User Guide | CG000772 | Rev A

GEM-X Universal 5' Gene Expression v3 4-plex

On-chip Multiplexing (OCM) with Feature Barcode technology for CRISPR Screening

For use with:

GEM-X Universal 5’ Gene Expression v3 4-plex

16 samples PN-1000780

GEM-X OCM 5' Chip Kit v3 4-plex

2 chips PN-1000748

Chromium GEM-X Single Cell 5' Feature Barcode Kit v3

16 rxns PN-1000703

Chromium Single Cell V(D)J Amplification Kits

Human 16 rxns TCR PN-1000252 / BCR PN-1000253

Mouse 16 rxns TCR PN-1000254 / BCR PN-1000255

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

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

Notices

Document Number

CG000772 | Rev A

Legal Notices

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Document Revision Summary

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Title

GEM-X Universal 5' Gene Expression v3 4-plex On-chip Multiplexing (OCM) with Feature Barcode technology for CRISPR Screening

Revision

N/A to Rev A

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November 14, 2024

Table of Contents

Introduction

GEM-X Universal 5' Gene Expression v3 4-plex Reagent Kits 8
10x Genomics Accessories 12
Third-Party Items 13
Protocol Steps & Timing 14
Stepwise Objectives 16
CRISPR Screening Overview 25

Tips & Best Practices

Step 1: GEM Generation and Barcoding

1.0 Get Started 39
1.1 Prepare Master Mix 40
Assemble GEM-X OCM Chip 40
Sample Multiplexing Guidelines 43
1.2 Load GEM-X OCM Chip 45
1.3 Run the Chromium X Series Instrument 48
1.4 Transfer GEMs 49
1.5 GEM-RT Incubation 51

Step 2: Post GEM–RT Cleanup & cDNA Amplification

2.0 Get Started 53
2.1 Post GEM-RT Cleanup – Dynabeads 55
Step Overview (steps 2.2 & 2.3) 58
2.2 cDNA Amplification 59
2.3 cDNA Cleanup –SPRIselect 61
2.3A Pellet Cleanup (for V(D)J & 5' Gene Expression) 62
2.3B Transferred Supernatant Cleanup (for CRISPR Screening) 63
2.4 Post cDNA Amplification QC & Quantification 64

Step 3: V(D)J Amplification from cDNA

3.0 Get Started 67
3.1 V(D)J Amplification 1 68
3.2 Post V(D)J Amplification 1 Cleanup Double Sided – SPRIselect 69
3.3 V(D)J Amplification 2 70

Table of Contents

3.4 Post V(D)J Amplification 2 Cleanup Double Sided – SPRIselect 71
3.5 Post V(D)J Amplification QC & Quantification 72

Step 4: V(D)J Library Construction
4.0 Get Started 76
4.1 Fragmentation, End Repair & A-tailing 78
4.2 Adaptor Ligation 79
4.3 Post Ligation Cleanup – SPRIselect 79
4.4 Sample Index PCR 81
4.5 Post Sample Index PCR Cleanup – SPRIselect 82
4.6 Post Library Construction QC 83

Step 5: 5' Gene Expression Library Construction
5.0 Get Started 85
Step Overview (Step 5.1d) 86
5.1 GEX Fragmentation, End Repair & A-tailing 87
5.2 GEX Post Fragmentation, End Repair & A-tailing Double Sided – SPRIselect 88
5.3 GEX Adaptor Ligation 89
5.4 GEX Post Ligation Cleanup – SPRIselect 89
5.5 GEX Sample Index PCR 91
5.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect 92
5.7 Post Library Construction QC 93

Step 6: CRISPR Screening Library Construction
6.0 Get Started 95
6.1 Guide RNA cDNA Cleanup – SPRIselect 96
6.2 Feature PCR 96
6.3 Post Feature PCR Cleanup – SPRIselect 97
6.4 Sample Index PCR 98
6.5 Post Sample Index PCR Double Sided Size Selection – SPRIselect 99
6.6 Post Library Construction QC & Quantification 100

Table of Contents

Step 7: Sequencing

- Sequencing Libraries 102
- Sequencer Compatibility 103
- Sample Indices 103
- Library Sequencing Depth & Run Parameters 103
- Library Loading 104
- Library Pooling 104
- Data Analysis and Visualization 105

Troubleshooting

- GEM Generation & Barcoding 107
- Chromium X Series Errors 110

Appendix

- GEM-X OCM Chip Loading Overview 112
- Post Library Construction Quantification using KAPA qPCR 113
- Library Traces 114
- Oligonucleotide Sequences 117

Introduction

GEM-X Universal 5' Gene Expression v3 4-plex Reagent Kits 8

10x Genomics Accessories 12

Third-Party Items 13

Protocol Steps & Timing 14

Stepwise Objectives 16

CRISPR Screening Overview 25

GEM-X Universal 5' Gene Expression v3 4-plex Reagent Kits

Refer to SDS for handling and disposal information

GEM-X Universal 5' Gene Expression Kit v3 4-plex 16 samples PN-1000780

Chromium GEM-X
Single Cell 5' GEM Kit v3
4 rxns, PN-1000697
Shipped on dry ice
Store at -20°C

	#	PN
RT Reagent E	1	2001106
RT Enzyme E	1	2001146
Poly-dT RT Primer B	1	2001110
Reducing Agent B	1	2000087
Cleanup Buffer	1	2000088
cDNA Primers	1	2000089
Amp Mix	1	2000103

10x GENOMICS

Library Construction Kit C
4 rxns, PN-1000689
Shipped on dry ice
Store at -20°C

	#	PN
Fragmentation Enzyme	1	2000090
Fragmentation Buffer	1	2000091
Ligation Mix	1	2001109
DNA Ligase	1	220110

10x GENOMICS

GEM-X OCM
5' Gel Bead Kit v3 4-plex
16 samples, PN-1000750
Shipped on dry ice
Store at -80°C

	#	PN
GEM-X OCM 5' Gel Bead v3 (16 samples)	2	2001127

10x GENOMICS

Dynabeads™ MyOne™ SILANE
PN-2000048
Shipped at ambient temperature
Store at 4°C

	#	PN
Dynabeads MyOne SILANE	1	2000048

If constructing Gene Expression libraries with V(D)J (T-cell receptor or B-cell receptor) libraries, additional Library Construction Kit C will be required. Refer to the 10x Genomics Support website for more details

GEM-X OCM 5' Chip Kit v3 4-plex, 2 chips PN-1000748

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
5' OCM Chip & Gaskets
Shipped at ambient temperature
Store at ambient temperature

	#	PN
GEM-X 5' OCM Chip	2	2001100
Chip Gasket X/iX, 2-pack	2	3000656

10x GENOMICS

Chromium GEM-X Single Cell 5' Feature Barcode Kit v3, 16 rxns PN-1000703

Chromium GEM-X
Single Cell 5' Feature Barcode Kit v3
16 rxns, PN-1000703
Shipped on dry ice
Store at -20°C

	#	PN
CRISPR Poly-dT Primer Mix B	1	2001145
Feature cDNA Primers 4	1	2000277
Feature SI Primers 4	1	2000592
Amp Mix	2	2000047

10x GENOMICS

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

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

	#	PN
Dual Index Plate TT Set A	1	3000431

Chromium Single Cell V(D)J Amplification Kits, Human

Chromium
Single Cell Human TCR Amplification
16 rxns, PN-1000252
Shipped on dry ice
Store at -20°C

		#	PN
●	Human T Cell Mix 1 v2	1	2000242
●	Human T Cell Mix 2 v2	1	2000246
○	Amp Mix	2	2000047

10x GENOMICS

Chromium
Single Cell Human BCR Amplification
16 rxns, PN-1000253
Shipped on dry ice
Store at -20°C

		#	PN
●	Human B Cell Mix 1 v2	1	2000254
●	Human B Cell Mix 2 v2	1	2000255
○	Amp Mix	2	2000047

10x GENOMICS

Chromium Single Cell V(D)J Amplification Kits, Mouse

Chromium
Single Cell Mouse TCR Amplification
16 rxns, PN-1000254
Shipped on dry ice
Store at -20°C

		#	PN
●	Mouse T Cell Mix 1 v2	1	2000256
●	Mouse T Cell Mix 2 v2	1	2000257
○	Amp Mix	2	2000047

10x GENOMICS

Chromium
Single Cell Mouse BCR Amplification
16 rxns, PN-1000255
Shippedon dry ice
Store at -20°C

		#	PN
●	Mouse B Cell Mix 1 v2	1	2000258
●	Mouse B Cell Mix 2 v2	1	2000259
○	Amp Mix	2	2000047

10x GENOMICS

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 Immune Profiling workflow requires third-party reagents, kits, and equipment in addition to those provided by 10x Genomics. All third-party reagents and consumables should be obtained prior to starting this library construction workflow.

Refer to the GEM-X 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.

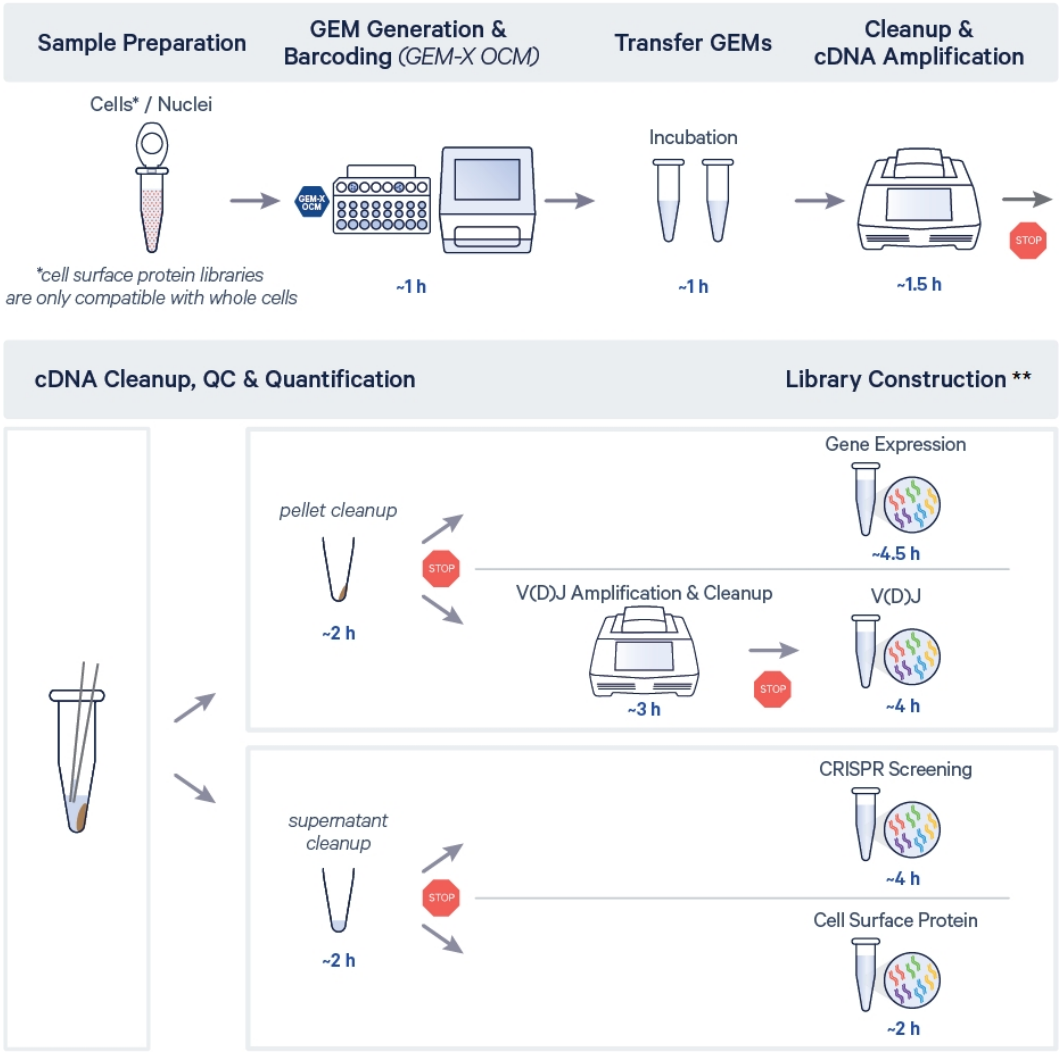
Protocol Steps & Timing

Steps	Timing	Stop & Store
Sample Preparation	variable*	
	*Refer to the appropriate Demonstrated Protocols for details.	
Step 1: GEM Generation and Barcoding (page 38)		
1.1 Prepare Master Mix (page 40)	20 min	
1.2 Load GEM-X OCM Chip (page 45)	10 min	
1.3 Run the Chromium X Series Instrument (page 48)	4 min	
1.4 Transfer GEMs (page 49)	3 min	
1.5 GEM-RT Incubation (page 51)	55 min	STOP 4°C ≤72 h/-20°C ≤1 week
Step 2: Post GEM–RT Cleanup & cDNA Amplification (page 52)		
2.1 Post GEM-RT Cleanup – Dynabeads (page 55)	45 min	
2.2 cDNA Amplification (page 59)	40 min	STOP 4°C ≤72 h/-20°C ≤1 week
2.3 cDNA Cleanup –SPRIselect (page 61) 2.3A Pellet Cleanup 2.3B Supernatant Cleanup	 30 min 20 min	STOP 4°C ≤72 h/-20°C ≤4 weeks
2.4 Post cDNA Amplification QC & Quantification (page 64)	50 min	
Step 3: V(D)J Amplification from cDNA (page 66)		
3.1 V(D)J Amplification 1 (page 68)	50 min	
3.2 Post V(D)J Amplification 1 Cleanup Double Sided – SPRIselect (page 69)	30 min	
3.3 V(D)J Amplification 2 (page 70)	25 min	
3.4 Post V(D)J Amplification 2 Cleanup Double Sided – SPRIselect (page 71)	30 min	
3.5 Post V(D)J Amplification QC & Quantification (page 72)	40 min	STOP 4°C ≤72 h
Step 4: V(D)J Library Construction (page 75)		
4.1 Fragmentation, End Repair & A-tailing (page 78)	50 min	
4.2 Adaptor Ligation (page 79)	30 min	
4.3 Post Ligation Cleanup – SPRIselect (page 79)	25 min	
4.4 Sample Index PCR (page 81)	40 min	STOP 4°C ≤72 h
4.5 Post Sample Index PCR Cleanup – SPRIselect (page 82)	30 min	STOP 4°C ≤72 h/-20°C long term

Steps	Timing	Stop & Store
4.6 Post Library Construction QC (page 83)	50 min	
Step 5: 5' Gene Expression Library Construction (page 84)		
5.1 GEX Fragmentation, End Repair & A-tailing (page 87)	50 min	
5.2 GEX Post Fragmentation, End Repair & A-tailing Double Sided – SPRIselect (page 88)	30 min	
5.3 GEX Adaptor Ligation (page 89)	25 min	
5.4 GEX Post Ligation Cleanup – SPRIselect (page 89)	30 min	
5.5 GEX Sample Index PCR (page 91)	40 min	STOP 4°C ≤72 h
5.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect (page 92)	30 min	STOP 4°C ≤72 h/-20°C long term
5.7 Post Library Construction QC (page 93)	50 min	
Step 6: CRISPR Screening Library Construction (page 94)		
6.1 Guide RNA cDNA Cleanup – SPRIselect (page 96)	40 min	
6.2 Feature PCR (page 96)	40 min	
6.3 Post Feature PCR Cleanup – SPRIselect (page 97)	40 min	
6.4 Sample Index PCR (page 98)	40 min	
6.5 Post Sample Index PCR Double Sided Size Selection – SPRIselect (page 99)	20 min	STOP 4°C ≤72 h/-20°C long term
6.6 Post Library Construction QC & Quantification (page 100)	50 min	

Stepwise Objectives

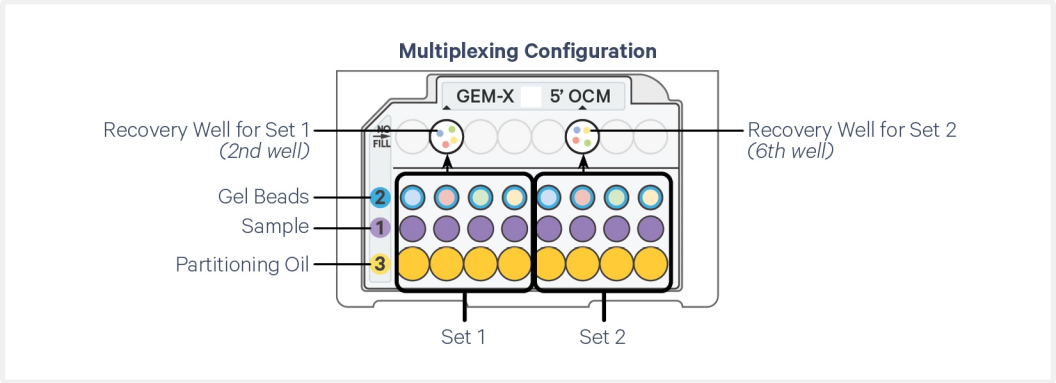
Workflow Overview



***See the following pages for information on the specific library types that can be generated by referring to this user guide.*

The GEM-X Universal 5' Gene Expression v3 4-plex assay with Feature Barcode technology 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 CRISPR-mediated perturbations, along with immune repertoire and gene expression from the same cell by profiling 500-5,000 individual cells per sample. For CRISPR screening, a PCR-based approach is used to capture the single-guide RNA (sgRNA), which is reverse transcribed. To profile the immune repertoire of cells, full-length (5' UTR to constant region), paired T-cell receptor (TCR) and/or B-cell receptor (BCR) transcripts from 500-5,000 individual cells per sample are assessed.

A pool of ~900,000 barcodes are sampled separately to index each cell's transcriptome 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/DNA (from poly-adenylated mRNA /single-guide RNAs/sgRNAs), where DNA derived from a cell share a common barcode. From each pool, the indicated dual indexed library types are generated and sequenced, and 10x Barcodes are used to associate the individual reads back to the individual partitions and samples.



This document outlines the protocols to generate the following libraries:

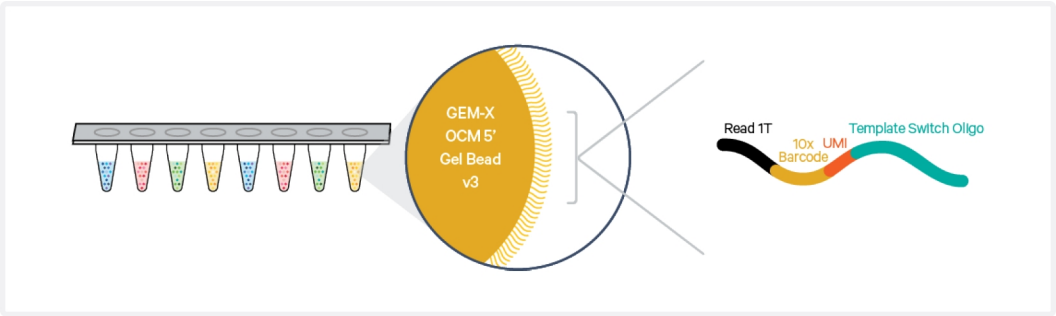
- Single Cell V(D)J libraries from V(D)J-amplified cDNA derived from poly-adenylated mRNA
- Single Cell 5' Gene Expression libraries from amplified cDNA derived from poly-adenylated mRNA
- Single Cell 5' CRISPR Screening libraries from amplified DNA derived from sgRNA molecules

Generating and sequencing a 5' Gene Expression library along with the V(D)J library is highly recommended for efficient data analysis. V(D)J only analysis is enabled but not officially supported in the software. See Sequencing on page 101 for details.

GEM-X OCM 5' Gel Beads v3 4-plex

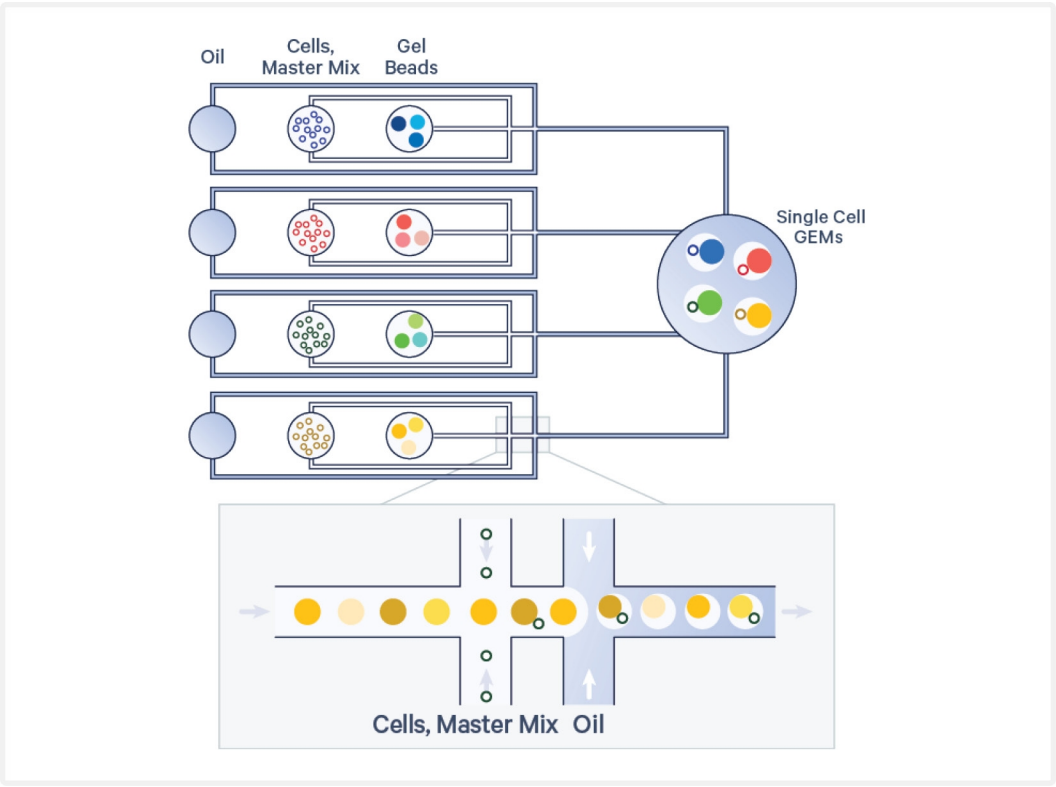
For each multiplexing set, the 4 color coded gel beads, each with a unique list of ~900,000 barcodes, are loaded in consecutive wells of the chip and are associated with a specific sample.

Gel Bead Primers



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 in to 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 remainders largely contain a single cell.

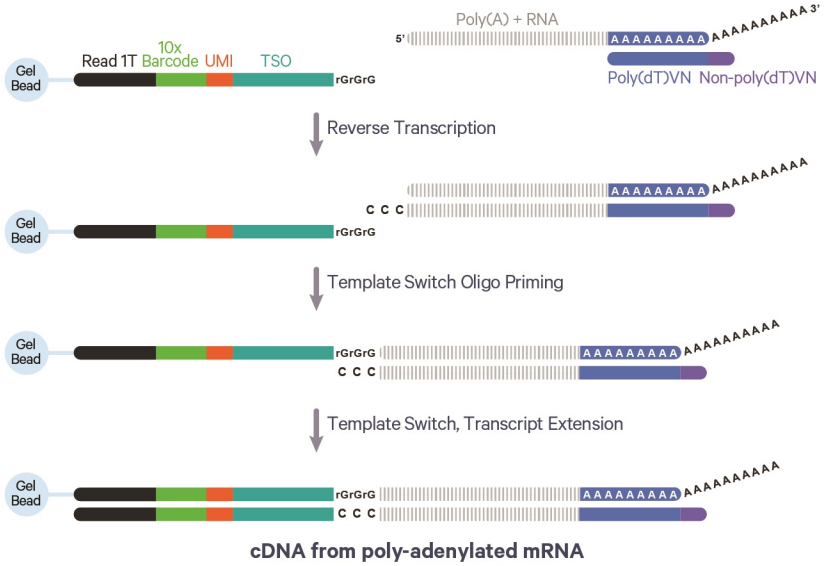


Immediately following GEM generation, GEMs (containing ~3,600,000 unique barcodes from the 4 pooled samples) are transferred from the two recovery wells to two separate consecutive tubes of a tube strip. The Gel Bead is dissolved and any co-partitioned cell is lysed. Gel Bead primers containing (i) an Illumina TruSeq Read 1 sequence (read 1 sequencing primer), (ii) a 16 nt 10x Barcode, (iii) a 12 nt unique molecular identifier (UMI), and (iv) a 13 nt template switch oligo (TSO) are released and mixed with both the cell lysate and a Master Mix containing reverse transcription (RT) reagents and primer mix (poly(dT) + CRISPR primers).

Incubation of the GEMs simultaneously produces 10x Barcoded, full-length cDNA from poly-adenylated mRNA (A) and barcoded DNA from the sgRNA protospacer (Feature Barcode) cDNA, designed to target gene/s of interest (B).

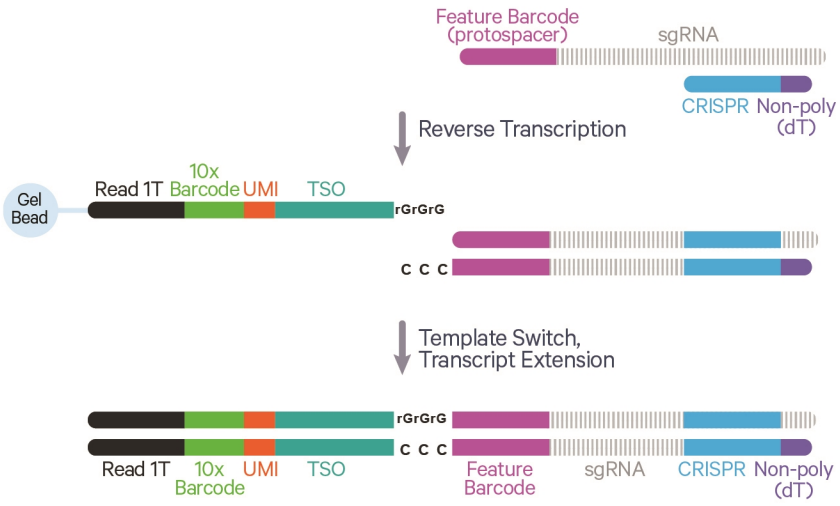
Inside Individual GEMs

A. cDNA from Poly-adenylated mRNA



cDNA from poly-adenylated mRNA

B. cDNA from the sgRNA protospacer (Feature Barcode)

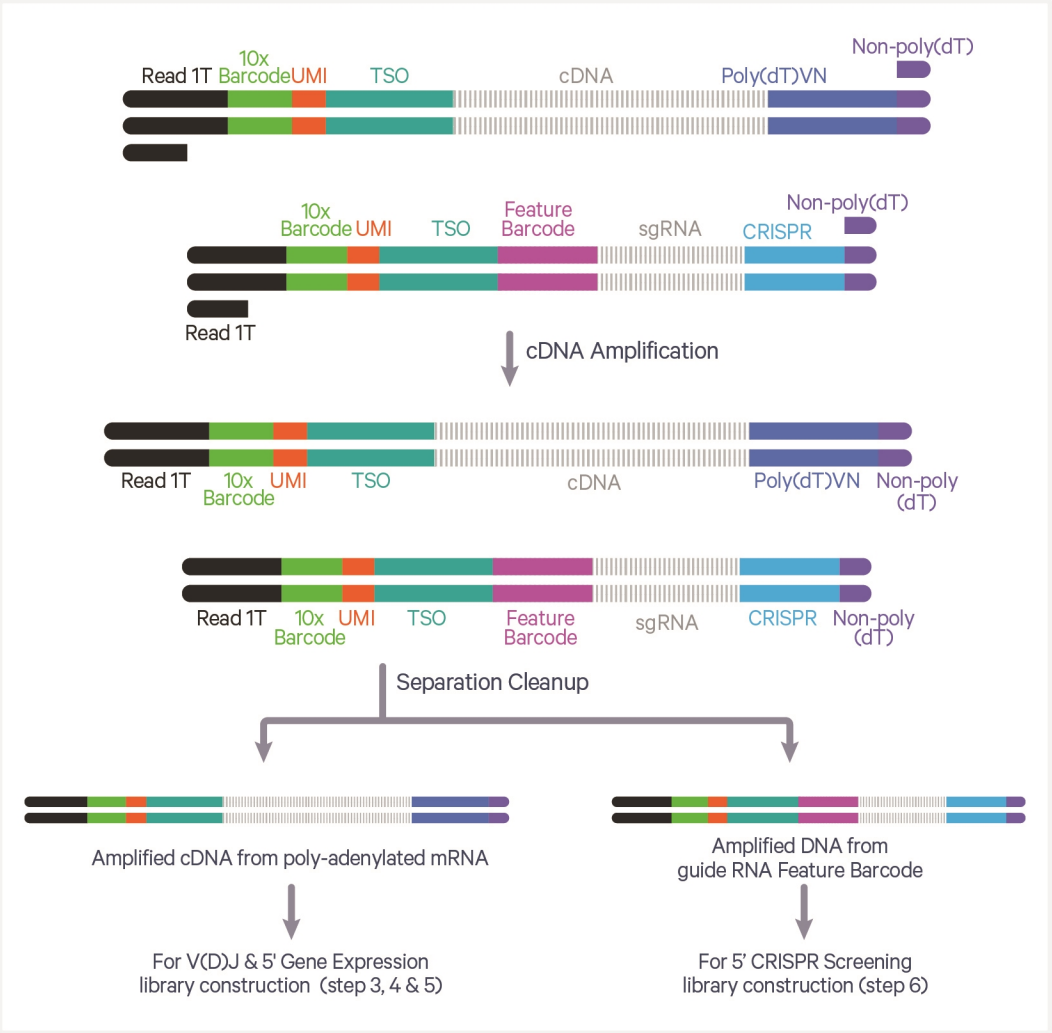


cDNA from the sgRNA protospacer (Feature Barcode)

Step 2: Post GEM-RT Cleanup & cDNA Amplification

GEMs are broken and barcoded cDNA/DNA is pooled after the GEM-RT reactions are complete. Silane magnetic beads are used to purify the 10x Barcoded first-strand cDNA from the post GEM-RT reaction mixture, which includes leftover biochemical reagents and primers. 10x Barcoded, full-length

cDNA is then amplified via PCR with primers against common 5' and 3' ends added during GEM-RT. Amplification generates sufficient material to construct multiple libraries from the same cells, e.g. both T cell and/or B cell libraries (steps 3 and 4), 5' Gene Expression libraries (step 5), and 5' CRISPR Screening libraries (step 6). The amplified cDNA from poly-adenylated mRNA and the amplified DNA from sgRNA Feature Barcode (protospacer) are separated by size selection for generating V(D)J and/or 5′ Gene Expression libraries, and CRISPR Screening libraries, respectively.



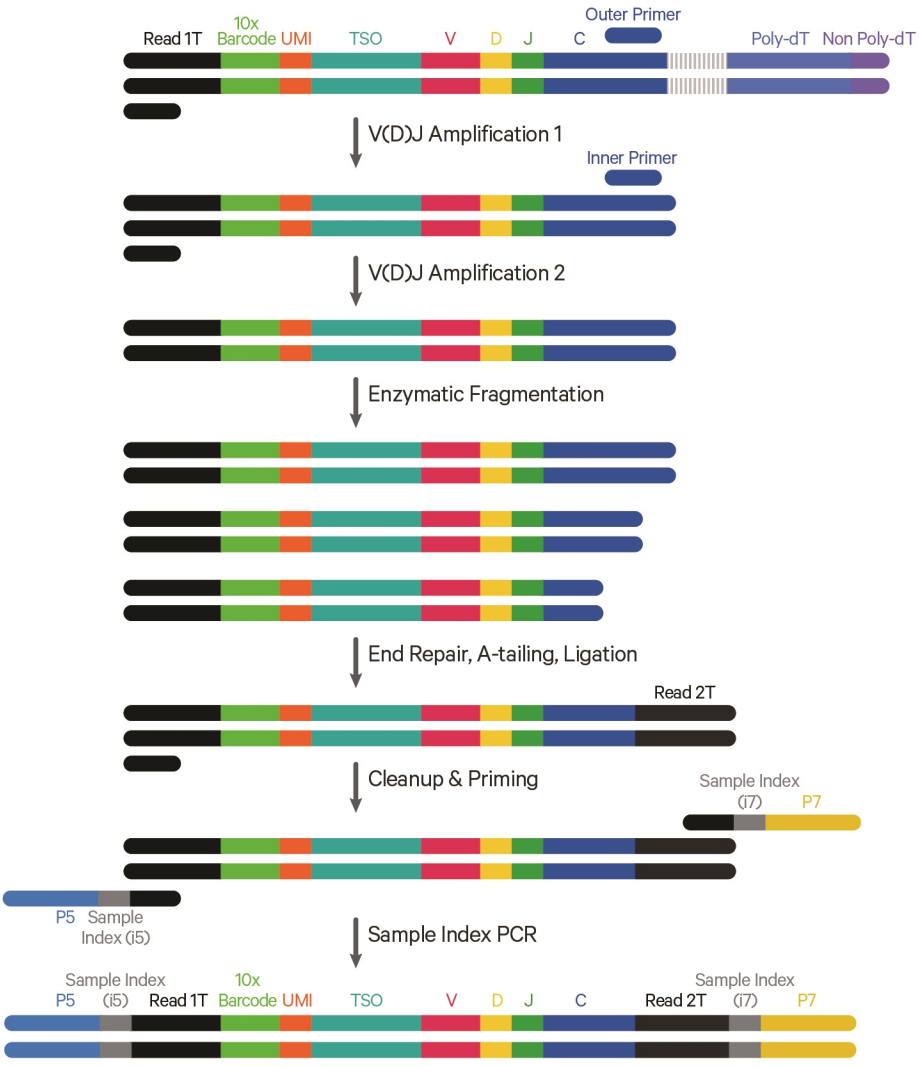
Step 3: V(D)J Amplification from cDNA

Amplified full-length cDNA from poly-adenylated mRNA is used to enrich full-length V(D)J segments (10x Barcoded) via PCR amplification with primers specific to either the TCR or BCR constant regions. If both T and B cells are expected to be present in the partitioned cell population, TCR and BCR transcripts can be amplified in separate reactions from the same amplified cDNA material.

Step 4: V(D)J Library Construction

Enzymatic fragmentation and size selection are used to generate variable length fragments that collectively span the V(D)J segments of the amplified TCR and/or BCR transcripts prior to library construction. P5, P7, i5 and i7 sample indexes, and an Illumina R2 sequence (read 2 primer sequence) are added via End Repair, A-tailing, Adaptor Ligation, and Sample Index PCR. The final libraries contain the P5 and P7 primers used in Illumina amplification.

Pooled Amplified cDNA Processed in Bulk



Step 5: 5' Gene Expression (GEX) Library Construction

Amplified full-length cDNA from poly-adenylated mRNA is used to generate 5′ Gene Expression library. Enzymatic fragmentation and size selection are used to optimize the cDNA amplicon size prior to 5’ gene expression library

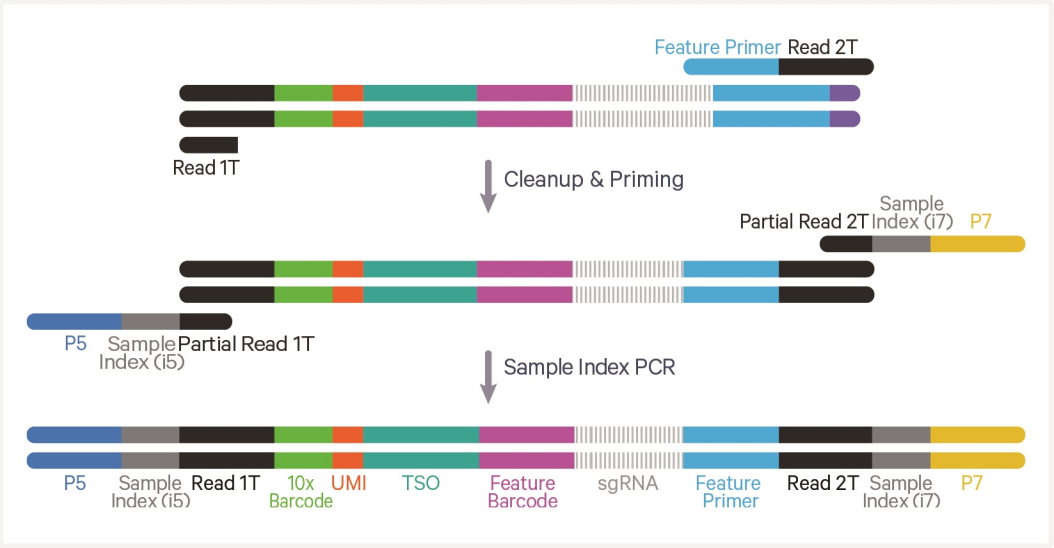
construction. P5, P7, i5 and i7 sample indexes, and Illumina R2 sequence (read 2 primer sequence) are added via End Repair, A-tailing, Adaptor Ligation, and Sample Index PCR. The final libraries contain the P5 and P7 primers used in Illumina amplification.

Pooled Amplified cDNA Processed in Bulk

Step 6: CRISPR Screening Library Construction

Amplified cDNA from sgRNA molecules is used to generate CRISPR Screening libraries. 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.

Pooled Amplified DNA Processing in Bulk (dual index)



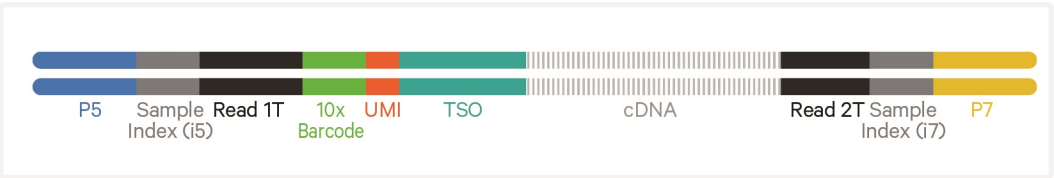
Step 7: Sequencing

The configuration of sequencing-ready libraries generated using the GEM-X Universal 5' assay are illustrated below.

Sequencing-ready dual index libraries can be sequenced at the recommended depth & run parameters. Sequencer compatibility, sample indices, library loading and pooling for sequencing are summarized in the Sequencing step.

Chromium Single Cell V(D)J Library

Chromium Single Cell 5' Gene Expression Library



Sequence data from the 5' Gene Expression library is required for the analysis of CRISPR Screening libraries.

Chromium Single Cell 5' CRISPR Screening Library

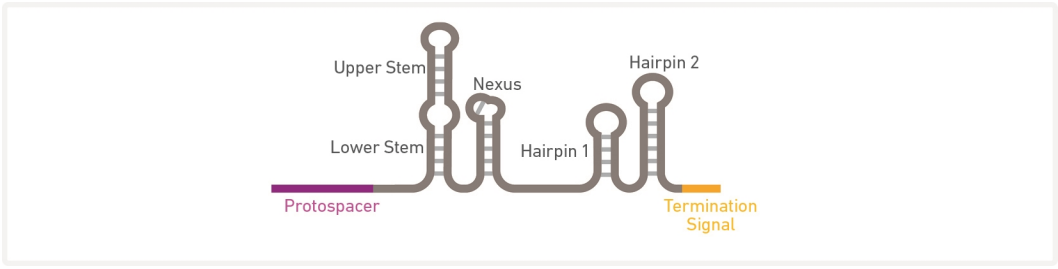
See Oligonucleotide Sequences on page 117

CRISPR Screening Overview

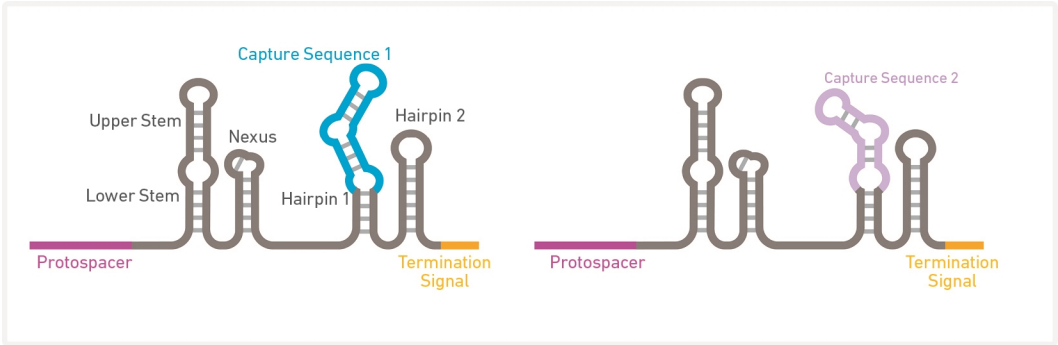
The Chromium Single Cell Immune Profiling Solution with Feature Barcode technology provides a high-throughput and scalable approach to obtain gene expression profiles along with perturbation phenotypes via direct capture of poly-adentylated mRNAs and single-guide RNAs (sgRNAs) from the same single cell (see Stepwise Objectives on page 16). For compatibility with the Chromium Single Cell 5' CRISPR Screening assay, sgRNAs should be engineered for use with standard Cas9 systems with a protospacer on the 5' end (panel A). Compatibility of the assay can be verified by ensuring primer binding is possible with the sgRNA of interest. The assay is also compatible with sgRNA engineered with either Capture Sequence 1 or Capture Sequence 2 within the sgRNA hairpin structure (panel B), or immediately before the sgRNA termination signal (panel C), elongating the 3'-end of the sgRNA. Alternate sgRNA structures for use with other Cas enzymes may be compatible, but have not been tested by 10x Genomics.

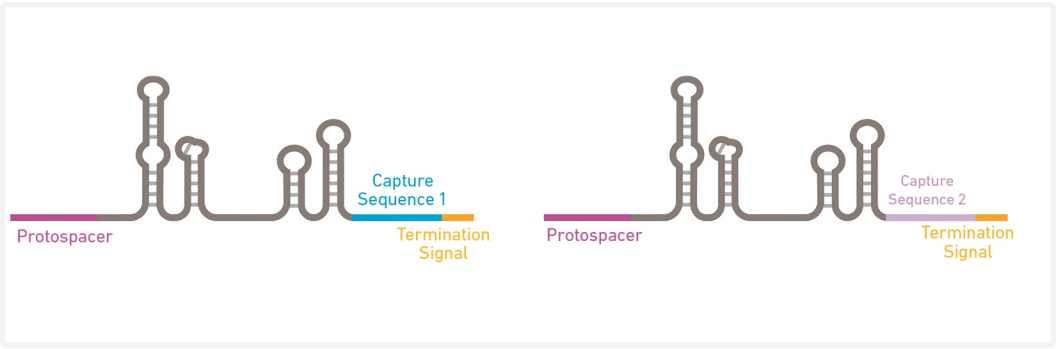
sgRNA compatible with Single Cell 5' CRISPR Assay

A.



B.





Experimental Planning Guide: Refer to the Chromium Single Cell CRISPR Screening – Experimental Planning Guide (Document CG000398).

Performing sgRNA QC by qPCR, NGS, or other methods is recommended prior to proceeding with the Single Cell Immune Profiling and CRISPR Screening Solution.

10x GENOMICS

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/ µl	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

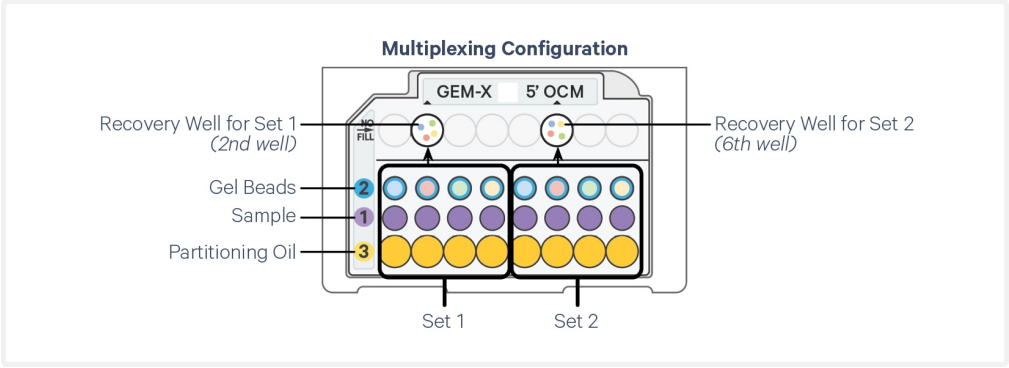
- Follow manufacturer's calibration and maintenance schedules.
- Pipette accuracy is particularly important when using SPRIselect reagents.

GEM-X OCM Chip

- GEM-X 5' 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 43 before loading the chip.



Chromium X Series Chip Holder

- Chromium X/iX or X Series Chip Holders encase GEM-X 5' 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 43 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 5' 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 45 for specific instructions.

Chromium X Series Instrument Firmware

- To run the GEM-X 5' 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.
- 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).

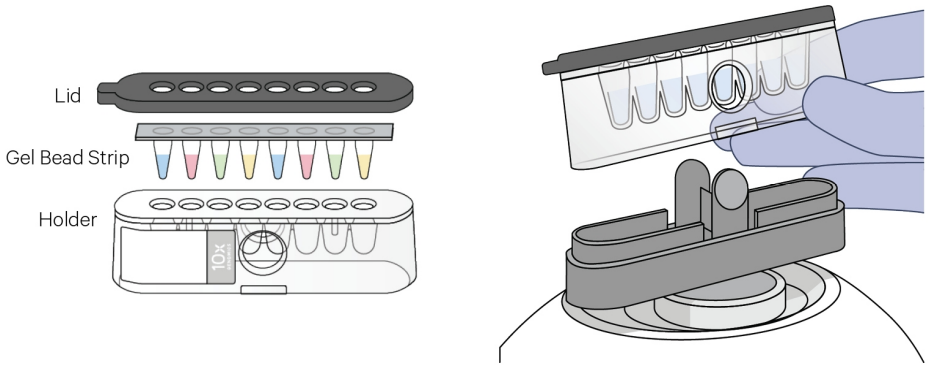
Gel Bead Handling

- 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**.

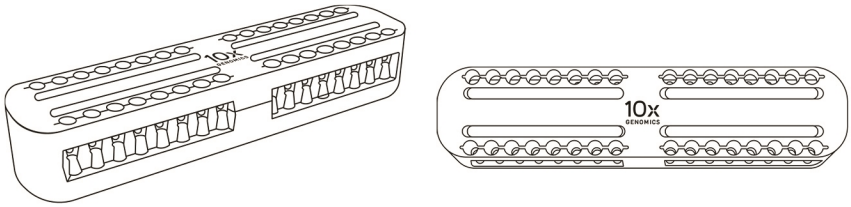
- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**.
- Note the color coded Gel Beads in the tube strip, starting with blue in the first tube followed by red, green, and yellow (see illustration below). When loading the chip, follow the guidance for loading 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.

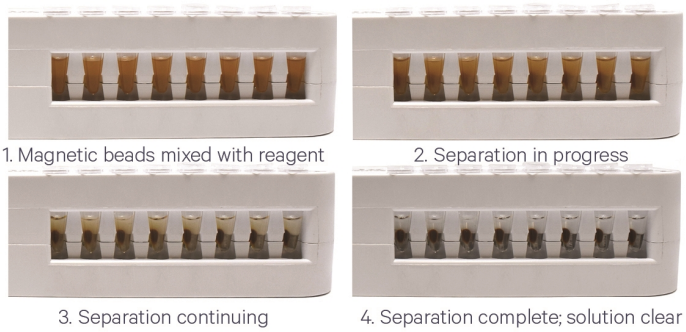


Magnetic Bead Cleanup Steps

- 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

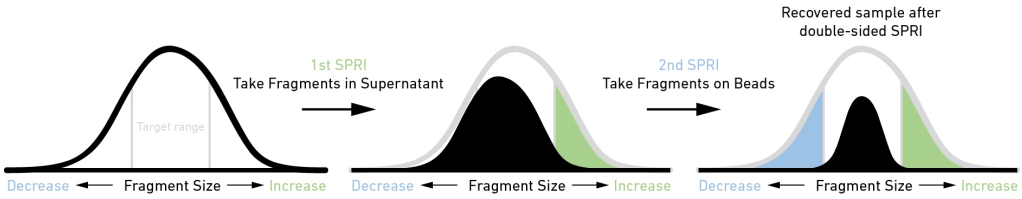
- After aspirating the desired volume of SPRIselect reagent, examine the pipette tips before dispensing to ensure the correct volume is transferred.
- Pipette mix thoroughly as insufficient mixing of sample and SPRIselect reagent will lead to inconsistent results.
- Use fresh preparations of 80% ethanol.

Tutorial — SPRIselect Reagent:DNA Sample Ratios

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

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

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**).

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

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

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

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 39

1.1 Prepare Master Mix 40

Assemble GEM-X OCM Chip 40

Sample Multiplexing Guidelines 43

1.2 Load GEM-X OCM Chip 45

1.3 Run the Chromium X Series Instrument 48

1.4 Transfer GEMs 49

1.5 GEM-RT Incubation 51

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. Chromium iX and Xo are not compatible without a licensing upgrade.

Item		10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐	GEM-X OCM 5' Gel Bead v3	2001127	Equilibrate to room temperature for **30 min** before loading the chip.	−80°C
☐	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
☐	CRISPR Poly-dT Primer Mix B	2001145	Vortex, verify no precipitate, centrifuge briefly.	−20°C
☐	Reducing Agent B	2000087	Vortex, verify no precipitate, centrifuge briefly.	−20°C
Place on Ice				
☐	Cell Suspension	—		—
☐	RT Enzyme E	2001146	Centrifuge briefly before adding to the mix.	−20°C
☐	1X PBS	—		—
Obtain				
☐	Partitioning Oil B	2001213	—	Ambient
☐	GEM-X 5' OCM Chip *Verify name and PN*	2001100	See Tips & Best Practices.	Ambient
☐	X/iX Chip Gasket	3000656	See Tips & Best Practices.	Ambient
☐	Chromium X/iX or Chromium X Series Chip Holder	3000598	See Tips & Best Practices.	Ambient
☐	10x Vortex Adapter	330002	See Tips & Best Practices.	Ambient
☐	50% glycerol solution *If loading only one set (each set = 4 samples)*	—	See Tips & Best Practices.	—

1.1 Prepare Master Mix

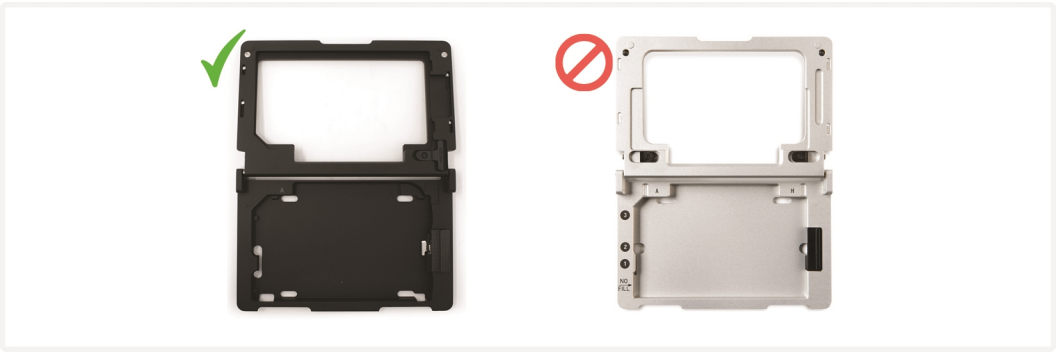
a. Prepare Master Mix on ice. Pipette mix 15x and centrifuge briefly.

Master Mix *Add reagents in the order listed*	PN	4X + 10% (µl)	8X + 10% (µl)
RT Reagent E	2001106	18.9	37.8
CRISPR Poly-dT Primer Mix B	2001145	2.6	5.3
Reducing Agent B	2000087	2.2	4.4
RT Enzyme E	2001146	7.9	15.8
Total	–	**31.7**	**63.4**

b. Add **7.2 µl** 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 27 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 5' 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					
	1.5					
200	4.1	8.3				
	5.7	1.5				
300	2.8	5.5				
	7.0	4.3				
400	2.1	4.1	8.3			
	7.7	5.7	1.5			
500	1.7	3.3	6.6			
	8.1	6.5	3.2			
600	1.4	2.8	5.5	8.3		
	8.4	7.0	4.3	1.5		
700	1.2	2.4	4.7	7.1	9.4	
	8.6	7.4	5.1	2.7	0.4	
800	1.0	2.1	4.1	6.2	8.3	
	8.8	7.7	5.7	3.6	1.5	
900	0.9	1.8	3.7	5.5	7.3	9.2
	8.9	8.0	6.1	4.3	2.5	0.6
1000	0.8	1.7	3.3	5.0	6.6	8.3
	9.0	8.1	6.5	4.8	3.2	1.5
1100	0.8	1.5	3.0	4.5	6.0	7.5
	9.0	8.3	6.8	5.3	3.8	2.3
1200	0.7	1.4	2.8	4.1	5.5	6.9
	9.1	8.4	7.0	5.7	4.3	2.9
1300	0.6	1.3	2.5	3.8	5.1	6.3
	9.2	8.5	7.3	6.0	4.7	3.5
1400	0.6	1.2	2.4	3.5	4.7	5.9
	9.2	8.6	7.4	6.3	5.1	3.9
1500	0.6	1.1	2.2	3.3	4.4	5.5
	9.2	8.7	7.6	6.5	5.4	4.3
1600	0.5	1.0	2.1	3.1	4.1	5.2
	9.3	8.8	7.7	6.7	5.7	4.6
1700	0.5	1.0	1.9	2.9	3.9	4.9
	9.3	8.8	7.9	6.9	5.9	4.9
1800	0.5	0.9	1.8	2.8	3.7	4.6
	9.3	8.9	8.0	7.0	6.1	5.2
1900	0.4	0.9	1.7	2.6	3.5	4.3
	9.4	8.9	8.1	7.2	6.3	5.5
2000	0.4	0.8	1.7	2.5	3.3	4.1
	9.4	9.0	8.1	7.3	6.5	5.7

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)

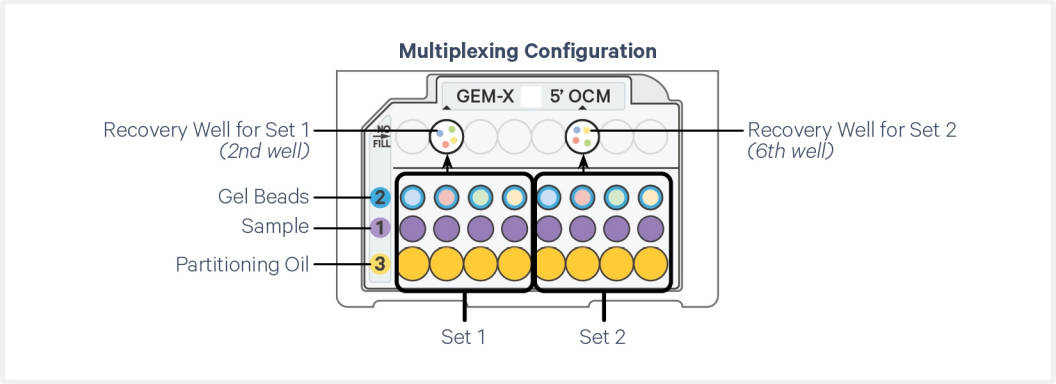
Sample Multiplexing Guidelines

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.



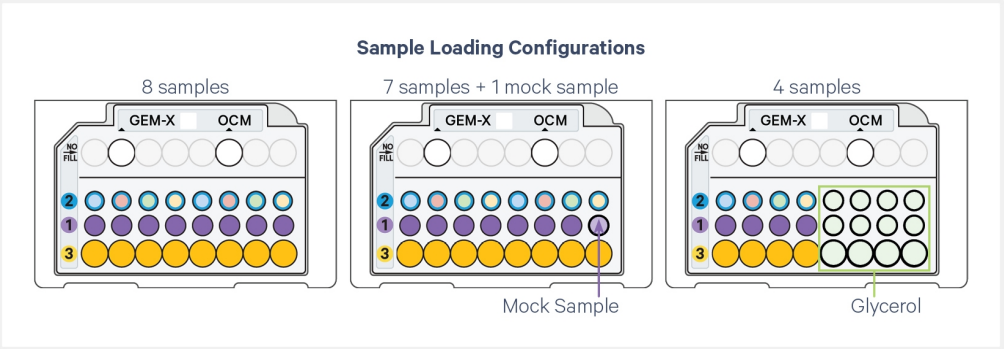
Chip loading overview

- **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, green, and yellow. 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

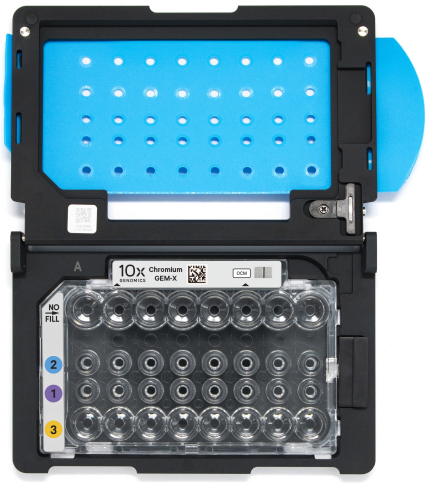
TIPS

- 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**.

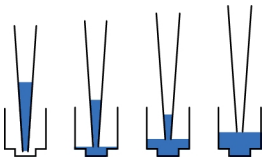
Color Legend
2: Gel beads (4 colors - blue, red, green, & yellow)
1: Sample
3: Oil

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 40* *for details.*

GEM-X 5' OCM Chip, gasket attached
Representative chip image



Pipette technique



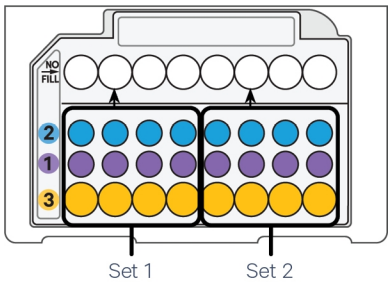
a. If loading only one set (each set = 4 samples), add 50% glycerol solution to each unused well in row 1, 2, and 3 of the second set

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 43 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

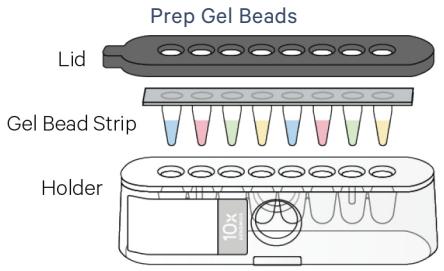
DO NOT add 50% glycerol solution to the wells in top row labeled NO FILL.
DO NOT use any substitute for 50% glycerol solution.

Glycerol
in GEM-X 5' OCM Chip



b. Prepare Gel Beads

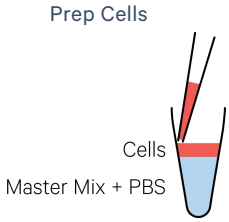
- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**. Gel Beads are color coded to indicate two sets of 4 Gel Beads each, where each color represents a unique set of barcodes.
- Centrifuge the Gel Bead strip for **~5 sec**.
- Confirm there are no bubbles at 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.



Colors indicate Gel beads with different barcode whitelists.

c. Prepare Master Mix + Cell Suspension

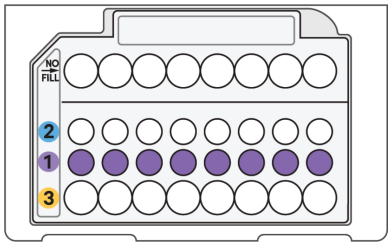
- Refer to the Cell Suspension Volume Calculator Table.
- Add the appropriate volume of 1X PBS to Master Mix. Pipette mix 5x. Centrifuge briefly.
- Add corresponding volume of single cell suspension into the Master Mix + PBS (total 17 µl in each tube).
- Gently pipette mix the cell suspension before adding to the Master Mix.
- If running less than 4 samples in a multiplexing set, prepare 17 µl mock sample (add 1X PBS 9.8 µl to Master Mix 7.2 µl).



d. Load Row Labeled 1

- Using a multichannel pipette (P20), gently mix the Master Mix + Cell Suspension.
- Using the same pipette tip, dispense **15 µl** 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 µl** mock sample (1X PBS + Master Mix) in the designated well.

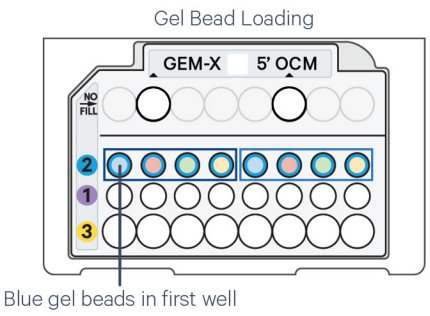
15 µl Master Mix + Cell Suspension in GEM-X 5' OCM Chip



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 5' OCM Chip

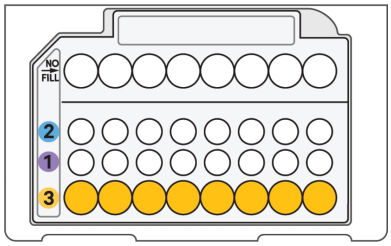


f. Load Row Labeled 3

- 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 5' OCM Chip

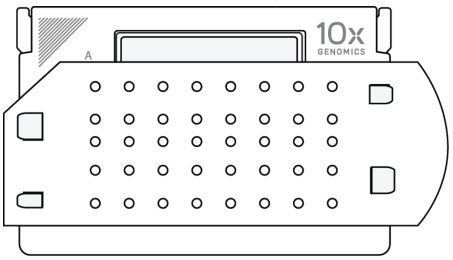


g. Prepare for Run

- 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.*

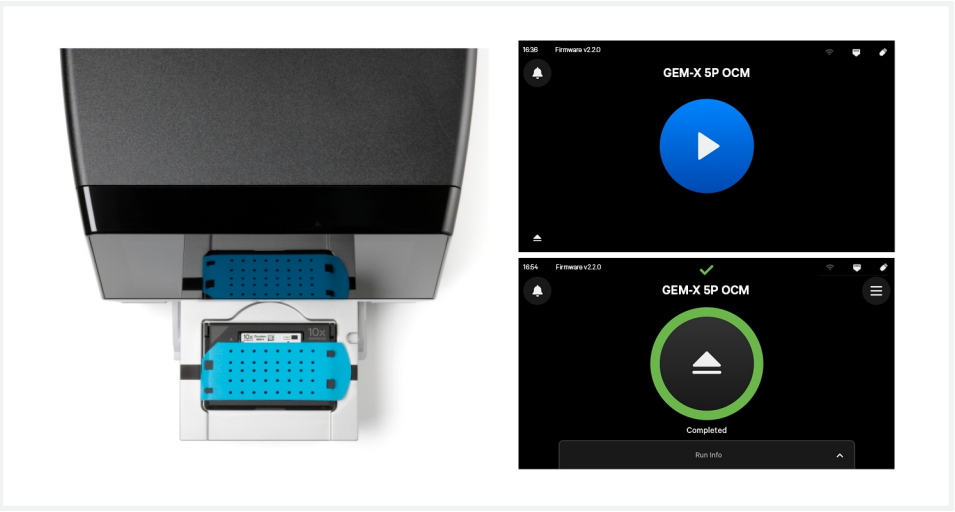
GEM-X 5' OCM Chip, closed



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.

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 5P 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 106).

Inspect/photograph while horizontal

Fold lid back to 45°; inspect/photograph again

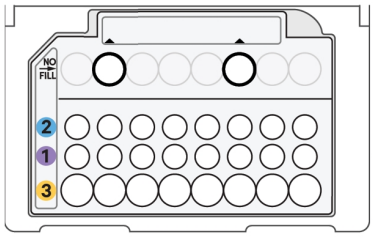
Slowly aspirate GEMs keeping the pipette tip at an angle

Inspect/photograph in pipette tip against black background

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 106 for additional guidance for clogs/emulsion faliures.

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.

10x GENOMICS

Step 2:

Post GEM–RT Cleanup & cDNA Amplification

2.0 Get Started 53

2.1 Post GEM-RT Cleanup – Dynabeads 55

Step Overview (steps 2.2 & 2.3) 58

2.2 cDNA Amplification 59

2.3 cDNA Cleanup –SPRIselect 61

2.3A Pellet Cleanup (for V(D)J & 5' Gene Expression) 62

2.3B Transferred Supernatant Cleanup (for CRISPR Screening) 63

2.4 Post cDNA Amplification QC & Quantification 64

2.0 Get Started

Item			10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
☐	◯	Reducing Agent B	2000087	Thaw, vortex, verify no precipitate, centrifuge briefly.	−20°C
☐	●	cDNA Primers	2000089	Thaw, vortex, centrifuge briefly.	−20°C
☐		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
☐		Beckman Coulter SPRIselect Reagent	—	Manufacturer's recommendations.	—
☐		Agilent Bioanalyzer High Sensitivity Kit *If used for QC & quantification*	—	Manufacturer's recommendations.	—
☐		DNA High Sensitivity Reagent Kit *If LabChip used for QC & quantification*	—	Manufacturer's recommendations.	—
☐		Agilent TapeStation ScreenTape & Reagents *If used for QC & quantification*	—	Manufacturer's recommendations.	—
Place on Ice					
☐	◯ ⚠	Amp Mix *DO NOT use the Library Amp Mix PN-2000531 (if provided) at this step to avoid a significant decrease in assay performance*	2000047 /2000103	Vortex, centrifuge briefly.	−20°C
Thaw at 65°C					
☐	●	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					
☐	◯	Recovery Agent	220016	—	Ambient
☐		10x Magnetic Separator B	2001212	—	Ambient
☐		Qiagen Buffer EB	—	Manufacturer's recommendations.	—

Item		10x PN	Preparation & Handling	Storage
☐	Bio-Rad 10% Tween 20	—	Manufacturer's recommendations.	—
☐	Prepare 80% Ethanol *Prepare 15 ml for 8 reactions.*	—	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**.

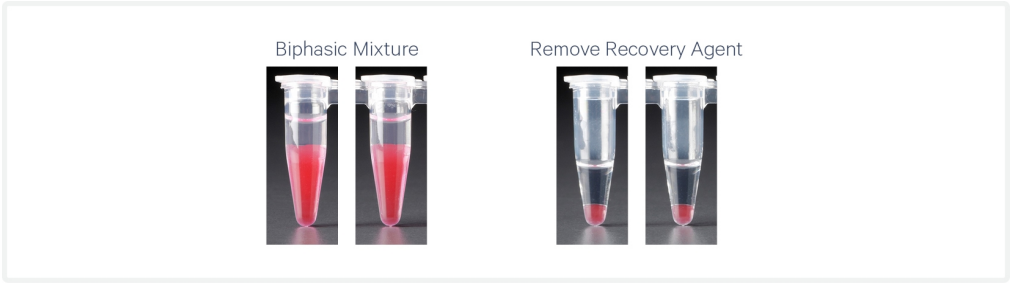
The resulting biphasic mixture contains Recovery Agent/Partitioning Oil (pink) and aqueous phase (clear), with no persisting emulsion (opaque).

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:

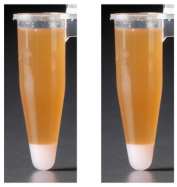
Vortex the Dynabeads thoroughly (**≥30 sec**) **immediately** before adding to the mix.

- 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 *Add reagents in the order listed*		PN	1X (µl)	2X + 10% (µl)	4X + 10% (µl)
○	**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 µl** to each tube. Pipette mix 10x (pipette set to 200 µl). Before adding ensure that there are no clumps.

Add Dynabeads Cleanup Mix



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 *Add reagents in the order listed*		PN	1X (µl)	10X (µl)
	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 µl** 80% ethanol to the pellet while on the magnet. Wait **30 sec**.

j. Remove the ethanol.

k. Add **200 µl** 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.

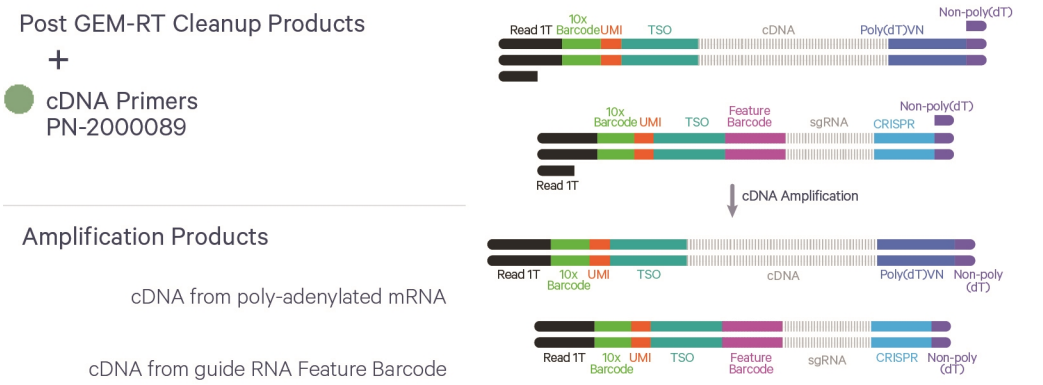
q. Incubate **1 min** at **room temperature**.

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

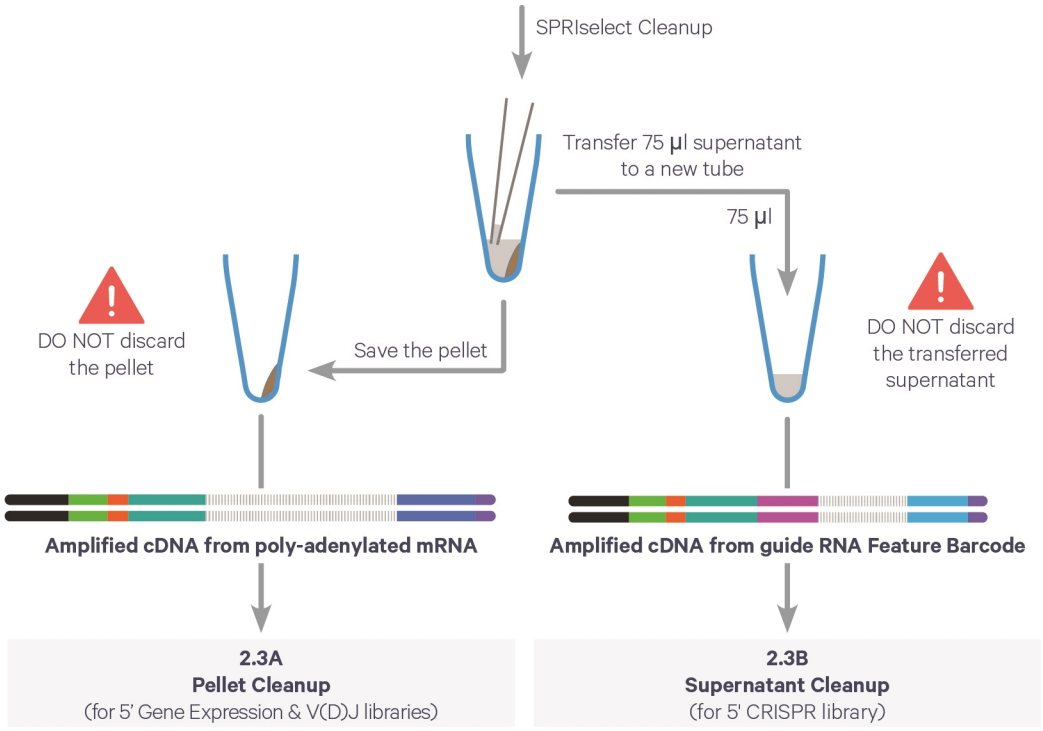
s. Transfer **35 µl** sample to a new tube strip.

Step Overview (steps 2.2 & 2.3)

Amplification Products Generated in Step 2.2 – cDNA Amplification



Step 2.3 – cDNA Cleanup – SPRIselect Overview



2.2 cDNA Amplification

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

cDNA Amplification Reaction Mix *Add reagents in the order listed*		PN	1X (µl)	2X + 10% (µl)	4X + 10% (µl)
○	**Amp Mix** DO NOT use the Library Amp Mix PN-2000531 (if provided)	2000047/ 2000103	50	110	220
●	**cDNA Primers**	2000089	15	33	66
	Total		**65**	**143**	**286**

b. Add **65 µl** cDNA Amplification Reaction Mix to **35 µl** sample (Post-GEM-RT Cleanup, step 2.1s).

c. Pipette mix 15x (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	~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.

Version Specific The number of total cycles is based on the targeted cell recovery and is independent of the RNA content of the sample.

Targeted Cell Recovery (from a set with up to 4 samples)	Total Cycles
<500	17
501–2,000	15

Targeted Cell Recovery (from a set with up to 4 samples)	Total Cycles
2,001–6,000	13
>6,000	12

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

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 into a new tube strip without disturbing the pellet. Maintain at **room temperature**. DO NOT discard the transferred supernatant (cleanup for CRISPR Screening-step 2.3B).

e. Remove the remaining supernatant from the pellet without disturbing the pellet. DO NOT discard the pellet (cleanup for V(D)J & 5′ Gene Expression library construction). **Immediately** proceed to Pellet Cleanup (step 2.3A).

2.3A Pellet Cleanup (for V(D)J & 5' Gene Expression)

i. Add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.

ii. Remove the ethanol.

iii. **Repeat** steps i and ii for a total of 2 washes.

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

v. Remove any remaining ethanol. Air dry for **2 min**. DO NOT exceed **2 min** as this will decrease elution efficiency.

vi. Remove from the magnet. Add **40.5 µl** Buffer EB. Pipette mix 15x (pipette set to 35 µl).

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

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

ix. Transfer **40 µl** sample to a new tube strip.

x. 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 V(D)J Amplification & 5′ Gene Expression Library Construction.

2.3B Transferred Supernatant Cleanup (for CRISPR Screening)

i. Vortex to resuspend the SPRIselect reagent. Add **30 µl** SPRIselect reagent (1.2X) to **75 µl** of the transferred supernatant and pipette mix 15x (pipette set to 100 µl).

ii. Incubate for **5 min** at **room temperature**.

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

iv. Remove supernatant.

v. Add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.

vi. Remove the ethanol.

vii. **Repeat** steps v and vi for a total of 2 washes.

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

ix. Remove any remaining ethanol. Air dry for **2 min**. DO NOT exceed **2 min** as this will decrease elution efficiency.

x. Remove from the magnet. Add **50.5 µl** Buffer EB. Pipette mix 15x (pipette set to 35 µl).

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

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

xiii. Transfer **50 µl** sample to a new tube strip.

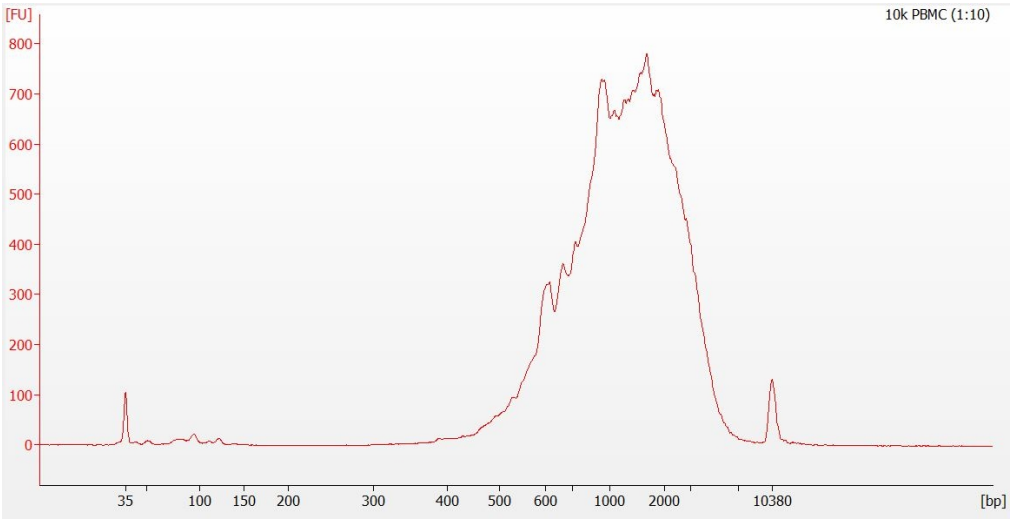
STOP xiv. Store at **4°C** for up to **72 h** or at **−20°C** for up to **4 weeks**, or proceed directly to CRISPR Screening Library Construction.

2.4 Post cDNA Amplification QC & Quantification

a. Run 1 µl (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

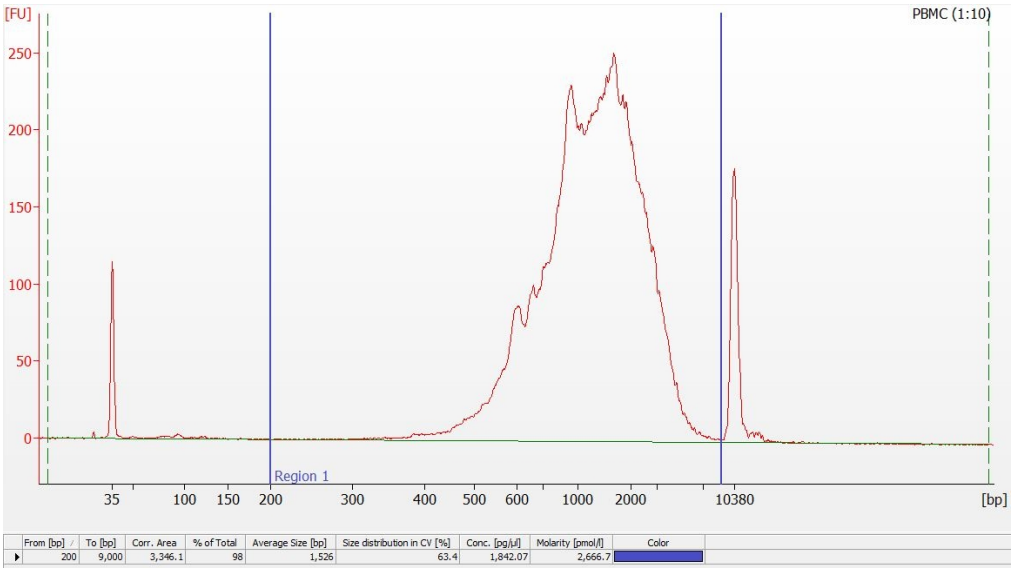
LabChip

See Appendix on page 111 for representative traces.

Example Calculation

i. Select Region: Under the “Electropherogram” view, choose the “Region Table.” Manually select the region of ~200 – ~9000 bp.

ii. Note Concentration [pg/µl]



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.

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

Example Calculation of cDNA Yield

Concentration: **1842.07 pg/µl** Elution volume:40 µl; Dilution Factor:10

Total Yield

$$= \frac{\text{Conc'n (pg/µl) x Elution Vol. (µl) x Dilution Factor}}{\text{1000 (pg/ng)}}$$

$$= \frac{\text{1842.07 x 40 x 10}}{\text{1000 (pg/ng)}}$$

= 736.83 ng

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

= Total cDNA x 0.25 = 736.83 ng x 0.25 = **184.21 ng**

Refer to step 5.5 for appropriate number of Sample Index PCR cycles based on carry forward cDNA product yield.

10x GENOMICS

Step 3:

V(D)J Amplification from cDNA

3.0 Get Started 67

3.1 V(D)J Amplification 1 68

3.2 Post V(D)J Amplification 1 Cleanup Double Sided – SPRIselect 69

3.3 V(D)J Amplification 2 70

3.4 Post V(D)J Amplification 2 Cleanup Double Sided – SPRIselect 71

3.5 Post V(D)J Amplification QC & Quantification 72

3.0 Get Started

	Item		10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
	For Human Samples (Choose B or T-cell primers based on desired amplification products)				
☐		Human T Cell Mix 1 v2	2000242	Thaw, vortex, centrifuge briefly	–20°C
☐		Human T Cell Mix 2 v2	2000246	Thaw, vortex, centrifuge briefly	–20°C
☐		Human B Cell Mix 1 v2	2000254	Thaw, vortex, centrifuge briefly	–20°C
☐		Human B Cell Mix 2 v2	2000255	Thaw, vortex, centrifuge briefly	–20°C
	For Mouse Samples (Choose B or T-cell primers based on desired amplification products)				
☐		Mouse T Cell Mix 1 v2	2000256	Thaw, vortex, centrifuge briefly	–20°C
☐		Mouse T Cell Mix 2 v2	2000257	Thaw, vortex, centrifuge briefly	–20°C
☐		Mouse B Cell Mix 1 v2	2000258	Thaw, vortex, centrifuge briefly	–20°C
☐		Mouse B Cell Mix 2 v2	2000259	Thaw, vortex, centrifuge briefly	–20°C
	For All Samples				
☐		Beckman Coulter SPRIselect Reagent	—	Manufacturer's recommendations.	—
☐		Agilent Bioanalyzer High Sensitivity Kit *If used for QC and quantification*	—	Manufacturer's recommendations.	—
☐		DNA High Sensitivity Reagent Kit *If LabChip used for QC*	—	Manufacturer's recommendations.	—
☐		Agilent TapeStation ScreenTape & Reagents *If used for QC and quantification*	—	Manufacturer's recommendations.	—
Place on Ice					
☐	 	Amp Mix *Retrieve from Single Cell V(D)J Amplification Kits* DO NOT use the Library Amp Mix (PN 2000531) at this step to avoid a significant decrease in assay performance	2000047	Vortex, centrifuge briefly.	–20°C
Obtain					
☐		10x Magnetic Separator B	2001212	See Tips & Best Practices.	Ambient
☐		Qiagen Buffer EB	—	Manufacturer's recommendations.	Ambient
☐		Prepare 80% Ethanol *Prepare 15 ml for 8 reactions.*	—	Prepare fresh.	Ambient

3.1 V(D)J Amplification 1

a. Place a tube strip on ice and transfer **2 µl** sample (post cDNA Amplification & QC, step 2.3A) to the same tube.

b. Prepare V(D)J Amplification 1 Reaction Mix on ice. Vortex and centrifuge briefly.

V(D)J Amplification 1 Rxn Mix *Add reagents in the order listed*		PN	1X (µl)	2X + 10% (µl)	4X + 10% (µl)
○	**Amp Mix**	2000047	50	110	220
●	**T Cell Mix 1 v2** **or** **B Cell Mix 1 v2**	Human 2000242/ Mouse 2000256 or Human 2000254/ Mouse 2000258	48	105.6	211.2
	Total		**98**	**215.6**	**431.2**

c. Add **98 µl** V(D)J Amplification 1 Reaction Mix to each tube containing **2 µl** sample.

d. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.

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

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~20-30 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	62°C	00:00:30
4	72°C	00:01:00
5 ⚠ Different cycle numbers for T & B cells	T Cell: Go to Step 2, 11x (total 12 cycles) *B Cell: Go to Step 2, 7x (total 8 cycles)	
6	72°C	00:01:00
7	4°C	Hold

*When working with multiple samples with a low clonotype diversity like cell lines, the B-cell enrichment cycles can be reduced to a total of 6 cycles.

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

3.2 Post V(D)J Amplification 1 Cleanup Double Sided – SPRIselect

a. Vortex to resuspend SPRIselect Reagent. Add **50 µl** SPRIselect Reagent **(0.5X)** to each sample. Pipette mix 15x (pipette set to 140 µl).

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

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

DO NOT discard supernatant.

d. Transfer **145 µl** supernatant to a new tube strip.

e. Vortex to resuspend SPRIselect Reagent. Add **30 µl** SPRIselect Reagent **(0.8X)** to each sample. Pipette mix 15x (pipette set to 150 µl).

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

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

h. Remove **170 µl** supernatant. DO NOT discard any beads.

i. Add **200 µl** 80% ethanol. 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 wash. DO NOT over-dry beads to ensure maximum elution efficiency.

n. Remove from the magnet. Add **35.5 µl** Buffer EB. Pipette mix 15x.

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

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

q. Transfer **35 µl** sample to a new tube strip.

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

3.3 V(D)J Amplification 2

a. Prepare V(D)J Amplification 2 Reaction Mix on ice. Vortex and centrifuge briefly.

V(D)J Amplification 2 Reaction Mix *Add reagents in the order listed*		PN	1X (µl)	2X + 10% (µl)	4X + 10% (µl)
○	Amp Mix	2000047	50	110	220
●	T Cell Mix 2 v2	Human 2000246/ Mouse 2000257	15	33	66
	or	or			
●	B Cell Mix 2 v2	Human 2000255/ Mouse 2000259			
	Total		65	143	286

b. Add **65 µl** V(D)J Amplification 2 Reaction Mix to each tube containing **35 µl** sample.

c. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.

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

	Reaction Volume	Run Time
105°C	100 µl	~25-30 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	62°C	00:00:30
4	72°C	00:01:00
5 ⚠ Different cycle numbers for T & B cells	T Cell: Go to Step 2, 9x (total 10 cycles) *B Cell: Go to Step 2, 7x (total 8 cycles)	
6	72°C	00:01:00
7	4°C	Hold

*When working with multiple samples with a low clonotype diversity like cell lines, the B-cell enrichment cycles can be reduced to a total of 6 cycles.

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

3.4 Post V(D)J Amplification 2 Cleanup Double Sided – SPRIselect

a. Vortex to resuspend SPRIselect Reagent. Add **50 µl** SPRIselect Reagent **(0.5X)** to each sample. Pipette mix 15x (pipette set to 145 µl).

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

c. Place tube strip on the magnet•**High** until the solution clears. DO NOT discard supernatant.

d. Transfer **145 µl** supernatant to a new tube strip.

e. Vortex to resuspend SPRIselect Reagent. Add **30 µl** SPRIselect Reagent **(0.8X)** to each sample. Pipette mix 15x (pipette set to 150 µl).

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

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

h. Remove **170 µl** supernatant. DO NOT discard any beads.

i. Add **200 µl** 80% ethanol. 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 wash. DO NOT over-dry beads to ensure maximum elution efficiency.

n. Remove from the magnet. Add **40.5 µl** Buffer EB. Pipette mix 15x.

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

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

q. Transfer **40 µl** sample to a new tube strip.

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

3.5 Post V(D)J Amplification QC & Quantification

a. Run **1 µl** sample (see below for dilution) on an Agilent Bioanalyzer High Sensitivity chip.

Version Specific

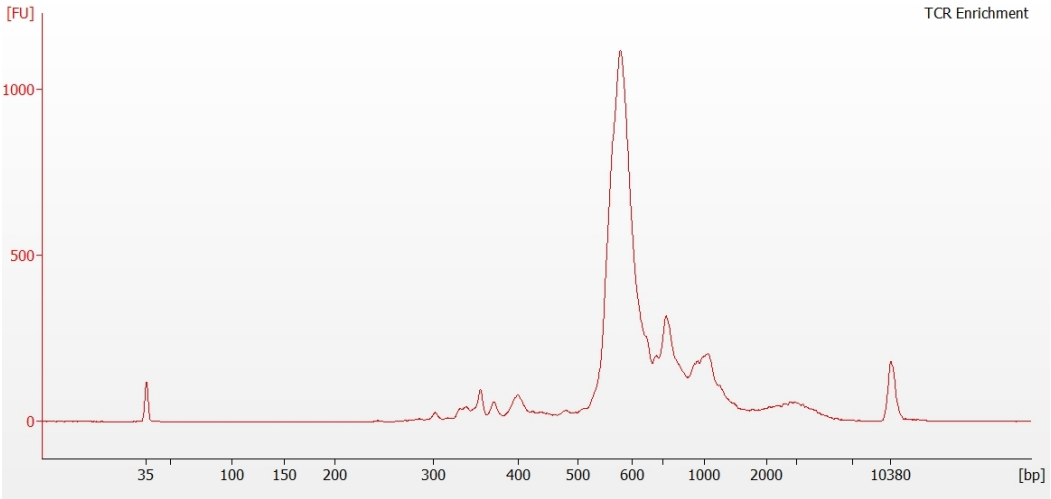
Sample Dilution is dependent on the sample type.

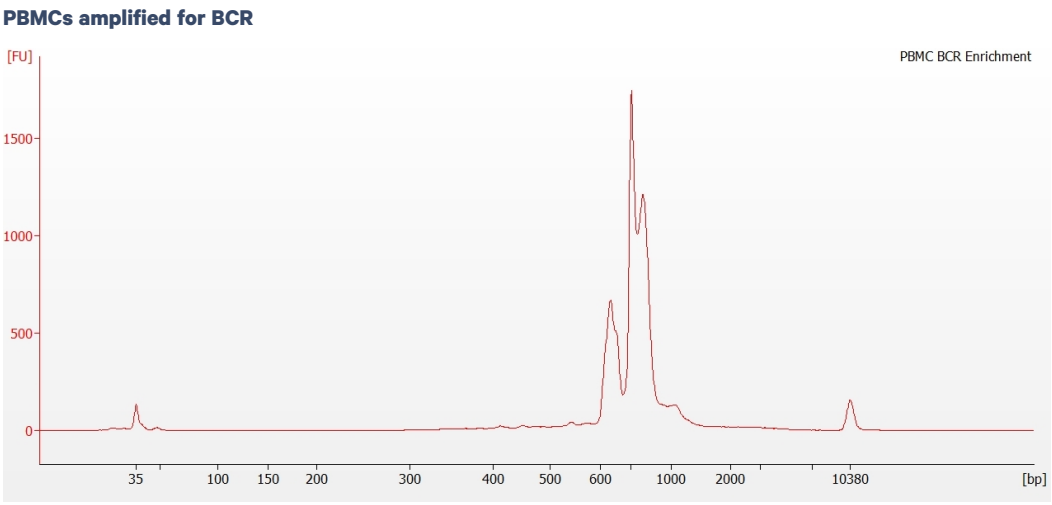
Sample Type	Dilution
PBMCs amplified for TCR	1:5
PBMCs amplified for BCR	1:2
Purified primary T cells (TCR)	1:10
Purified primary B cells (BCR)	1:5
High RNA content cells (i.e. Cell Lines)	1:15

Samples of RNA-rich cells may require additional dilution in nuclease-free water. The number of distinct peaks may vary. Higher molecular weight product (2,000-9,000 bp) may be present. This does not affect sequencing.

Representative Traces

PBMCs amplified for TCR





Determine yield for each sample. See example calculation in the following page.

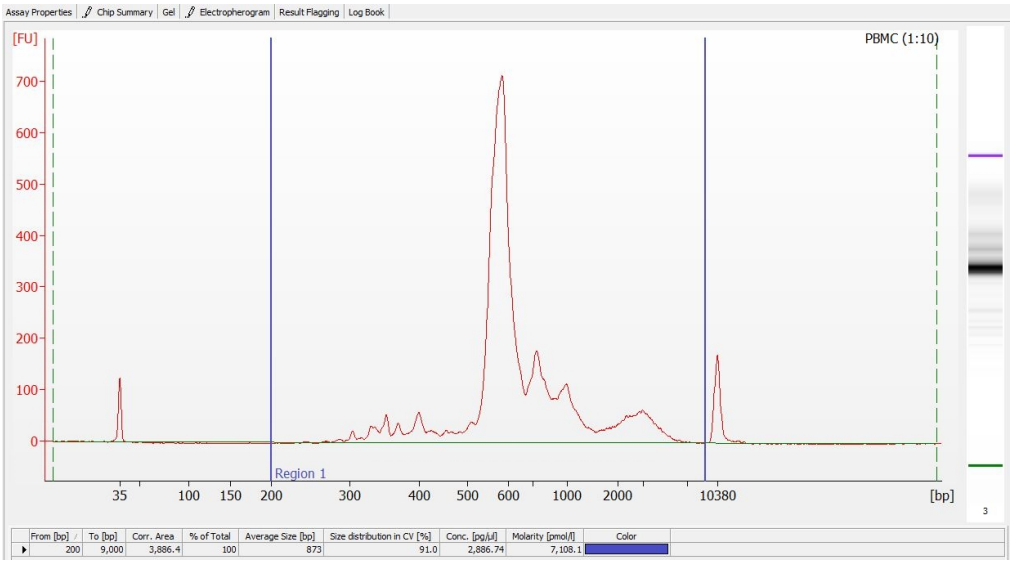
Alternate QC Methods:

- Agilent TapeStation
- LabChip
- See Appendix on page 111 for representative traces

Example Calculation

i. Select Region: Under the “Electropherogram” view, choose the “Region Table.” Manually select the region of ~200 – ~9000 bp.

ii. Note Concentration [pg/µl]



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 V(D)J amplified product concentration in ng/µl.

Version Specific

Carry forward ONLY 25% of total V(D)J-amplified product yield into V(D)J Library Construction.

Example Calculation of V(D)J-amplified Product Yield

Concentration: **2886.74 pg/µl** Elution volume: 40 µl; Dilution Factor: 10

Total Yield

$$= \frac{\text{Conc'n (pg/µl) x Elution Vol. (µl) x Dilution Factor}}{\text{1000 (pg/ng)}}$$

$$= \frac{\text{2886.74 x 40 x 10}}{\text{1000 (pg/ng)}}$$

=1154.70 **ng**

Carrying Forward ONLY 25% of total V(D)J amplified-product for V(D)J Library

= Total cDNA x 0.25 = 1154.70 ng x 0.25

=**288.68 ng**

Refer to step 4.5 for appropriate number of Sample Index PCR cycles based on carry forward V(D)J-amplified product yield.

10x GENOMICS

Step 4:

V(D)J Library Construction

4.0 Get Started 76

4.1 Fragmentation, End Repair & A-tailing 78

4.2 Adaptor Ligation 79

4.3 Post Ligation Cleanup – SPRIselect 79

4.4 Sample Index PCR 81

4.5 Post Sample Index PCR Cleanup – SPRIselect 82

4.6 Post Library Construction QC 83

4.0 Get Started

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

Item		10x PN	Preparation & Handling	Storage
□	Qiagen Buffer EB	—	Manufacturer's recommendations.	Ambient
□	Prepare 80% Ethanol *Prepare 15 ml for 8 reactions*	—	Prepare fresh.	Ambient

4.1 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 *Pre-cool block prior to preparing the Fragmentation Mix*	4°C	Hold
Fragmentation	32°C	00:02:00
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. Pipette mix and centrifuge briefly.

Fragmentation Mix	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 V(D)J-amplified product from V(D)J Amplification (step 3.4) to a tube strip. Note that only 10 µl (25%) V(D)J-amplified sample is sufficient for generating V(D)J 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 V(D)J 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.

4.2 Adaptor Ligation

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

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

b. Remove the sample from the thermal cycler.

c. Add **50 µl** Adaptor Ligation Mix to **50 µl** sample. Pipette mix 15x (pipette set to 90 µl). Centrifuge briefly.

d. 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 µl	15 min

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

4.3 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**.

j. Remove from the magnet. Add **30.5 µl** Buffer EB. Pipette mix 15x. If beads still appear clumpy, continue pipette mixing until fully resuspended.

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.

4.4 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** Library Amp Mix (PN-2000531) or Amp Mix (PN-2000047/2000103) 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

STOP

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

The table recommends a starting point for optimization. The total cycles should be optimized based on 25% carry forward V(D)J-amplified product yield/input calculated during Post V(D)J Amplification QC & Quantification (step 3.5).

Version Specific

Recommended Cycle Numbers

cDNA Input	Total Cycles
<25 ng	9
25-150 ng	8
151-500 ng	7
501-1,000 ng	6
>1,500 ng	5

4.5 Post Sample Index PCR Cleanup – SPRIselect

a. Vortex to resuspend the 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 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 remaining ethanol. Air dry for **2 min**.

j. Remove from the magnet. Add **35.5 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 **35 µl** to a new tube strip.

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

4.6 Post Library Construction QC

Library QC

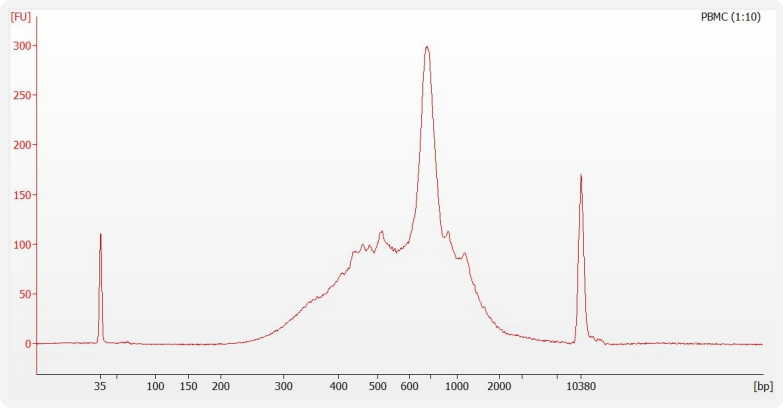
Use Agilent Bioanalyzer, Perkin Elmer LabChip, Agilent Tapestation, or Fragment Analyzer for QC.

a. Run **1 µl** sample at **1:10** dilution on an Agilent Bioanalyzer High Sensitivity chip.

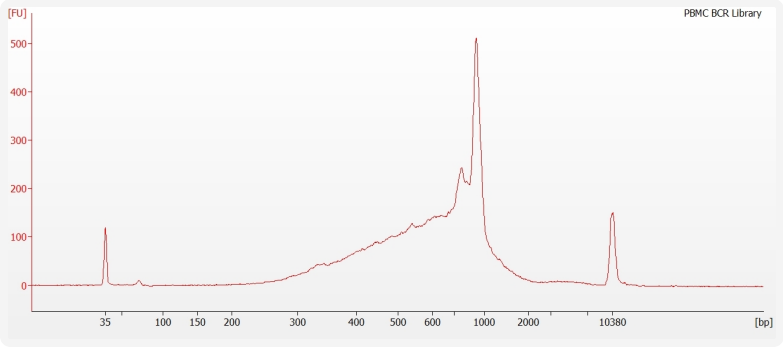
b. Select the region between 200-2,000 bp to determine average size of the library. This will be used as the insert size for library quantification.

Representative Traces

PBMCs amplified for TCR



PBMCs amplified for BCR



- See Appendix on page 111 for representative traces

Library 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 113

10x GENOMICS

Step 5:

5' Gene Expression Library Construction

5.0 Get Started 85

Step Overview (Step 5.1d) 86

5.1 GEX Fragmentation, End Repair & A-tailing 87

5.2 GEX Post Fragmentation, End Repair & A-tailing Double Sided – SPRIselect 88

5.3 GEX Adaptor Ligation 89

5.4 GEX Post Ligation Cleanup – SPRIselect 89

5.5 GEX Sample Index PCR 91

5.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect 92

5.7 Post Library Construction QC 93

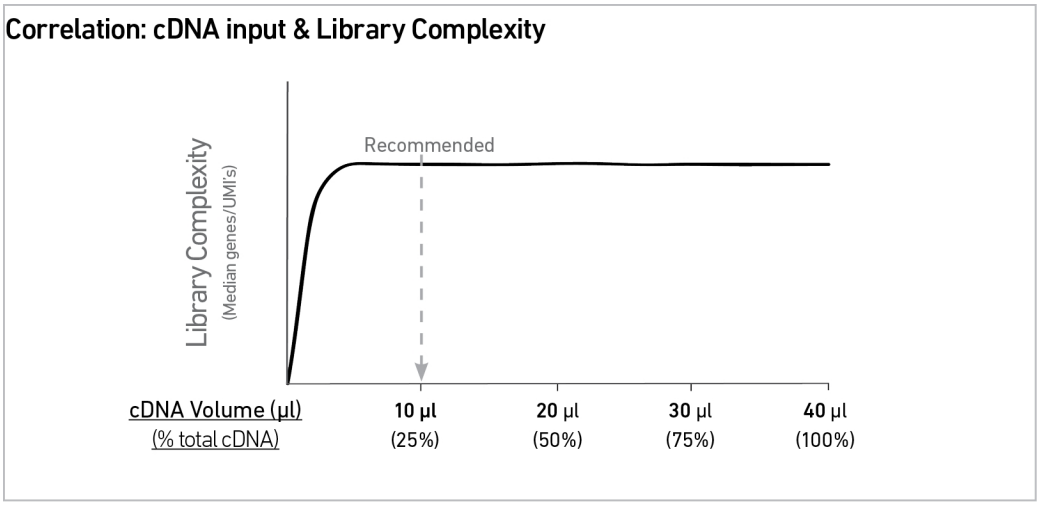
5.0 Get Started

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

Step Overview (Step 5.1d)

Correlation between input & library complexity

A Single Cell Gene Expression library is generated using a fixed proportion (10 µl, 25%) of the total cDNA (40 µl) obtained at step . The complexity of this library will be comparable to one generated using a higher proportion (>25%) of the cDNA. The remaining proportion (30 µl, 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**).



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 5.5d) should be optimized based on carrying forward a fixed proportion (10 µl, 25%) of the total cDNA yield calculated during Post cDNA Amplification QC & Quantification (step 2.4).

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

5.1 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 *Pre-cool block prior to preparing the Fragmentation Mix*	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 *Add reagents in the order listed*	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.

5.2 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.

5.3 GEX Adaptor Ligation

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

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

b. Add **50 µl** Adaptor Ligation Mix to **50 µl** sample. Pipette mix 15x (pipette set to 90 µl). 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 µl	15 min

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

5.4 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.

5.5 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).

Version Specific

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

STOP

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

5.6 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.

5.7 Post Library Construction QC

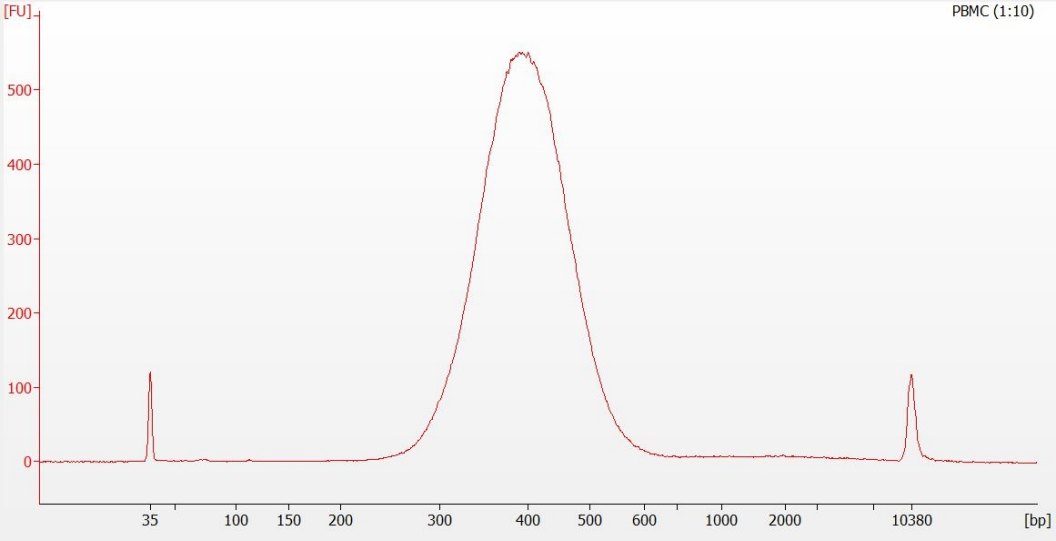
Library QC

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.

b. 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



See Appendix on page 111 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 113

10x GENOMICS

Step 6:

CRISPR Screening Library Construction

6.0 Get Started 95

6.1 Guide RNA cDNA Cleanup – SPRIselect 96

6.2 Feature PCR 96

6.3 Post Feature PCR Cleanup – SPRIselect 97

6.4 Sample Index PCR 98

6.5 Post Sample Index PCR Double Sided Size Selection – SPRIselect 99

6.6 Post Library Construction QC & Quantification 100

6.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐	Feature SI Primers 4 *Verify name & PN.* *Use indicated primer only*	2000592	—	−20°C
☐	Dual Index Plate TT Set A *Verify name & PN.* *Use indicated plate only*	3000431	—	−20°C
☐	Beckman Coulter SPRIselect Reagent	—	Manufacturer's recommendations.	—
☐	Agilent Bioanalyzer High Sensitivity Kit *If used for QC*	—	Manufacturer's recommendations.	—
☐	DNA High Sensitivity Reagent Kit If LabChip used for QC	—	Manufacturer's recommendations.	—
☐	Agilent TapeStation ScreenTape & Reagents *If used for QC*	—	Manufacturer's recommendations.	—
Place on Ice				
☐	Amp Mix *Retrieve from Chromium GEM-X Single Cell* 5' Feature Barcode Kit	2000047	Centrifuge briefly.	−20°C
☐	KAPA Library Quantification Kit for Illumina Platforms	—	Manufacturer's recommendations.	—
Obtain				
☐	10x Magnetic Separator B	2001212	See Tips & Best Practices.	Ambient
☐	Qiagen Buffer EB	—	Manufacturer's recommendations.	Ambient
☐	Prepare 80% Ethanol *Prepare 20 ml for 8 reactions.*	—	Prepare fresh.	Ambient

6.1 Guide RNA cDNA Cleanup – SPRIselect

a. Vortex to resuspend SPRIselect Reagent. Add **50 µl** SPRIselect Reagent **(1.0X)** to **50 µl** Transfered Supernatant Cleanup from step 2.3B. Pipette mix 15x (pipette set to 60 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 **40.5 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 **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 **a week**, or proceed to the next step.

6.2 Feature PCR

a. Prepare Feature PCR Mix on ice. Vortex and centrifuge briefly.

Feature PCR Mix *Add reagents in the order listed*	PN	1X (µl)	2X + 10% (µl)	4X + 10% (µl)
○ **Amp Mix**	2000047	50	110	220
● **Feature SI Primers 4**	2000592	45	99	198
Total		**95**	**209**	**418**

b. Transfer **ONLY 5 µl** from Guide RNA cDNA Cleanup to a new tube strip.

- Note that only **5 µl** of the Guide RNA cDNA Cleanup sample transfer is sufficient for generating CRISPR Screening 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 CRISPR Screening libraries.

c. Add **95 µl** Feature PCR Mix to **5 µl** sample.

d. Pipette mix 15x (pipette set to 90 µl). Centrifuge briefly.

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

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~20 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	62°C	00:00:10
4	72°C	00:00:10
5	Go to step 2, repeat 9X for a total 10 cycles	
6	72°C	00:01:00
7	4°C	Hold

6.3 Post Feature PCR Cleanup – SPRIselect

a. Vortex to resuspend the SPRIselect reagent. Add **100** SPRIselect Reagent **(1X)** 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.

d. Remove the supernatant.

e. Add **300 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 mins 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** to a new tube strip.

6.4 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-200047) to **30 µl** sample (Post Feature PCR Cleanup).

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	~25 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	62°C	00:00:10
4	72°C	00:00:10
5	Go to step 2, repeat 9X for a total of 10 cycles	
6	72°C	00:01:00
7	4°C	Hold

6.5 Post Sample Index PCR Double Sided Size Selection – SPRIselect

a. Vortex to resuspend the SPRIselect reagent. Add **70 µl** SPRIselect Reagent **(0.7X)** 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 **30 µl** SPRIselect Reagent **(1.0X)** to each sample. 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 the supernatant.

i. Add **300 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. Air dry for **1 min**.

n. Remove from the magnet. Add **40.5 µl** Buffer EB. Pipette mix 15x.

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

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

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

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

6.6 Post Library Construction QC & Quantification

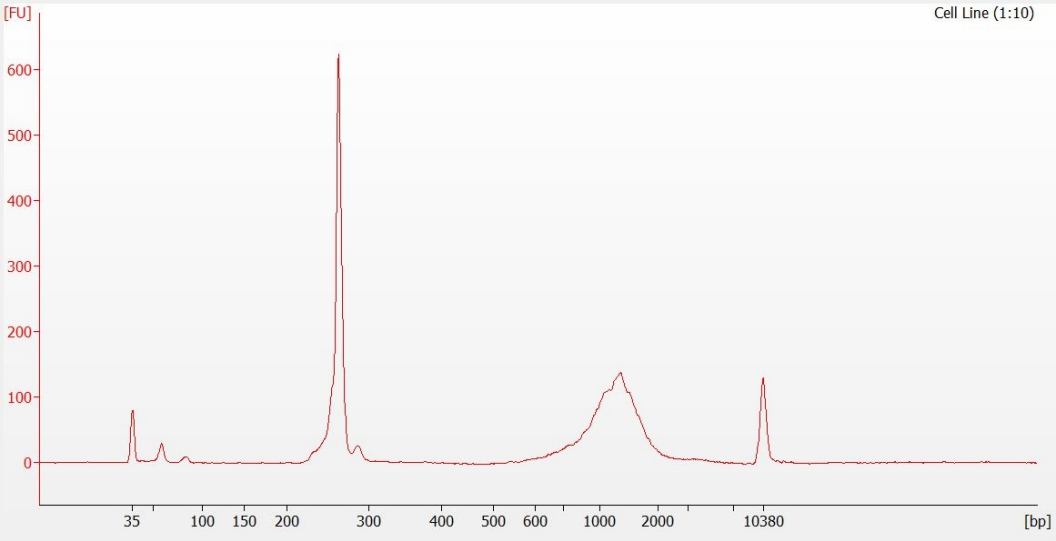
Library QC

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 200-400 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 111 for representative traces

Library 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 113

10x GENOMICS

Step 7:

Sequencing

Sequencing Libraries	102
Sequencer Compatibility	103
Sample Indices	103
Library Sequencing Depth & Run Parameters	103
Library Loading	104
Library Pooling	104
Data Analysis and Visualization	105

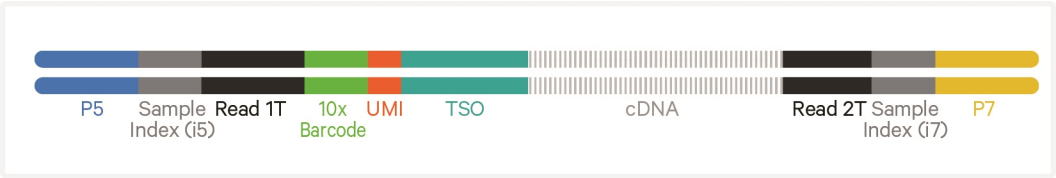
Sequencing Libraries

Chromium Single Cell V(D)J & Gene Expression Libraries

Chromium Single Cell 5' Gene Expression and V(D)J 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 and V(D)J libraries.

Chromium Single Cell V(D)J Library

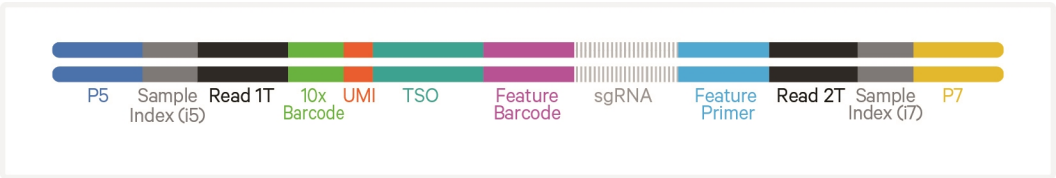
Chromium Single Cell 5' Gene Expression Library



Chromium Single Cell 5' CRISPR Screening Libraries

Chromium Single Cell 5' CRISPR Screening 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 CRISPR Screening libraries.

Chromium Single Cell 5' CRISPR Screening Library



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

Generating and sequencing a 5' Gene Expression library along with the V(D)J library is highly recommended as it greatly enhances cell calling when run

with "cellranger multi" command and facilitates downstream troubleshooting during data analysis. If data from GEX library is not required, shallow sequencing (5,000 read pairs/cell) can be used for GEX library as opposed to the typical sequencing recommendation (20,000 read pairs/cell). The minimum GEX sequencing depth needed for accurate cell calling may vary based on the sample type and data quality. V(D)J only analysis is enabled but not officially supported in the software.

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

** 5' CRISPR libraries have not been tested on this sequencer.*

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 36).

Library Sequencing Depth & Run Parameters

Parameter	Description
Sequencing Depth	Minimum 5,000 read pairs/cell for V(D)J library Minimum 20,000 read pairs/cell for 5' Gene Expression library Minimum 5,000 read pairs/cell for 5' CRISPR Screening library
Sequencing Type	Paired-end, dual indexing
Sequencing Read	Recommended Number of Cycles
Read 1 i7 Index i5 Index Read 2	28 cycles 10 cycles 10 cycles 90 cycles

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.

V(D)J, 5' Gene Expression, and CRISPR Screening libraries

GEM-X

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

DO NOT sequence CRISPR Screening libraries alone.

Libraries	Sequencing Depth (read pairs per cell)	Library Pooling Ratio
V(D)J library	5,000	1
5' Gene Expression library	20,000	4
5' CRISPR Screening 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.

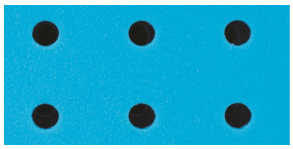
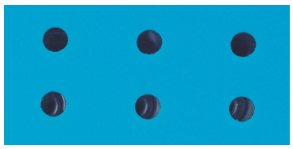
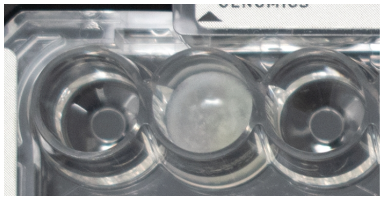
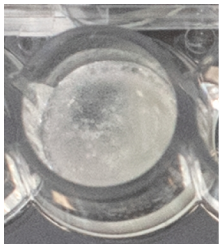
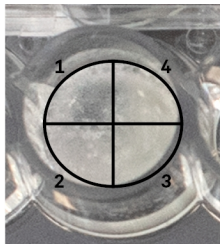
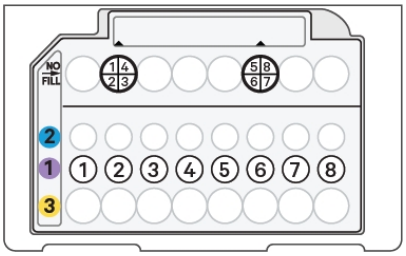
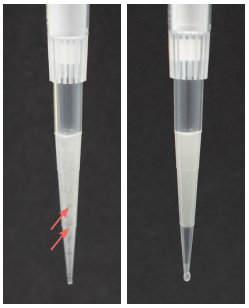
- Input: .cloupe, .vloupe
- Output: Data visualization, including t-SNE and UMAP projections, custom clusters, differentially expressed genes
- Operating System: MacOS, Windows

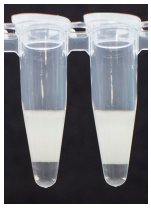
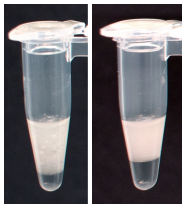
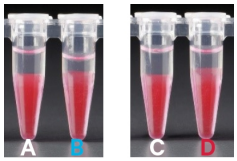
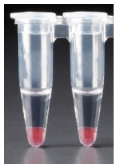
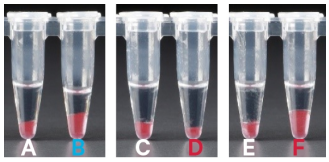
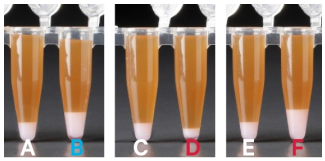
Troubleshooting

GEM Generation & Barcoding 107

Chromium X Series Errors 110

GEM Generation & Barcoding

STEP	Normal	Impacted
1.2 **Load Chromium GEM-X Chip**	 Gasket holes are aligned with the sample and gel bead wells.	 Gasket holes are misaligned with the gel bead wells. Open and close the chip holder slowly once. *One of the gasket rows (second from top) will not align with the GEM-X chip. This is normal & will not impact the assay.*
1.4d **After Chip is removed from the instrument and the wells are exposed**	 Recovery well shows consistent volume and opacity. **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.	   *The quadrant numbers correlate with the sample well numbers.* 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.
1.4e **Transfer GEMs from Chip**	 Consistent volume, opacity, no air trapped. Emulsion density (oil settling) differences seen only if recovery is delayed; likely no impact on assay.	 First pipette tip shows wetting failure. Second pipette tip shows a clog.

STEP	Normal	Impacted
2.1a **After transfer of the GEMs to tubes**	 GEMs transferred immediately to the tubes. Over time some settling is normal.	 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.
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**	 All liquid levels are similar in the aqueous layer (clear) and Recovery Agent/ Partitioning Oil B (pink).	 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).
2.1c **After aspiration of Recovery Agent/ Partitioning Oil**	 All liquid volumes are similar in the aqueous layer (clear) and residual Recovery Agent/ Partitioning Oil B (pink).	 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).
2.1d **After addition of Dynabeads Cleanup Mix**	 All liquid volumes are similar after addition of the Dynabeads Cleanup Mix.	 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.

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

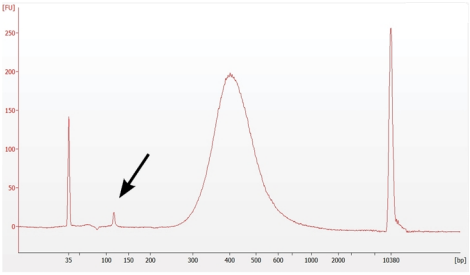
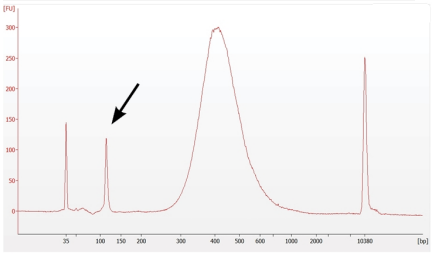
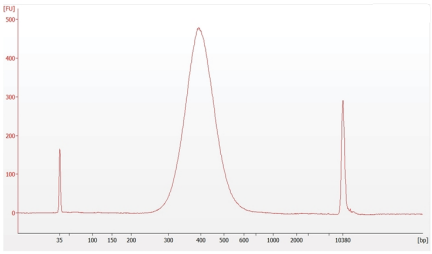
If a channel clog or wetting failure is documented in one or more samples in a set:

- 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
5.7 Post Library Construction QC (Gene Expression)	 Minor peaks below 200 bp may be observed.	**Libraries before repeating step 5.6**  **Libraries after repeating step 5.6**  If peaks below 200 bp are more prominent, repeat step 5.6 Post Sample Index PCR Double Sided Size Selection - SPRIselect.

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

GEM-X OCM Chip Loading Overview 112

Post Library Construction Quantification using KAPA qPCR 113

Library Traces 114

Oligonucleotide Sequences 117

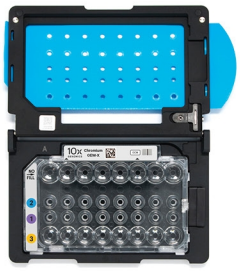
GEM-X OCM Chip Loading Overview

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 45 for details.

Steps

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

Representative Images



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 µl to row labeled 1
   - Load 18 µl to row labeled 2
   - Load 70 µl to row labeled 3

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

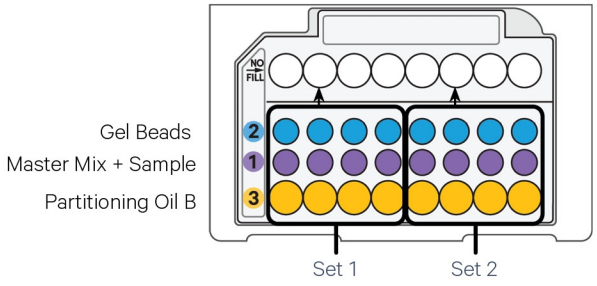
c. **Prepare GEM Master Mix + Sample**

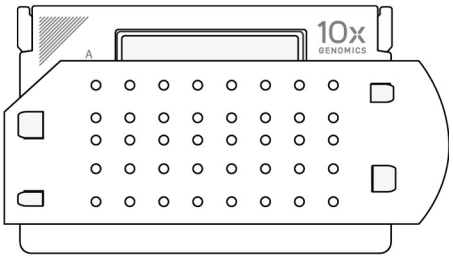
d. **Load Row Labeled 1**
   - Mix GEM Master Mix + Sample
   - Load 15 µl to row labeled 1
   - If running less than 4 samples in a multiplexing set, load 15 µl mock sample
   - Wait 30 sec

e. **Load Row Labeled 2**
   - Aspirate Gel Beads (blue in first tip)
   - Load 18 µl to row labeled 2
   - Wait 30 sec

f. **Load Row Labeled 3**
   - Load 70 µl Partitioning Oil B to row labeled 3.

g. **Close the lid and prepare for run.**





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)	

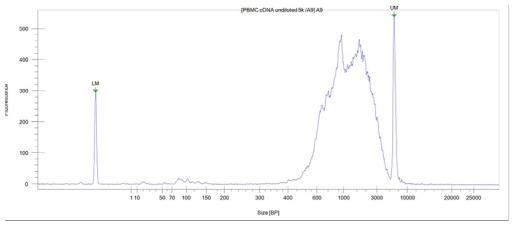
g. Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on insert size derived from the Bioanalyzer/TapeStation trace.

Library Traces

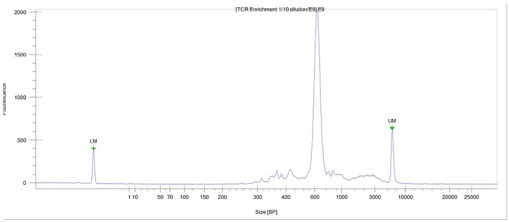
LabChip Traces

DNA High Sensitivity Reagent Kit was used.

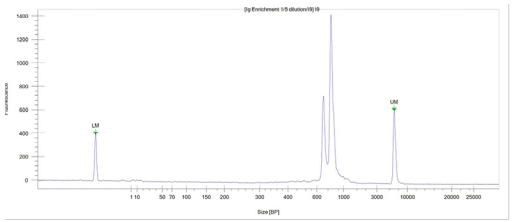
Post cDNA Amplification QC & Quantification



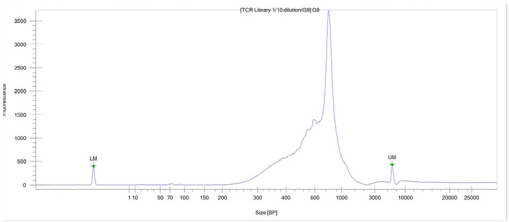
Post TCR Amplification QC



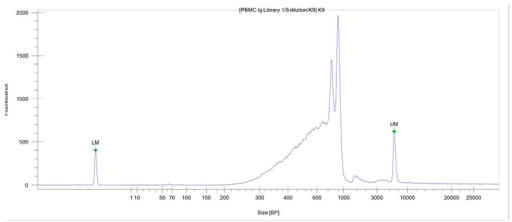
Post BCR Amplification QC



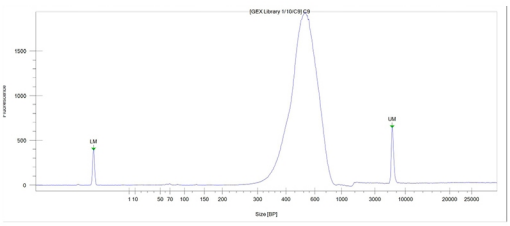
Post Library Construction QC (PBMCs amplified for TCR)



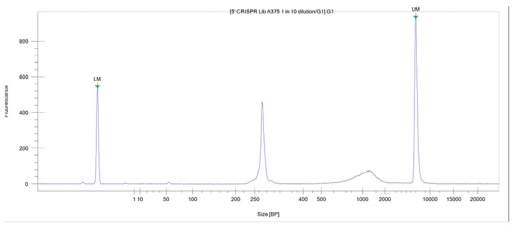
Post Library Construction QC (PBMCs amplified for BCR)



GEX- Post Library Construction QC

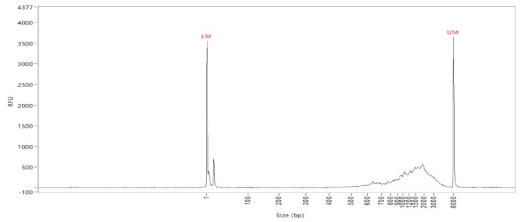


CRISPR Screening - Post Library Construction QC

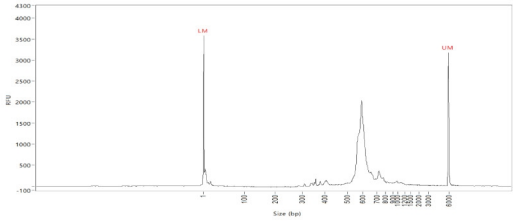


Fragment Analyzer Traces

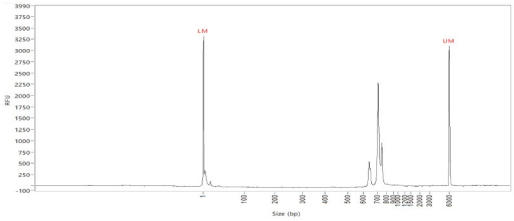
Post cDNA Amplification QC & Quantification



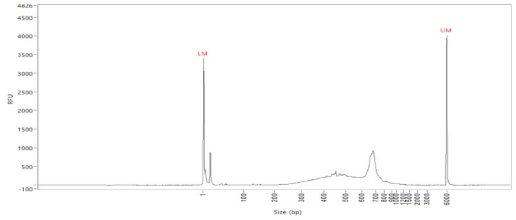
Post TCR Amplification QC



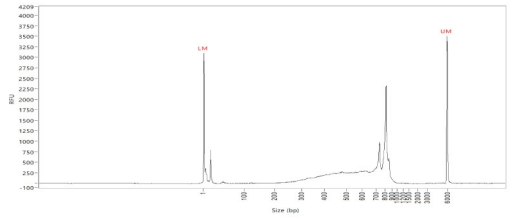
Post BCR Amplification QC



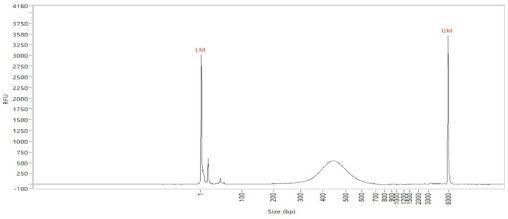
Post Library Construction QC (PBMCs amplified for TCR)



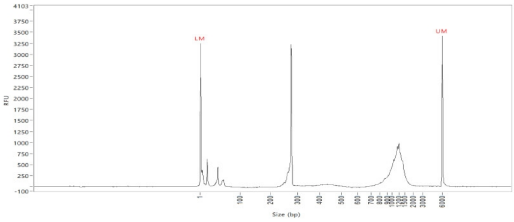
Post Library Construction QC (PBMCs amplified for BCR)



GEX- Post Library Construction QC

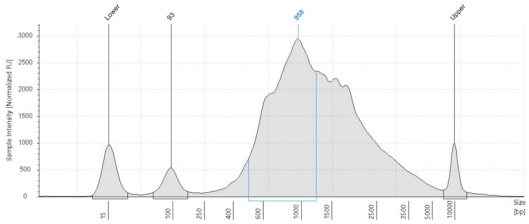


CRISPR Screening - Post Library Construction QC

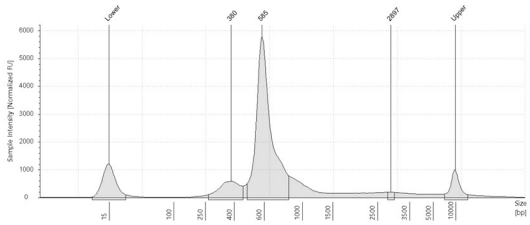


TapeStation Traces

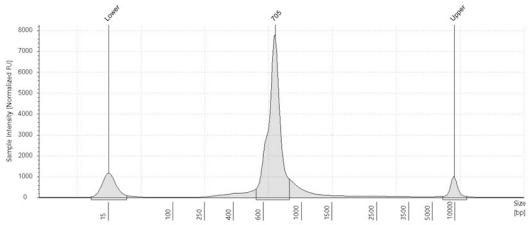
Post cDNA Amplification QC & Quantification



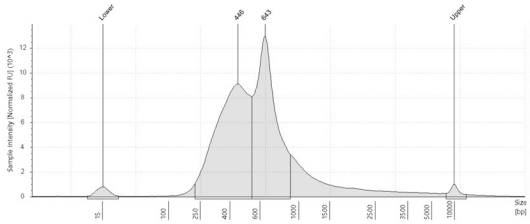
Post TCR Amplification QC



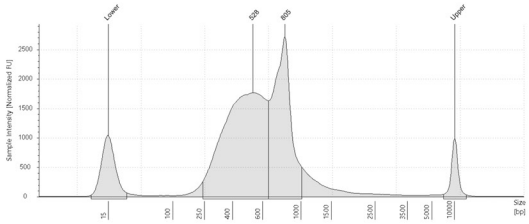
Post BCR Amplification QC



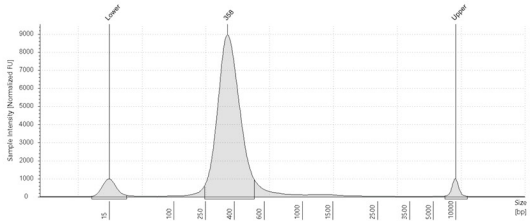
Post Library Construction QC (PBMCs amplified for TCR)



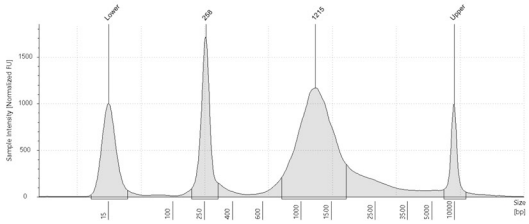
Post Library Construction QC (PBMCs amplified for BCR)



GEX- Post Library Construction QC



CRISPR Screening - Post Library Construction QC



Oligonucleotide Sequences

Gel Bead Primers

5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTTCTTATATrGrGrG-3'

Gene Expression Library

Sample Index PCR Product

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N12-TTTCTTATATGGG-cDNA_Insert-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'

3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12-AAAGAATATACCC-cDNA_Insert-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'

V(D)J Library

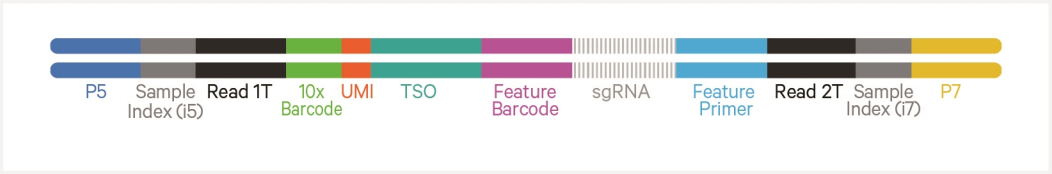
Sample Index PCR Product

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N12-TTTCTTATATGGG-Insert-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'

3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12-AAAGAATATACCC-Insert-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'

CRISPR Screening Library

Sample Index PCR Product



5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT- N16-N12-TTTCTTATATGGG-FeatureBarcode-SgRNA-AATAAGGCTAGTCCGTTATCAACTTG-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'

3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12-AAAGAATATACCC-FeatureBarcode- SgRNA-TTATTCCGATCAGGCAATAGTTGAAC-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-N10--TAGAGCATACGGCAGAAGACGAAC-5'

Additional Sequences

Protocol Step 3.1 – V(D)J Amplification 1			
Human T Cell Mix 1 v2 PN-2000242	Forward Primer: PCR Primer 5'-GATCTACACTCTTTCCCTACACGACGC-3'	Reverse Outer Primers: 5'-TGAAGGCGTTTGCACATGCA-3' 5'-TCAGGCAGTATCTGGAGTCATTGAG-3	Outer Primer
Human B Cell Mix 1 v2 PN-2000254	Forward Primer: PCR Primer 5'-GATCTACACTCTTTCCCTACACGACGC-3'	Reverse Outer Primers: 5'-CAGGGCACAGTCACATCCT-3' 5'-TGCTGGACCACGCATTTGTA-3' 5'-GGTTTTGTTGTCGACCCAGTCT-3' 5'-TTGTCCACCTTGGTGTTGCT-3' 5'-CATGACGTCCTTGGAAGGCA-3' 5'-TGTGGGACTTCCACTG-3' 5'-TTCTCGTAGTCTGCTTTGCTCAG-3'	Outer Primer
Mouse T Cell Mix 1 v2 PN-2000256	Forward Primer: PCR Primer 5'-GATCTACACTCTTTCCCTACACGACGC-3'	Reverse Outer Primers: 5'-CTGGTTGCTCCAGGCAATGG-3' 5'-TGTAGGCCTGAGGGTCCGT-3'	Outer Primer
Mouse B Cell Mix 1 v2 PN-2000258	Forward Primer: PCR Primer 5'-GATCTACACTCTTTCCCTACACGACGC-3'	Reverse Outer Primers: 5'-TCAGCACGGGACAAACTCTTCT-3' 5'-GCAGGAGACAGACTCTTCTCCA-3' 5'-AACTGGCTGCTCATGGTGT-3' 5'-TGGTGCAAGTGTGGTTGAGGT-3' 5'-TGGTCACTTGGCTGGTGGTG-3' 5'-CACTTGGCAGGTGAACTGTTTTCT-3' 5'-AACCTTCAAGGATGCTCTTGGGA-3' 5'-GGACAGGGATCCAGAGTTCCA-3' 5'-AGGTGACGGTCTGACTTGGC-3' 5'-GCTGGACAGGGCTCCATAGTT-3' 5'-GGCACCTTGTCCAATCATGTTCC-3' 5'-ATGTCGTTCATACTCGTCCTTGGT-3'	Outer Primer
Protocol Step 3.3 – V(D)J Amplification 2			
Human T Cell Mix 2 v2 PN-2000246	Forward Primer: PCR Primer 5'-GATCTACACTCTTTCCCTACACGACGC-3'	Reverse Inner Primers: 5'-AGTCTCTCAGCTGGTACACG-3' 5'-TCTGATGGCTCAAACACAGC-3'	Inner Primer
Human B Cell Mix 2 v2 PN-2000255	Forward Primer: PCR Primer 5'-GATCTACACTCTTTCCCTACACGACGC-3'	Reverse Inner Primers: 5'-GGGAAGTTTCTGGCGGTCA-3' 5'-GGTGGTACCCAGTTATCAAGCAT-3' 5'-GTGTCCCAGGTCACCATCAC-3' 5'-TCCTGAGGACTGTAGGACAGC-3' 5'-CACGCTGCTCGTATCCGA-3' 5'-TAGCTGCTGGCCGC-3' 5'-GCGTTATCCACCTTCCACTGT-3'	Inner Primer
Mouse T Cell Mix 2 v2 PN-2000257	Forward Primer: PCR Primer 5'-GATCTACACTCTTTCCCTACACGACGC-3'	Reverse Inner Primers: 5'-AGTCAAAGTCGGTGAACAGGCA-3' 5'-GGCCAAGCACACGAGGGTA-3'	Inner Primer
Mouse B Cell Mix 2 v2 PN-2000259	Forward Primer: PCR Primer 5'-GATCTACACTCTTTCCCTACACGACGC-3'	Reverse Inner Primers: 5'-TACACACCAGTGTGGCCTT-3' 5'-CAGGCCACTGTCACACCACT-3' 5'-CAGGTCACATTCATCGTGCCG-3' 5'-GAGGCCAGCACAGTGACCT-3' 5'-GCAGGGAAGTTCACAGTGCT-3' 5'-CTGTTTGAGATCAGTTTGCCATCCT-3' 5'-TGCGAGGTGGCTAGGTACTTG-3' 5'-CCCTTGACCAGGCATCC-3' 5'-AGGTCACGGAGGAACCAGTTG-3' 5'-GGCATCCCAGTGTCACCGA-3' 5'-AGAAGATCCACTTCACCTTGAAC-3' 5'-GAAGCACACGACTGAGGCAC-3'	Inner Primer

Step 2.2 cDNA Amplification		
CRISPR Poly-dT RT Primer Mix B PN-2001145	 	5'-AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3' 5'-AAGCAGTGGTATCAACGCAGAGTACCAAGTTGATAACGGACTAGCC-3'
Feature SI Primers 4 PN-2000592	Forward primer: 5'-GATCTACACTCTTTCCCTACACGACGC-3'	CRISPR Enrichment Primer:  5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAAGTTGATAACGGACTAGCCTTATT-3'