8-10
1,100-1,500 ng	6-8
>1,500 ng	5



- e. Store at **4°C** for up to **72 h** or proceed to the next step.

3.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect

- a. Vortex to resuspend the SPRIselect reagent. Add **60 µl** SPRIselect Reagent (**0.6X**) to each sample. Pipette mix 15x (pipette set to 150 µl).
- b. Incubate **5 min** at **room temperature**.
- c. Place the magnet **High** until the solution clears. DO NOT discard supernatant.
- d. Transfer **150 µl** supernatant to a new tube strip.
- e. Vortex to resuspend the SPRIselect reagent. Add **20 µl** SPRIselect Reagent (**0.8X**) to each transferred supernatant. Pipette mix 15x (pipette set to 150 µl).
- f. Incubate **5 min** at **room temperature**.
- g. Place the magnet **High** until the solution clears.
- h. Remove **165 µl** supernatant. DO NOT discard any beads.
- i. With the tube still in the magnet, add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.
- j. Remove the ethanol.
- k. **Repeat** steps i and j for a total of 2 washes.
- l. Centrifuge briefly. Place on the magnet **Low**.
- m. Remove remaining ethanol. DO NOT over dry beads to ensure maximum elution efficiency.
- n. Remove from the magnet. Add **35.5 µl** Buffer EB. Pipette mix 15x (pipette set to 35 µl).
- o. Incubate **2 min** at **room temperature**.
- p. Place on the magnet **Low** until the solution clears.
- q. Transfer **35 µl** to a new tube strip.
- r. Store at **4°C** for up to **72 h** or at **-20°C** for **long-term** storage.



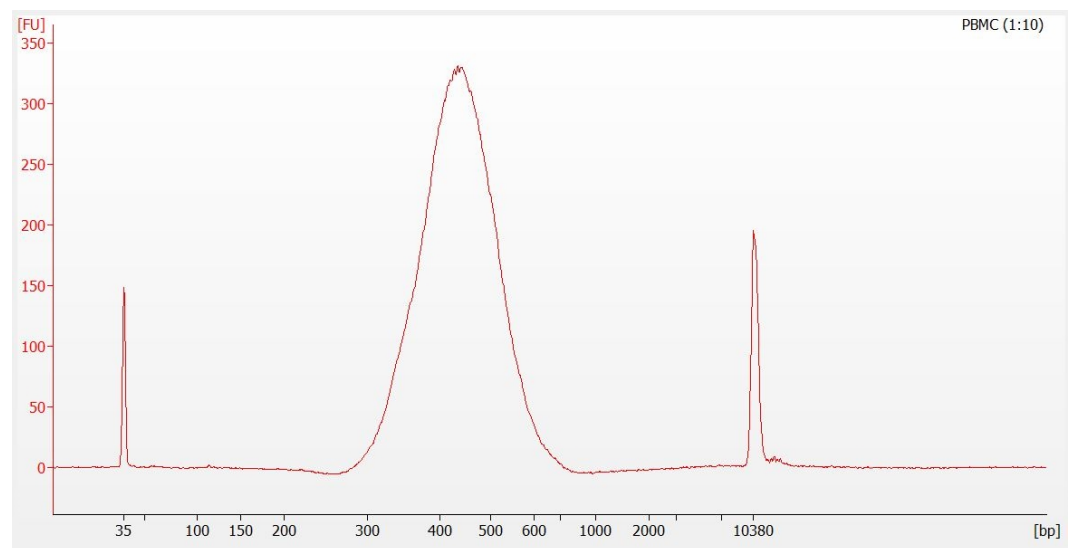
3.7 Post Library Construction QC

Library QC

Use Agilent Bioanalyzer, Perkin Elmer LabChip, Agilent Tapestation, or Fragment Analyzer for QC.

- a. Run **1 µl** sample at **1:10** dilution on an Agilent Bioanalyzer High Sensitivity chip.
- a. Select the region between 200-2,000 bp to determine average size of the library. This will be used as the insert size for library quantification.

Representative Trace



If additional peaks below 200 bp are observed, repeat step 3.6 Post Sample Index PCR Double Sided Size Selection - SPRIselect. Add nuclease-free water to bring the library volume to 100 µl before performing step 3.6a. Note that ~40% of material may be lost when repeating step 3.6. Alternatively, libraries that will be sequenced together can first be pooled and then used as input into step 3.6.

See [Appendix on page 89](#) for representative traces

Quantification

Library quantification should be performed prior to sequencing. For the most accurate quantitative assessment of libraries, a qPCR-based method should be used to ensure that the sequencing flowcell is loaded properly.



See [Post Library Construction Quantification using KAPA qPCR on page 91](#)

Step 4:

Cell Surface Protein Library Construction

4.0 Get Started	73
4.1 Sample Index PCR	74
4.2 Post Sample Index PCR Size Selection – SPRIselect	76
4.3 Post Library Construction QC	77

4.0 Get Started

Item			10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
<input type="checkbox"/>		Dual Index Plate NT Set A Verify name & PN. Use indicated plate only	3000483	—	–20°C
<input type="checkbox"/>		Beckman Coulter SPRIselect Reagent	—	Manufacturer’s recommendations.	—
<input type="checkbox"/>		Agilent Bioanalyzer High Sensitivity Kit If used for QC	—	Manufacturer’s recommendations.	—
<input type="checkbox"/>		DNA High Sensitivity Reagent Kit If LabChip used for QC	—	Manufacturer’s recommendations.	—
<input type="checkbox"/>		Agilent TapeStation ScreenTape & Reagents If used for QC	—	Manufacturer’s recommendations.	—
Place on Ice					
<input type="checkbox"/>		Amp Mix Retrieve from Chromium GEM-X Single Cell 3’ Feature Barcode Kit	2000047	Centrifuge briefly.	–20°C
<input type="checkbox"/>		KAPA Library Quantification Kit for Illumina Platforms	—	Manufacturer’s recommendations.	—
Obtain					
<input type="checkbox"/>		10x Magnetic Separator B	2001212	See Tips & Best Practices.	Ambient
<input type="checkbox"/>		Qiagen Buffer EB	—	Manufacturer’s recommendations.	Ambient
<input type="checkbox"/>		Prepare 80% Ethanol Prepare 20 ml for 8 reactions.	—	Prepare fresh.	Ambient

4.1 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-3000483 Dual Index Plate NT Set A well ID) used.

- b. Prepare Sample Index PCR Mix. Add reagents in the order listed.

Sample Index PCR Mix	PN	1X (μ l)	2X + 10% (μ l)	4X + 10% (μ l)
○ Amp Mix <i>Retrieve from Chromium GEM-X Single Cell 3' Feature Barcode Kit</i>	2000047	50	110	220
Buffer EB	—	25	55	110
Total		75	165	330


- c. Transfer **ONLY 5 μ l** from the Transferred Supernatant Cleanup (step 2.3B) to a new tube strip.
- Note that only 5 μ l sample transfer is sufficient for generating Cell Surface Protein library.
 - The remaining sample can be stored at **4°C** for up to **72 h** or at **-20°C** for up to **4 weeks**, for generating additional libraries.
- d. Add **75 μ l** Sample Index PCR Mix to **5 μ l** Transferred Supernatant Cleanup sample.
- e. Add **20 μ l** of an individual Dual Index NT Set A to each sample and record the well ID used. Pipette mix 5x (pipette set to 90 μ l). Centrifuge briefly.

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

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~35 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, repeat 9X for a total of 10 cycles*	
6	72°C	00:01:00
7	4°C	Hold

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

4.2 Post Sample Index PCR Size Selection – SPRIselect

- a. Vortex to resuspend the SPRIselect reagent. Add **120 µl** SPRIselect Reagent (**1.2X**) to each sample. Pipette mix 15x (pipette set to 150 µl).
- b. Incubate **5 min** at **room temperature**.
- c. Place on the magnet•**High** until the solution clears. Remove the supernatant.
- d. Add **300 µl** 80% ethanol to the pellet. Wait 30 sec.
- e. Remove the ethanol.
- f. Add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.
- g. Remove the ethanol.
- h. Centrifuge briefly. Place on the magnet•**Low**. Remove remaining ethanol.
- i. Remove from the magnet. Add **35.5 µl** Buffer EB. Pipette mix 15x.
- j. Incubate **2 min** at **room temperature**.
- k. Place on the magnet•**Low** until the solution clears.
- l. Transfer **35 µl** to a new tube strip.
-  m. Store at **4°C** for up to **72 h** or at **-20°C** for **long-term** storage.

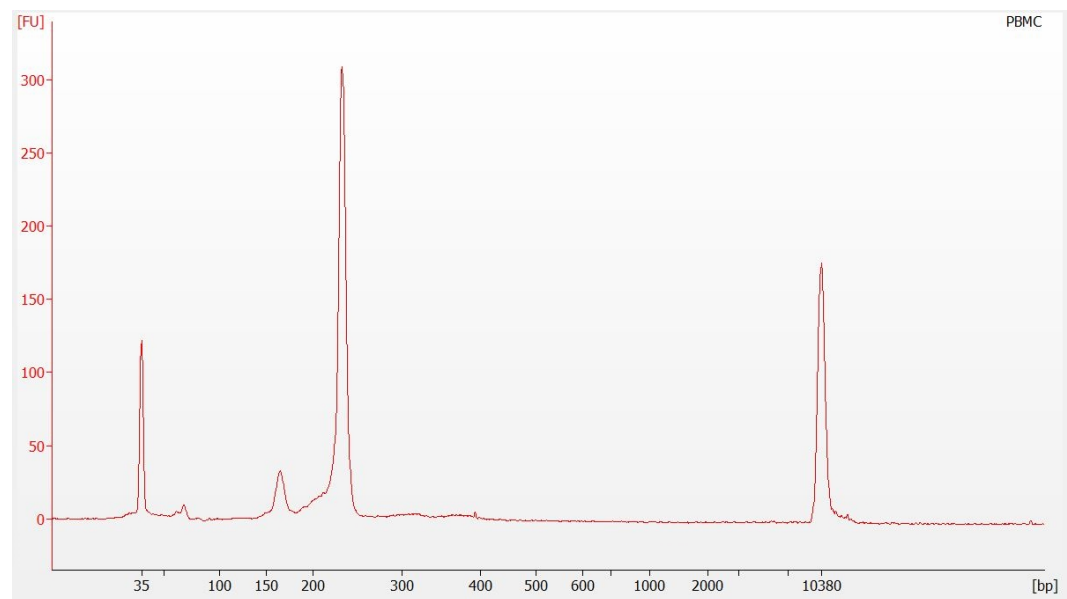
4.3 Post Library Construction QC

Library QC

Use Agilent Bioanalyzer, Perkin Elmer LabChip, Agilent Tapestation, or Fragment Analyzer for QC.

- a. Run **1 µl** sample at 1:10 dilution on an Agilent Bioanalyzer High Sensitivity chip.
- b. Select the region between 150-300 bp to determine average size of the library. This will be used as the insert size for library quantification.

Representative Trace



See [Appendix on page 89](#) for representative traces

Quantification

Library quantification should be performed prior to sequencing. For the most accurate quantitative assessment of libraries, a qPCR-based method should be used to ensure that the sequencing flowcell is loaded properly.

See [Post Library Construction Quantification using KAPA qPCR on page 91](#)

Step 5:

Sequencing

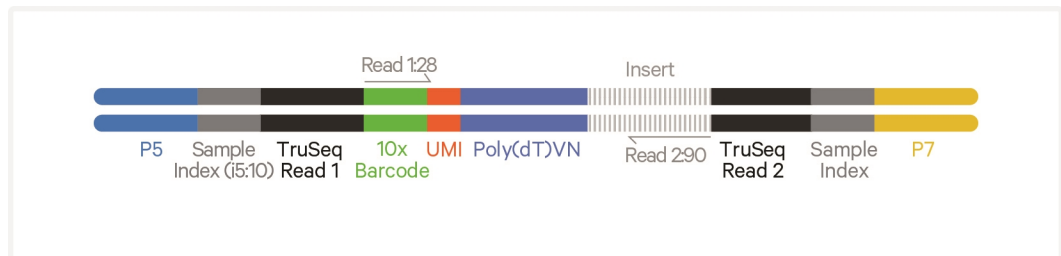
Sequencing Libraries	79
Sequencer Compatibility	79
Sample Indices	80
Library Sequencing Depth & Run Parameters	80
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Sequencing Libraries

Chromium Single Cell Gene Expression Libraries

Chromium Single Cell 3' Gene Expression Dual Index libraries comprise standard Illumina paired-end constructs which begin with P5 and end with P7. These libraries include 16 bp 10x Barcodes at the start of TruSeq Read 1 while i7 and i5 sample index sequences are incorporated as the sample index read. TruSeq Read 1 and TruSeq Read 2 are standard Illumina sequencing primer sites used in paired-end sequencing of Single Cell Gene Expression libraries.

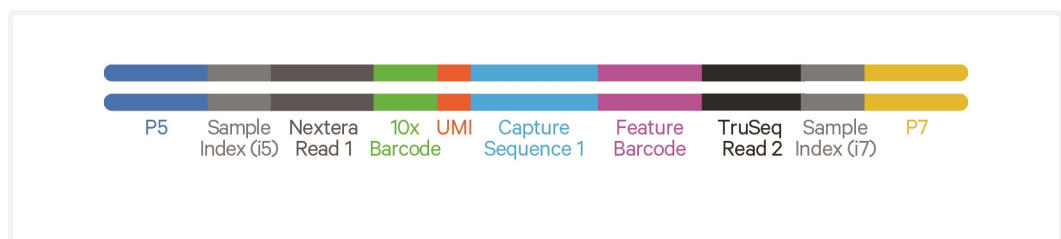
Chromium Single Cell 3' Gene Expression Library



Chromium Single Cell 3' Cell Surface Protein Libraries

Chromium Single Cell 3' Cell Surface Protein Dual Index libraries comprise standard Illumina paired-end constructs which begin with P5 and end with P7. These libraries include 16 bp 10x Barcodes at the start of Nextera Read 1 (Read 1N) while i7 and i5 sample index sequences are incorporated as the sample index read. Nextera Read 1 (Read 1N) and TruSeq Read 2 are standard Illumina sequencing primer sites used in paired-end sequencing of Cell Surface Protein libraries.

Chromium Single Cell 3' Cell Surface Protein Library



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

Sequencer Compatibility

10x Genomics libraries contain P5 and P7 adapters, 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.

Few of the compatible sequencers are listed below.

- MiSeq
- NextSeq 500/550/2000
- HiSeq 2500 (Rapid Run)
- HiSeq 3000/4000
- NovaSeq 6000
- NovaSeq X series

Some variation in assay performance is expected based on the sequencer choice. For more information on sequencing platform compatibility, refer to the 10x Genomics [Compatible Products](#) page and the 10x Genomics Support website.

Sample Indices

Each sample index in the relevant Dual Index Kit contains a mix of one unique i7 and one unique i5 sample index. If multiple samples are pooled in a sequence lane, the sample index name (i.e. the Dual Index plate well ID) is needed in the sample sheet used for generating FASTQs with "cellranger mkfastq". If multiple libraries are pooled in a sequence lane, a separate sample index is needed with each library (see [Sample Indices in Sample Index PCR on page 33](#)).

Library Sequencing Depth & Run Parameters

3' Gene Expression Library

Parameter	Description
Sequencing Depth	Minimum 20,000 read pairs per cell
Sequencing Type	Paired-end, dual indexing
Sequencing Read	Recommended Number of Cycles
Read 1	28 cycles
i7 Index	10 cycles
i5 Index	10 cycles
Read 2	90 cycles

Cell Surface Protein Library

Pooling Single Cell 3' Gene Expression & Cell Surface Protein libraries is recommended for sequencing to maintain nucleotide diversity.

Parameter	Description
Sequencing Depth	Minimum 5,000 read pairs per cell
Sequencing Type	Paired-end, dual indexing
Sequencing Read	Recommended Number of Cycles
Read 1	28 cycles
i7 Index	10 cycles
i5 Index	10 cycles
Read 2	90 cycles (Minimum required Read 2 length for Cell Surface Protein Libraries is 25 bp)

Library Loading

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

3' Gene Expression libraries alone or in combination with Cell Surface Protein libraries



Library Loading

Instrument	Loading Concentration (pM)*	PhiX (%)
MiSeq	12	1
NextSeq 500/550	1.6	1
NextSeq 1000/2000	650	1
HiSeq 2500 (RR)	12	1
HiSeq 4000	240	1
NovaSeq 6000 Standard & Xp workflow	150	1
NovaSeq X series	150-200	1

**Minor updates to the loading concentrations*

Library Pooling

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

Library Pooling Example

Libraries	Sequencing Depth (read pairs per cell)	Library Pooling Ratio
3' Gene Expression library	20,000	4
Cell Surface Protein library	5,000	1

Data Analysis and Visualization

Sequencing data may be analyzed using Cell Ranger 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 Gene Expression data to align reads, generate Feature Barcode matrices and perform clustering and gene expression analysis.

- Input: Binary base call (BCL) and FASTQ
- Output: BAM, MEX, CSV, HDF5, Web Summary, .cloupe/.loupe
- Operating System: Linux

Cloud Analysis

Cloud Analysis is currently only available for US & Canada customers.

Cloud Analysis allows users to run Cell Ranger analysis pipelines from a web browser while computation is handled in the cloud.

- Key features: scalable, highly secure, simple to set up and run
- Input: FASTQ
- Output: BAM, MEX, CSV, HDF5, Web Summary, .cloupe/.loupe

Loupe Browser

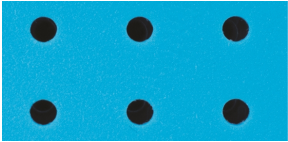
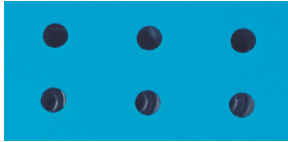
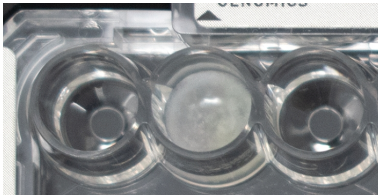
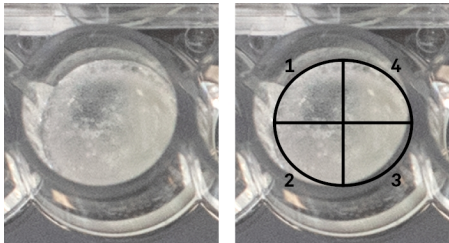
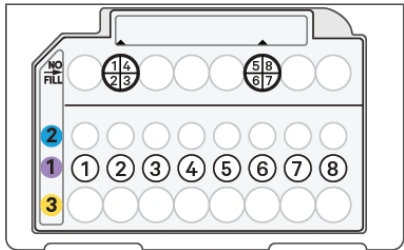
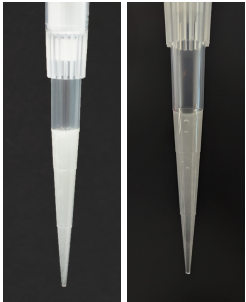
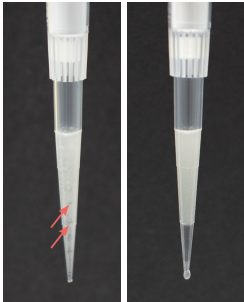
Loupe Browser is an interactive data visualization application that requires no prior programming knowledge.

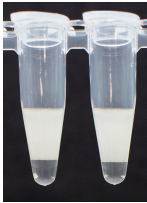
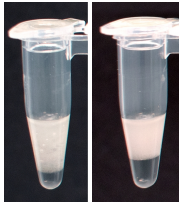

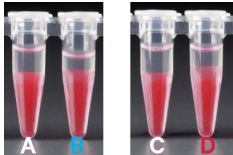

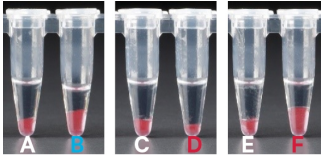

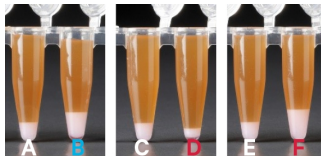
- Input: .cloupe, .vloupe
- Output: Data visualization, including t-SNE and UMAP projections, custom clusters, differentially expressed genes
- Operating System: MacOS, Windows

Troubleshooting

GEM Generation & Barcoding	85
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GEM Generation & Barcoding

STEP	Normal	Impacted
1.2 Load Chromium GEM-X Chip	 <p>Gasket holes are aligned with the sample and gel bead wells.</p>	 <p>Gasket holes are misaligned with the gel bead wells. Open and close the chip holder slowly once. <i>One of the gasket rows (second from top) will not align with the GEM-X chip. This is normal & will not impact the assay.</i></p>
1.4d After Chip is removed from the instrument and the wells are exposed	 <p>Recovery well shows consistent volume and opacity.</p>	  <p>Inspecting emulsions in chip wells & pipette tips is recommended for diagnosing failures. Occurrence of small bubbles in the emulsion is normal & does not indicate failure.</p> <p><i>The quadrant numbers correlate with the sample well numbers.</i></p> <p>Recovery well with wetting failure in one of the samples (top left - quadrant 1). This should not impact the other three samples and it is recommended to move forward with the next step.</p>
1.4e Transfer GEMs from Chip	 <p>Consistent volume, opacity, no air trapped. Emulsion density (oil settling) differences seen only if recovery is delayed; likely no impact on assay.</p>	 <p>First pipette tip shows wetting failure. Second pipette tip shows a clog.</p>

STEP	Normal	Impacted
2.1a After transfer of the GEMs to tubes	 <p>GEMs transferred immediately to the tubes. Over time some settling is normal.</p>	 <p>GEMs transferred immediately to the tubes. First tube shows a wetting failure. Second tube shows a clog. If excess oil is observed immediately after emulsion recovery, it would likely indicate a clog.</p>
Images shown below for step 2.1b-d are for illustrative purposes. The differences in volume may be more subtle. Therefore, emulsion QC is recommended in the chips/tips/tube. Diagnosing emulsion failures/clogs during subsequent steps may be challenging.		
2.1b After transfer of the GEMs + Recovery Agent	 <p>All liquid levels are similar in the aqueous layer (clear) and Recovery Agent/ Partitioning Oil B (pink).</p>	 <p>Tube B indicates a reagent clog. There is a decrease in aqueous layer (clear). Tube D indicates a wetting failure. There is abnormal volume of Recovery Agent/ Partitioning Oil B (pink).</p>
2.1c After aspiration of Recovery Agent/ Partitioning Oil	 <p>All liquid volumes are similar in the aqueous layer (clear) and residual Recovery Agent/ Partitioning Oil B (pink).</p>	 <p>Tube B indicates a reagent clog. There is a decrease in aqueous layer (clear). There is also a greater residual volume of Recovery Agent/ Partitioning Oil B (pink). Tubes D and F indicate a wetting failure. There is an abnormal residual volume of Recovery Agent/ Partitioning Oil B (pink).</p>
2.1d After addition of Dynabeads Cleanup Mix	 <p>All liquid volumes are similar after addition of the Dynabeads Cleanup Mix.</p>	 <p>Tube B indicates a reagent clog. There is an abnormal ratio of Dynabeads Cleanup Mix (brown) to Recovery Agent/ Partitioning Oil B (appears white). Tubes D and F indicate a wetting failure. An abnormal ratio of Dynabeads Cleanup Mix (brown) to Recovery Agent/ Partitioning Oil B (appears white) is observed.</p>



After GEM generation, it is strongly recommended to always take a picture of:

- Emulsion(s) in the chip (horizontal and at 45 degrees)
- Emulsion(s) in the pipette tip/tube
- The chip after emulsions have been recovered

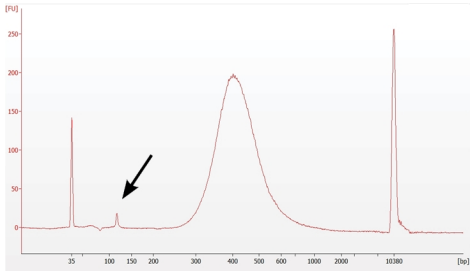
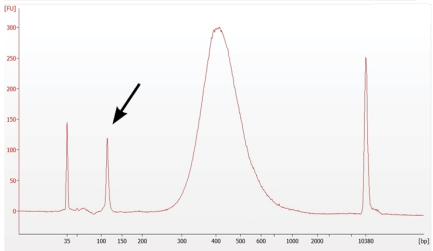
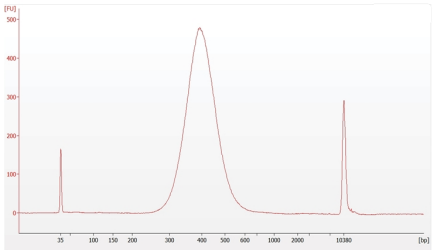
If a channel clog or wetting failure is documented in one or more samples in a set:

- Data can be derived from the remaining samples in the set by immediately proceeding with GEM-RT

OR

- Data from all the samples in a set can be derived by immediately restarting GEM generation. If restarting GEM generation for one set of samples, ensure that a thermal cycler is available for the second set as GEM-RT incubation should start within 5 min of GEM recovery.

Contact 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.

Step	Normal	Prominent Adaptor Dimer Peaks
3.7 Post Library Construction QC (Gene Expression)	 <p>Minor peaks below 200 bp may be observed.</p>	<p>Libraries before repeating step 3.6</p>  <p>Libraries after repeating step 3.6</p>  <p>If peaks below 200 bp are more prominent, repeat step 3.6 Post Sample Index PCR Double Sided Size Selection - SPRIselect.</p>

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 Instrument User Guide with Readiness Test (CG000396) for additional information and follow the instrument touchscreen prompts for execution. The instrument touchscreen will guide the user through recoverable errors.

Appendix

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Post Library Construction Quantification using KAPA qPCR	91
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GEM-X OCM Chip Loading Overview

This section provides a quick overview to the GEM-X OCM chip loading and does not include detailed instructions. Refer to [Load GEM-X OCM Chip on page 42](#) 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. If loading only one set (each set = 4 samples), add 50% glycerol solution to each unused well in row 1, 2, and 3 of the second set. If running fewer than 4 samples in a set, DO NOT load glycerol to the unused wells in that set.

- Load 15 μ l to row labeled 1
- Load 18 μ l to row labeled 2
- Load 70 μ l to row labeled 3

- b. Prepare Gel Beads

- Vortex for 30 sec
- Centrifuge for 5 sec

- c. Prepare GEM Master Mix + Sample

- d. Load Row Labeled 1

- Mix GEM Master Mix + Sample
- Load 15 μ l to row labeled 1
- If running less than 4 samples in a multiplexing set, load 15 μ l mock sample
- Wait 30 sec

- e. Load Row Labeled 2

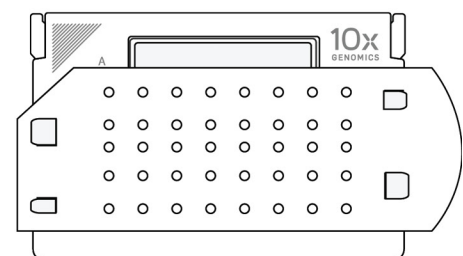
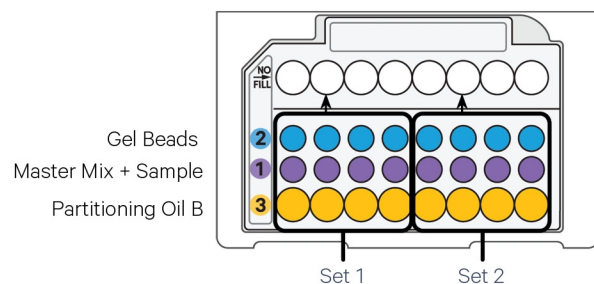
- Aspirate Gel Beads (blue in first tip)
- Load 18 μ l to row labeled 2
- Wait 30 sec

- f. Load Row Labeled 3

- Load 70 μ l Partitioning Oil B to row labeled 3.

- g. Close the lid and prepare for run.

Representative Images



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

Post Library Construction Quantification using KAPA qPCR

- 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)	

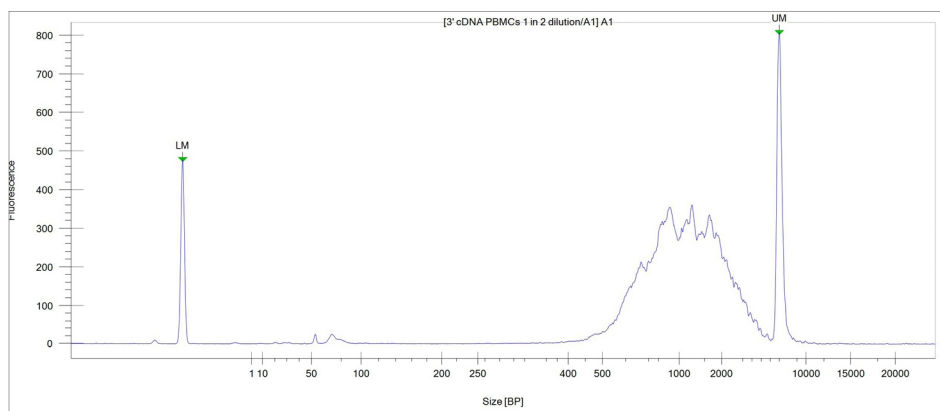
- g. Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on insert size derived from the Bioanalyzer/TapeStation trace.

Library Traces

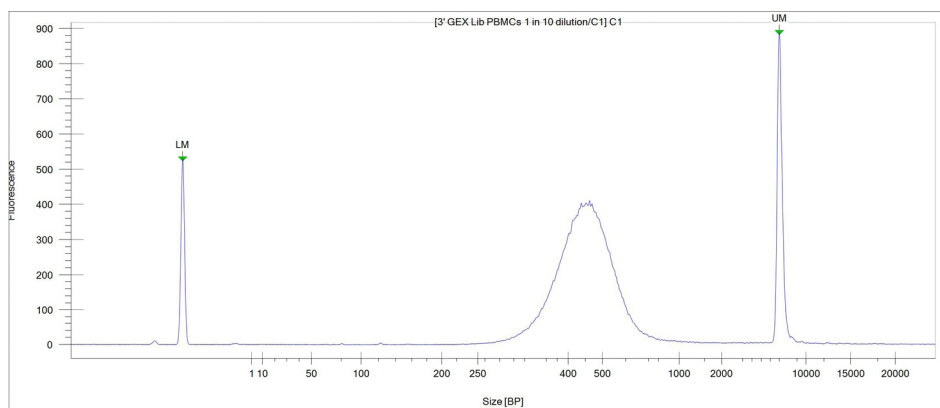
LabChip Traces

DNA High Sensitivity Reagent Kit was used.

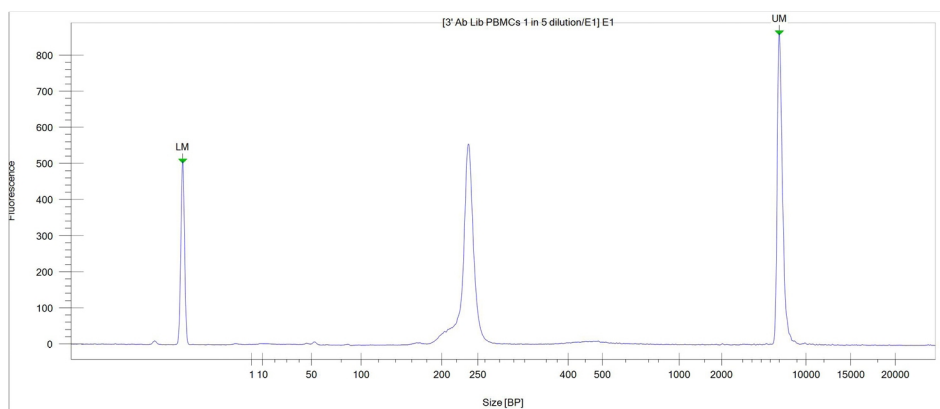
Post cDNA Amplification QC & Quantification



GEX- Post Library Construction QC

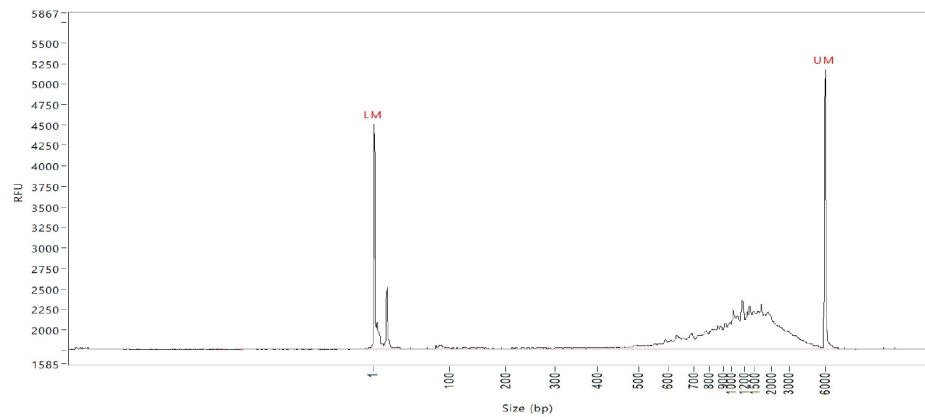


Cell Surface Protein - Post Library Construction QC

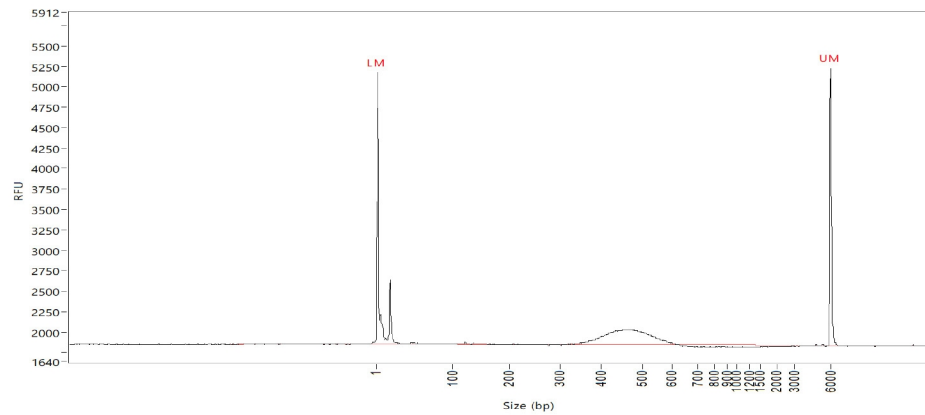


Fragment Analyzer Traces

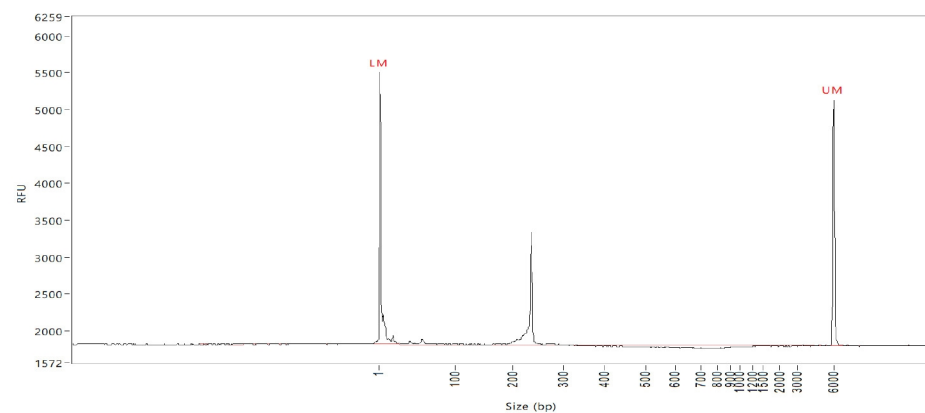
Post cDNA Amplification QC & Quantification



GEX - Post Library Construction QC

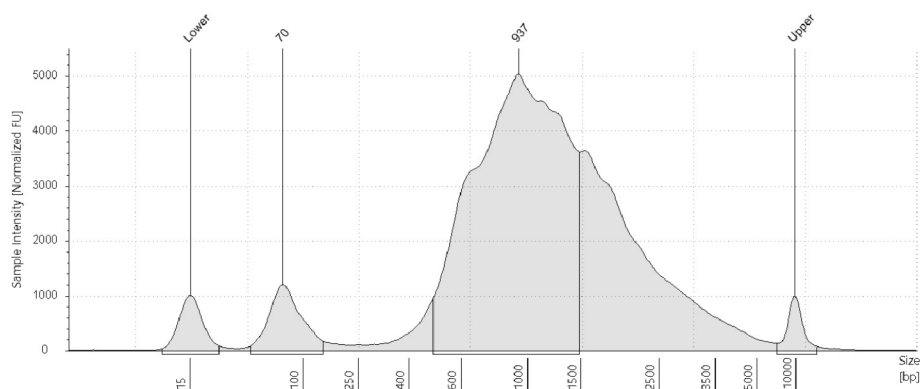


Cell Surface Protein - Post Library Construction QC

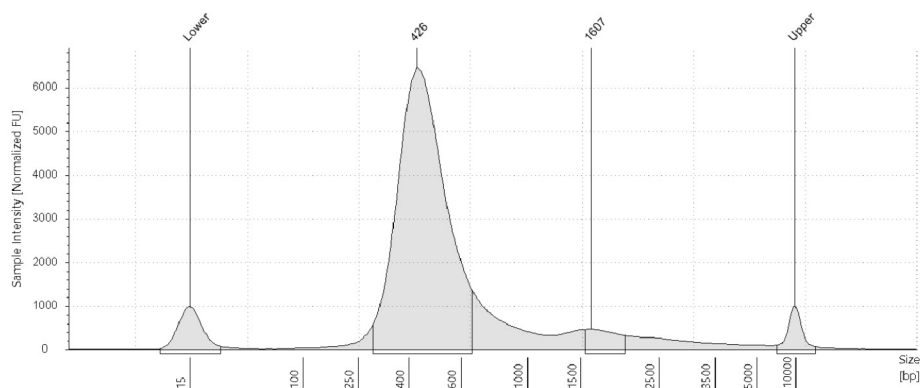


TapeStation Traces

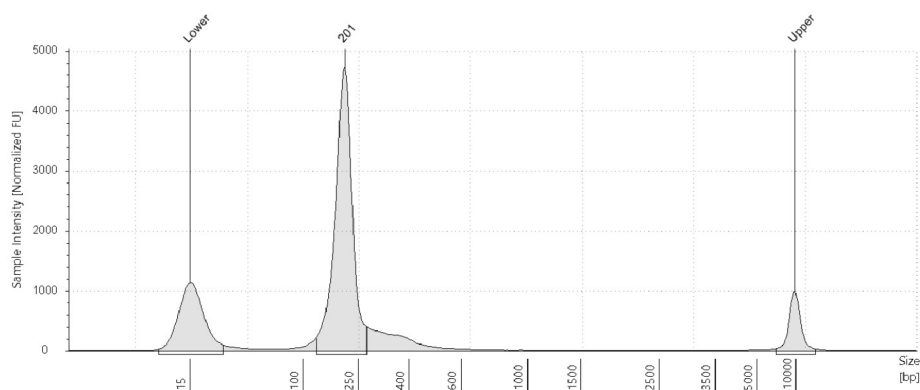
Post cDNA Amplification QC & Quantification



GEX - Post Library Construction QC

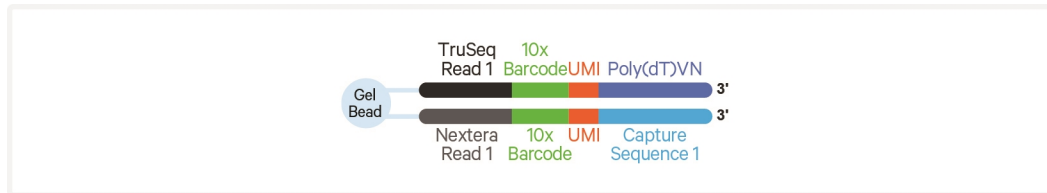


Cell Surface Protein - Post Library Construction QC



Oligonucleotide Sequences

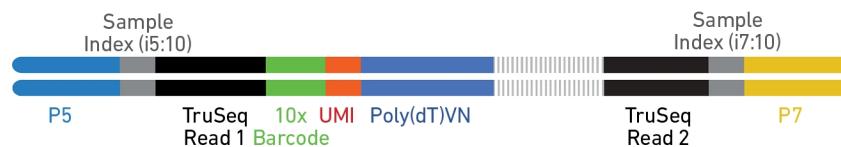
Gel Bead Primers



5'-CTACACGACGCTCTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-VN-3'
 5'-GTCAGATGTGTATAAGAGACAG-N16-N12-TTGCTAGGACCGGCCTTAAAGC-3'

Gene Expression Library

Sample Index PCR Product



5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N12-T30-VN-cDNA_Insert-
 AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-N10-ATCTCGTATGCGCTCTTCTGCTTG-3'

3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12-A30-BN-cDNA_Insert-
 TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAACGACGAAC-5'

Cell Surface Protein Library

Sample Index PCR Product



5'-AATGATACGGCGACCACCGAGATCTACAC-N10-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-N16-N12-TTGCTAGGACCGGCCTTAAAGC-N9-
 N15-N10-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-N10-ATCTCGTATGCGCTCTTCTGCTTG-3'

3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-AGCAGCCGTCGCAGTCTACACATATTCTCTGTC-N16-N12-AACGATCCTGGCCGAATTTCG-N9-
 N15-N10-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAACGACGAAC-5'