10x GENOMICS

User Guide | CG000735 | Rev A

Chromium GEM-X Single Cell 5' Reagent Kits v3

with Feature Barcode technology for CRISPR Screening

For use with:

Chromium GEM-X Single Cell 5' Kit v3
16 rxns PN-1000699 | 4 rxns PN-1000695

Library Construction Kit C
16 rxns PN-1000694

Chromium GEM-X Single Cell 5' Chip Kit
4 chips PN-1000698

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*

Notices

Document Number

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Document Revision Summary

Document Number

CG000735 | Rev A

Title

Chromium GEM-X Single Cell 5' Reagent Kits v3 with Feature Barcode technology for CRISPR Screening

Revision

N/A to Rev A

Revision Date

March 07, 2024

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Introduction

Chromium GEM-X Single Cell 5' Reagent Kits v3

Refer to SDS for handling and disposal information

Chromium GEM-X Single Cell 5' Kit v3, 16 rxns PN-1000699

Chromium GEM-X
Single Cell 5' GEM Kit v3
16 rxns, PN-1000701
Store at -20°C

	#	PN
RT Reagent E	1	2001106
RT Enzyme E	1	2001105
Poly-dT RT Primer B	1	2001110
Reducing Agent B	1	2000087
Cleanup Buffer	2	2000088
cDNA Primers	1	2000089
Amp Mix	1	2000047

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Library Construction Kit C
16 rxns, PN-1000694
Store at -20°C

	#	PN
Fragmentation Enzyme	1	2000090
Fragmentation Buffer	1	2000091
Ligation Mix	1	2001109
DNA Ligase	1	220110
Library Amp Mix	1	2000531

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Chromium GEM-X
Single Cell 5' Gel Bead Kit v3
16 rxns, PN-1000700
Store at -80°C

	#	PN
GEM-X Single Cell 5' Gel Bead v3 (16 rxns)	2	2001129

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

	#	PN
Dynabeads MyOne SILANE	1	2000048

Chromium GEM-X Single Cell 5' Kit v3, 4 rxns PN-1000695

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

	#	PN
Fragmentation Enzyme	1	2000104
Fragmentation Buffer	1	2000091
Ligation Mix	1	2001109
DNA Ligase	1	220131

10x GENOMICS

Chromium GEM-X
Single Cell 5' Gel Bead Kit v3
4 rxns, PN-1000696
Store at -80°C

	#	PN
GEM-X Single Cell 5' Gel Bead v3 (4 rxns)	1	2001129

10x GENOMICS

Dynabeads™ MyOne™ SILANE
PN-2000048
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

Chromium GEM-X Single Cell 5' Chip Kit v3, 4 chips PN-1000698

Chromium
Partitioning Oil B
Store at ambient temperature

		#	PN
	Partitioning Oil B	4	2001213

Chromium
Recovery Agent
Store at ambient temperature

		#	PN
	Recovery Agent	4	220016

Chromium GEM-X
5' Chip & Gaskets
Store at ambient temperature

	#	PN
GEM-X 5' Chip	4	2001098
Chip Gasket X/iX, 2-pack	2	3000656

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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
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

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

Dual Index Kit TT Set A
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
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

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Chromium
Single Cell Human BCR Amplification
16 rxns, PN-1000253
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

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

Chromium
Single Cell Mouse TCR Amplification
16 rxns, PN-1000254
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

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Chromium
Single Cell Mouse BCR Amplification
16 rxns, PN-1000255
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

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10x Genomics Accessories

Product	Part Number (Kit)	Part Number (Item)
10x Vortex Adapter	120251	330002
10x Magnetic Separator B*	1000709 (Chromium X/iX Accessory Kit)/ 1000707 (GEM-X Transition Kit)	2001212
Chromium X/iX Chip Holder		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 Chromium GEM-X Single Cell Gene Expression v4 & Immune Profiling v3 - Protocol Planner (CG000748) for a detailed list of the following third-party items:

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

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

Protocol Steps & Timing

Steps	Timing	Stop & Store
Sample Preparation	variable*	
	*Refer to the appropriate Demonstrated Protocols for details.	
Step 1: GEM Generation and Barcoding (page 34)		
1.1 Prepare Master Mix (page 36)	20 min	
1.2 Load GEM-X Chip (page 41)	10 min	
1.3 Run the Chromium X/iX (page 43)	6 min	
1.4 Transfer GEMs (page 44)	3 min	
1.5 GEM-RT Incubation (page 45)	55 min	STOP 4°C ≤72 h/-20°C ≤1 week
Step 2: Post GEM-RT Cleanup & cDNA Amplification (page 46)		
2.1 Post GEM-RT Cleanup – Dynabeads (page 49)	45 min	
2.2 cDNA Amplification (page 53)	40 min	STOP 4°C ≤72 h/-20°C ≤1 week
2.3 cDNA Cleanup –SPRIselect (page 55) 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 58)	50 min	
Step 3: V(D)J Amplification from cDNA (page 60)		
3.1 V(D)J Amplification 1 (page 63)	50 min	
3.2 Post V(D)J Amplification 1 Cleanup Double Sided – SPRIselect (page 64)	30 min	
3.3 V(D)J Amplification 2 (page 65)	25 min	
3.4 Post V(D)J Amplification 2 Cleanup Double Sided – SPRIselect (page 66)	30 min	
3.5 Post V(D)J Amplification QC & Quantification (page 67)	40 min	STOP 4°C ≤72 h
Step 4: V(D)J Library Construction (page 70)		
4.1 Fragmentation, End Repair & A-tailing (page 73)	50 min	
4.2 Adaptor Ligation (page 74)	30 min	
4.3 Post Ligation Cleanup – SPRIselect (page 74)	25 min	
4.4 Sample Index PCR (page 76)	40 min	STOP 4°C ≤72 h

Steps	Timing	Stop & Store
4.5 Post Sample Index PCR Cleanup – SPRIselect (page 77)	30 min	STOP 4°C ≤72 h/-20°C long term
4.6 Post Library Construction QC (page 78)	50 min	
Step 5: 5' Gene Expression Library Construction (page 79)		
5.1 GEX Fragmentation, End Repair & A-tailing (page 84)	50 min	
5.2 GEX Post Fragmentation, End Repair & A-tailing Double Sided – SPRIselect (page 85)	30 min	
5.3 GEX Adaptor Ligation (page 86)	25 min	
5.4 GEX Post Ligation Cleanup – SPRIselect (page 86)	30 min	
5.5 GEX Sample Index PCR (page 88)	40 min	STOP 4°C ≤72 h
5.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect (page 89)	30 min	STOP 4°C ≤72 h/-20°C long term
5.7 Post Library Construction QC (page 90)	50 min	
Step 6: CRISPR Screening Library Construction (page 91)		
6.1 Guide RNA cDNA Cleanup – SPRIselect (page 93)	40 min	
6.2 Feature PCR (page 93)	40 min	
6.3 Post Feature PCR Cleanup – SPRIselect (page 94)	40 min	
6.4 Sample Index PCR (page 95)	40 min	
6.5 Post Sample Index PCR Double Sided Size Selection – SPRIselect (page 96)	20 min	STOP 4°C ≤72 h/-20°C long term
6.6 Post Library Construction QC & Quantification (page 97)	50 min	

Stepwise Objectives

The Chromium GEM-X Single Cell Immune Profiling v3 with Feature Barcode technology offers comprehensive, scalable solutions to detect CRISPR-mediated perturbations along with the gene expression and immune repertoire information from the same single cell. 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 500-20,000 individual cells per sample can be assessed.

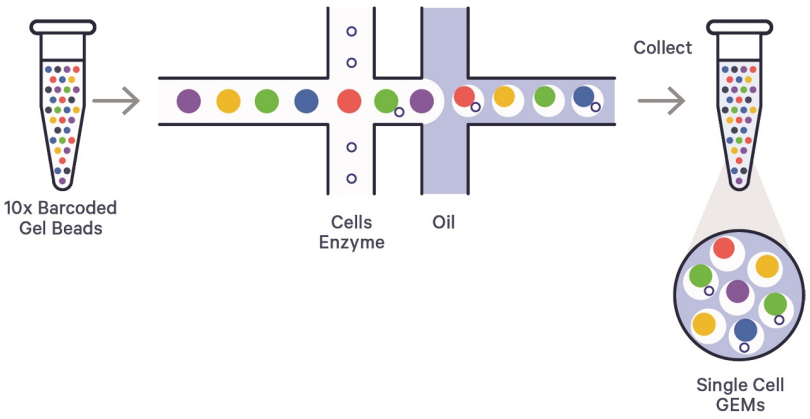
A pool of ~3,600,000 barcodes are sampled separately to index each cell's transcriptome along with CRISPR-mediated perturbations. This is done by partitioning thousands of cells into nanoliter-scale Gel Beads-in-emulsion (GEMs), where all generated cDNA/DNA (from single-guide RNAs/sgRNAs) share a common 10x Barcode. Libraries are generated and sequenced and 10x Barcodes are used to associate individual reads back to the individual partitions.

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

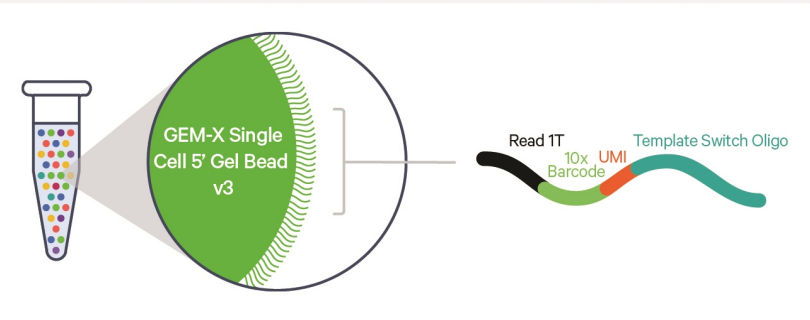
Step 1: GEM Generation & Barcoding

GEMs are generated by combining barcoded GEM-X Single Cell 5' Gel Bead v3, a Master Mix with cells and Partitioning Oil B onto GEM-X 5' Chip. To achieve single cell resolution, cells are delivered at a limiting dilution, such that the majority (~90-99%) of generated GEMs contain no cell, while the remainders largely contain a single cell.



Immediately following GEM generation, 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).

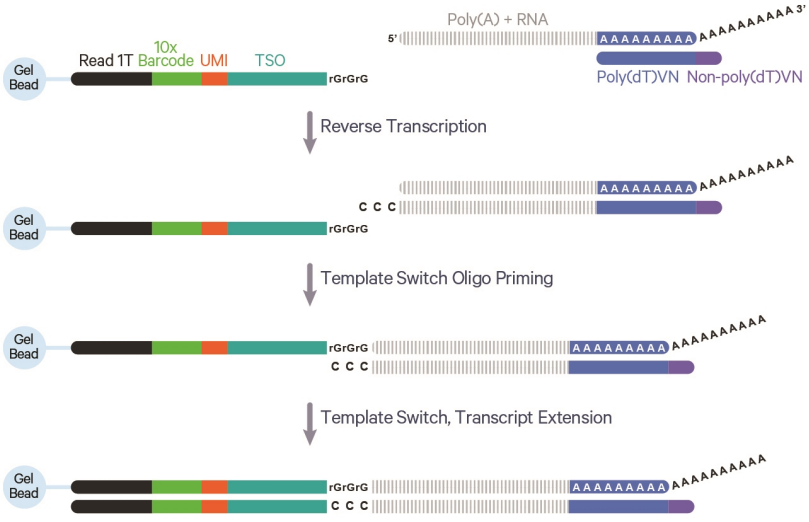
Gel Bead



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 taget gene/s of interest (B).

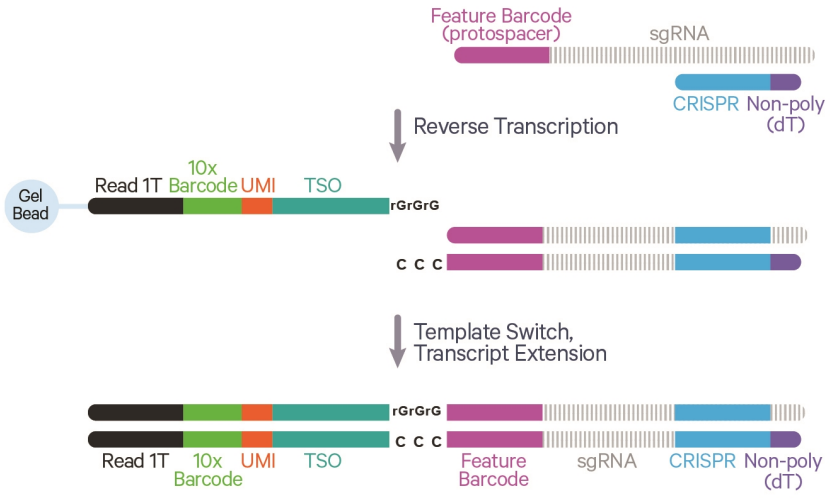
Inside Individual GEMs

A. cDNA from Poly-adenylated mRNA



cDNA from poly-adenylated mRNA

B. cDNA from the sgRNA protospacer (Feature Barcode)

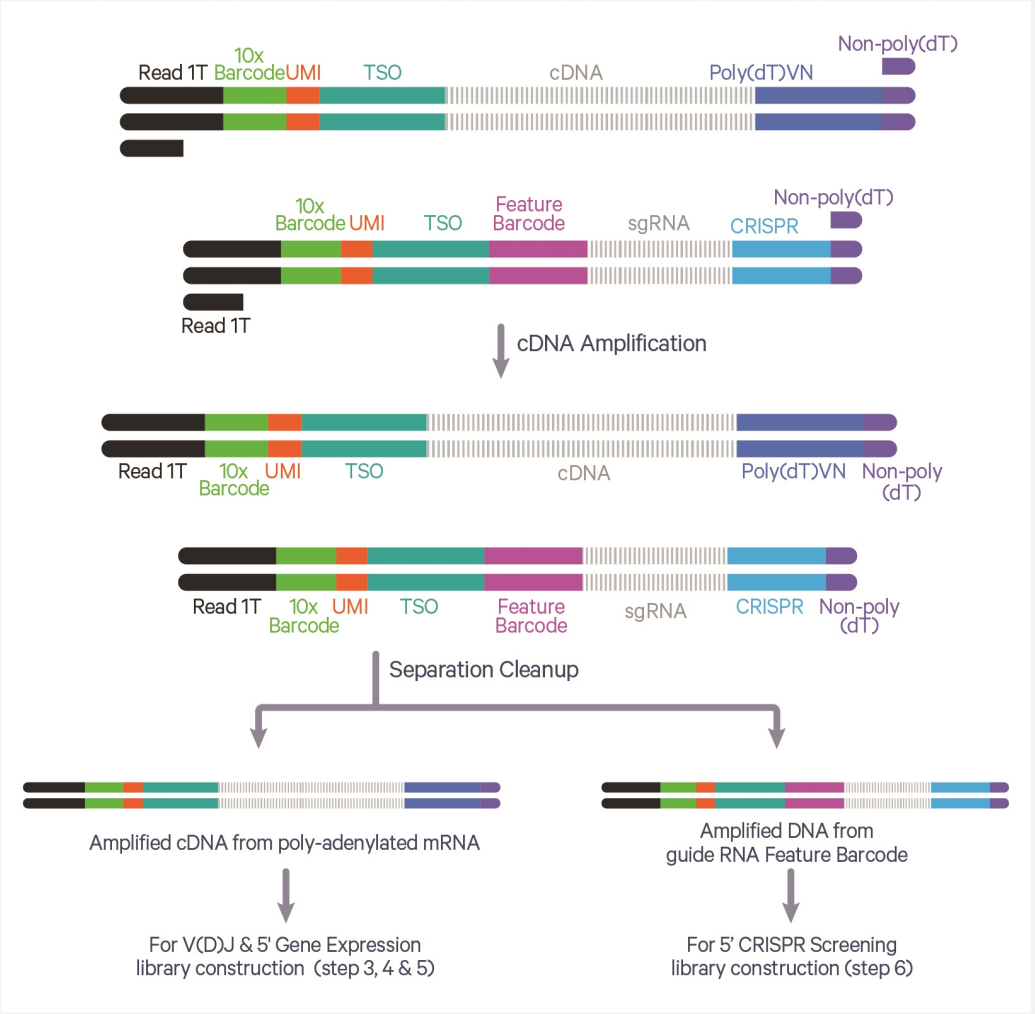


cDNA from the sgRNA protospacer (Feature Barcode)

Step 2: Post GEM-RT Cleanup & cDNA Amplification

GEMs are broken and pooled after the GEM-RT reaction mixtures are recovered. 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.



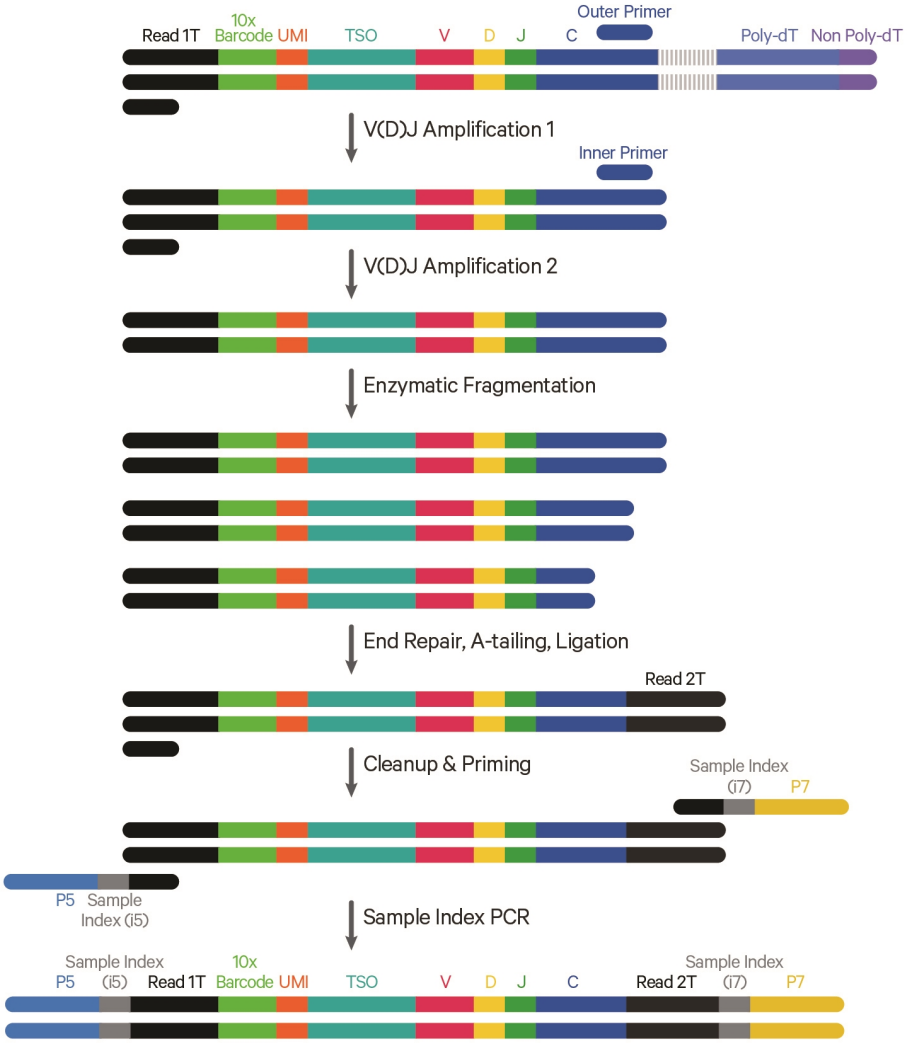
Step 3: V(D)J Amplification from cDNA

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

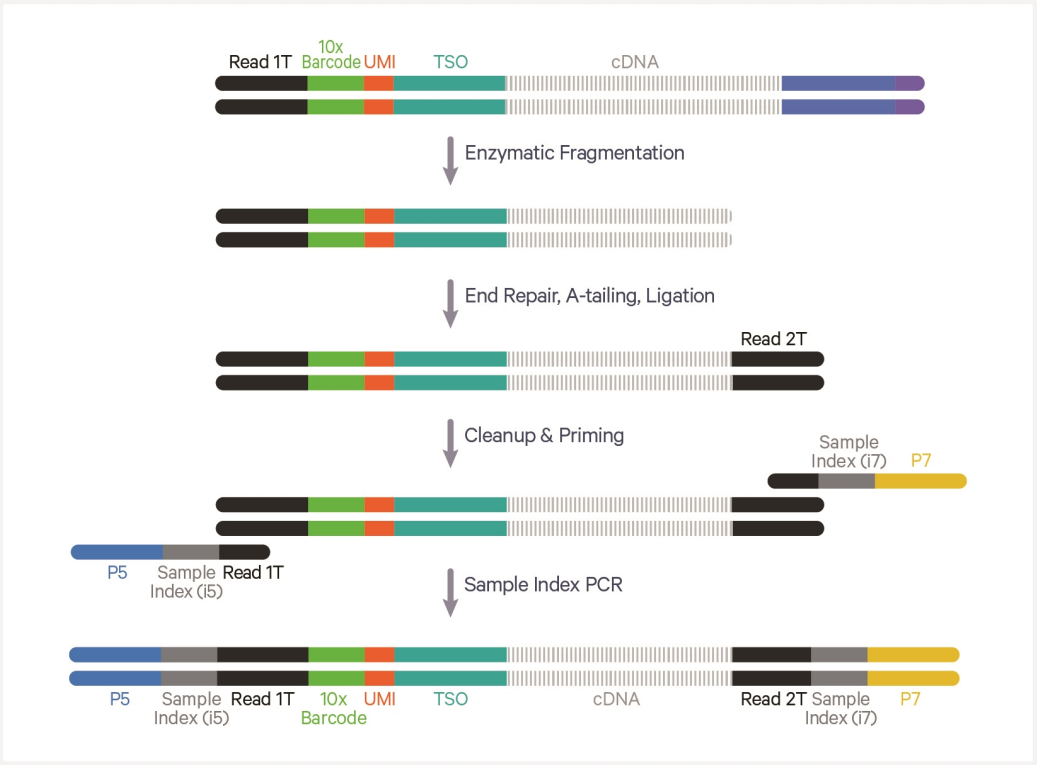


Step 5: 5' Gene Expression (GEX) Library Construction

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.

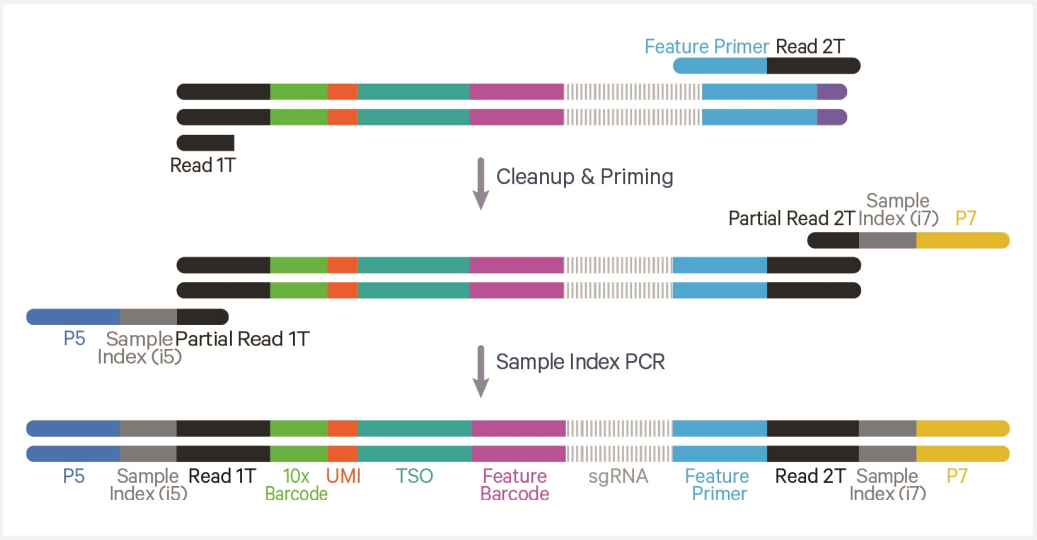
Pooled Amplified cDNA Processed in Bulk



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)

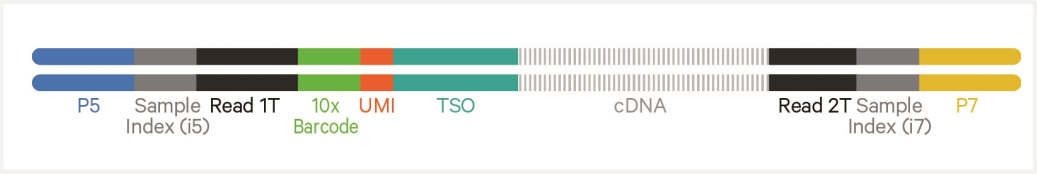


Step 7: Sequencing

Illumina-ready dual index libraries can be sequenced at the recommended depth & run parameters. Illumina 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

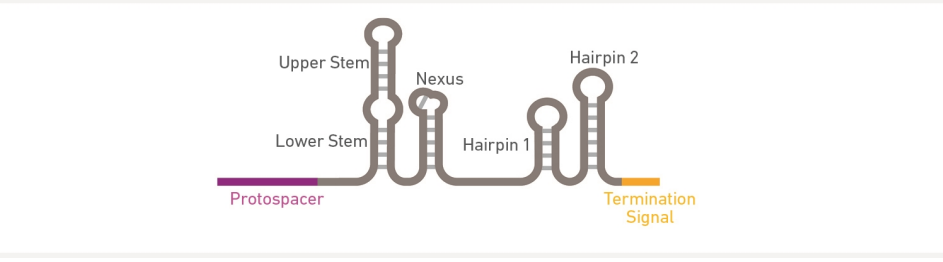
See Oligonucleotide Sequences on page 113

CRISPR Screening Overview

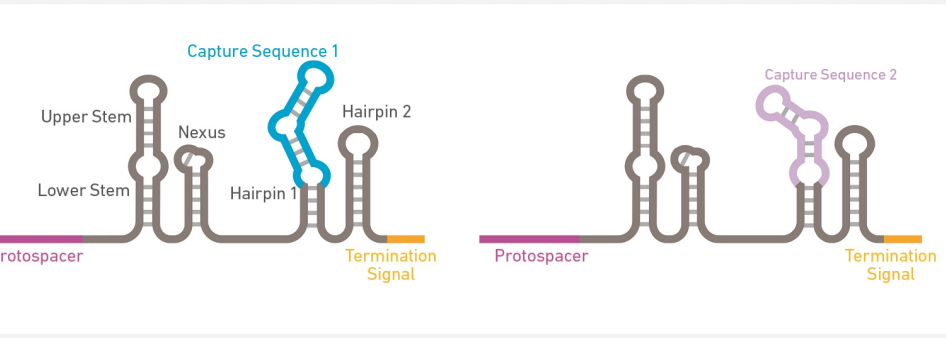
The Chromium Single Cell Immune Profiling Solution with Feature Barcode technology provides a high-throughput and scalable approach to obtain gene expression profiles along with perturbation phenotypes via direct capture of poly-adentylated mRNAs and single-guide RNAs (sgRNAs) from the same single cell (see Stepwise Objectives on page 15). 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.

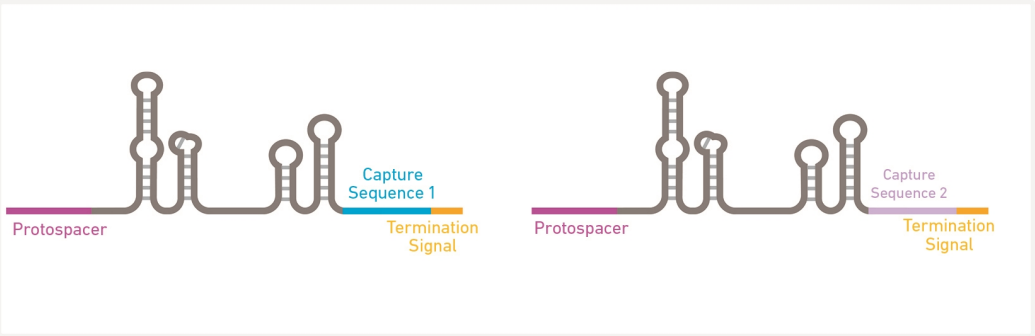
sgRNA compatible with Single Cell 5' CRISPR Assay

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.

Tips & Best Practices

Icons

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

GEM-X specific protocol step updates

Version specific protocol step updates

Emulsion-safe Plastics

- Use validated emulsion-safe plastic consumables when handling GEMs as some plastics can destabilize GEMs.
- Consult Chromium GEM-X Single Cell Gene Expression v3 & Immune Profiling v4 - 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	500-10,000 cells
1,300-1,600 cells/ µl	10,000-20,000 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 (%)	# of Cells Loaded	# of Cells Recovered
~0.20%	~725	~500
~0.40%	~1,450	~1,000
~0.80%	~2,900	~2,000
~1.20%	~4,350	~3,000

Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~1.60%	~5,800	~4,000
~2.00%	~7,250	~5,000
~2.40%	~8,700	~6,000
~2.80%	~10,150	~7,000
~3.20%	~11,600	~8,000
~3.60%	~13,050	~9,000
~4.00%	~14,500	~10,000
~4.40%	~15,950	~11,000
~4.80%	~17,400	~12,000
~5.20%	~18,850	~13,000
~5.60%	~20,300	~14,000
~6.00%	~21,750	~15,000
~6.40%	~23,200	~16,000
~6.80%	~24,650	~17,000
~7.20%	~26,100	~18,000
~7.60%	~27,550	~19,000
~8.00%	~29,000	~20,000

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

- 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.

Chromium X/iX Chip Holder

- Chromium X/iX Chip Holders encase GEM-X 5' Chips.
- The holder lid flips over to become a stand, holding the chip at 45 degrees for optimal recovery well content removal.
- Squeeze the slider on the back (right side) of the holder toward the middle to unlock the lid and return the holder to a flat position.

GEM-X Chip Handling

- Minimize exposure of reagents, chips, and gaskets to sources of particles and fibers, laboratory wipes, frequently opened flip-cap tubes, clothing

that sheds fibers, and dusty surfaces.

- After removing the chip from the sealed bag, use in **≤24 h**.
- Execute steps without pause or delay, unless indicated. When using multiple chips, load, run, and collect the content from one chip before loading the next.
- Fill all unused input wells in rows labeled 1, 2, and 3 on a chip with an appropriate volume of 50% glycerol solution before loading the used wells. DO NOT add glycerol to the wells in the top NO FILL row.
- Avoid contacting the bottom surface of the chip with gloved hands and other surfaces. Frictional charging can lead to inadequate priming of the channels, potentially leading to wetting failures.
- Minimize the distance that a loaded chip is moved to reach the Chromium X/iX.
- Keep the chip horizontal to prevent wetting the gasket with oil, which depletes the input volume and may adversely affect the quality of the resulting emulsion.

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' Chip is shown below.

GEM-X Chip Loading

- Place the assembled chip and holder flat (gasket attached) on the bench with the lid open.
- Dispense at the bottom of the wells without introducing bubbles.
- When dispensing Gel Beads into the chip, wait for the remainder to drain into the bottom of the pipette tips and dispense again to ensure complete transfer.
- Refer to Load GEM-X Chip on page 41 for specific instructions.

Chromium X/iX Firmware

- Firmware version 2.0.0 or higher is required in the Chromium X/iX to run the GEM-X chips.
- The current firmware version of the instrument can be located using the system menu.
- Consult the Chromium X Series (X/iX) User Guide (CG000396) for detailed instructions on updating the firmware.

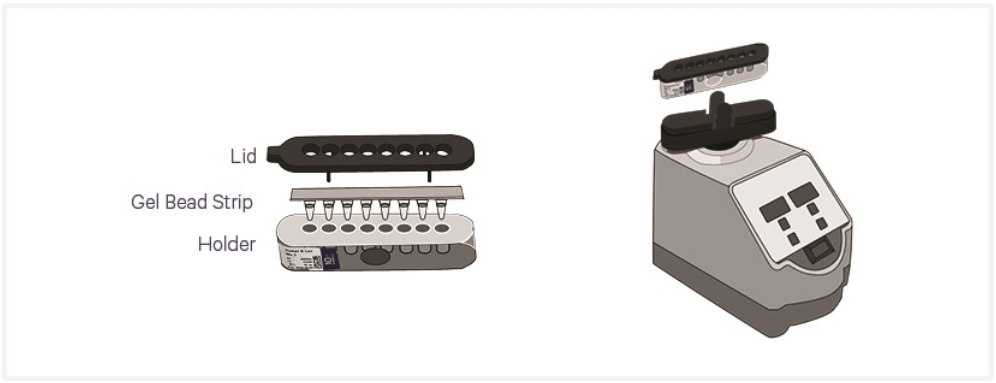
Gel Bead Handling

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

- Store unused Gel Beads at **−80°C** and avoid more than 12 freeze-thaw cycles.

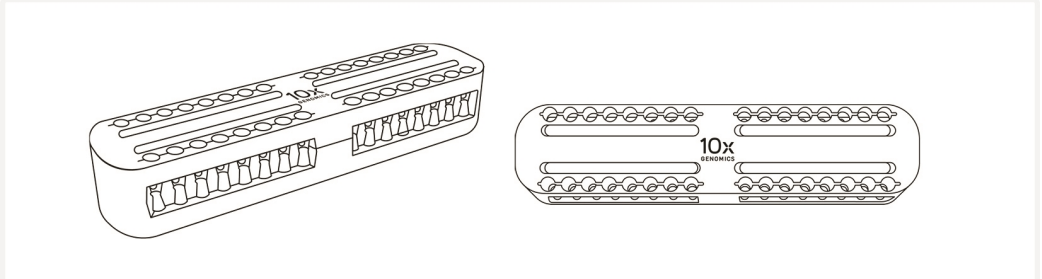
  DO NOT store Gel Beads at **−20°C**.

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



10x Magnetic Separator B

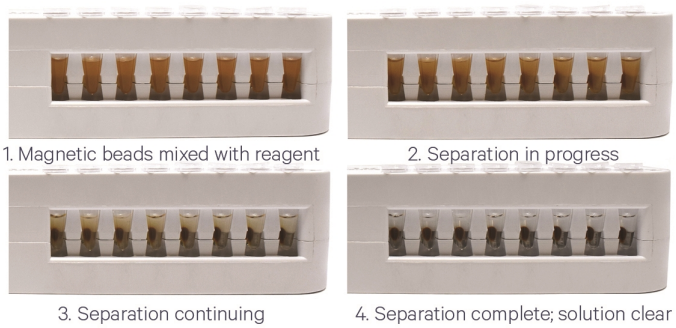
- Images below are illustrative - actual appearance of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.
- Offers two positions of the magnets (high and low) relative to a tube, depending on its orientation. Flip the magnetic separator over to switch between high (magnet•**High**) 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

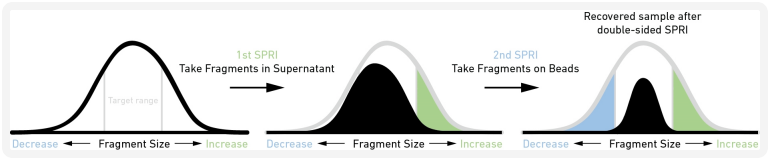
- After aspirating the desired volume of SPRIselect reagent, examine the pipette tips before dispensing to ensure the correct volume is transferred.
- Pipette mix thoroughly as insufficient mixing of sample and SPRIselect reagent will lead to inconsistent results.
- Use fresh preparations of 80% Ethanol.

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**).

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

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

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

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

Firmware Version v2.0.0 or higher is required in the Chromium X/iX used for this protocol.

Item		10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐	**GEM-X Single Cell 5' Gel Bead v3**	2001129	Equilibrate to room temperature **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**	2001105/ 2001146	Centrifuge briefly before adding to the mix.	–20°C
☐	**1X PBS**	—		—
Obtain				
☐	**Partitioning Oil B**	2001213	—	Ambient
☐	**GEM-X 5' Chip** *Verify name and PN*	2001098	See Tips & Best Practices.	Ambient
☐	**X/iX Chip Gasket**	3000656	See Tips & Best Practices.	Ambient
☐	**Chromium X/iX Chip Holder**	3000598	See Tips & Best Practices.	Ambient
☐	**10x Vortex Adapter**	330002	See Tips & Best Practices.	Ambient
☐	**50% glycerol solution** *If using < 8 reactions*	—	See Tips & Best Practices.	—

1.1 Prepare Master Mix

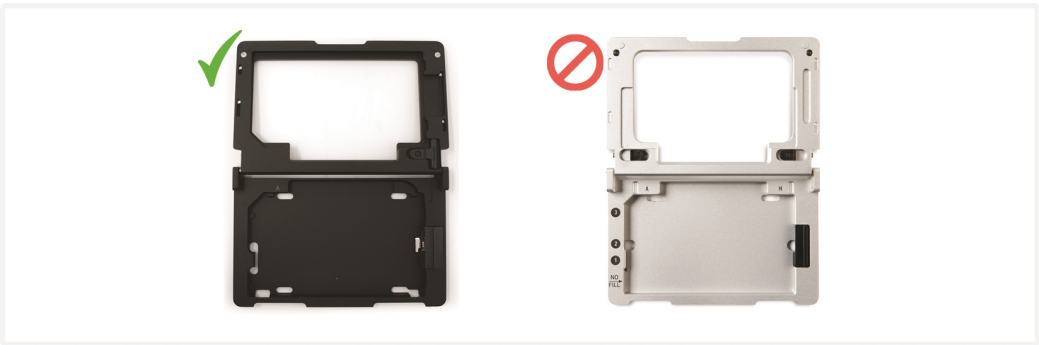
a. Prepare Master Mix on ice. Pipette mix 15x and centrifuge briefly.

Master Mix *Add reagents in the order listed*		PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
●	**RT Reagent E**	2001106	16.3	71.7	143.4
●	**CRISPR Poly-dT Primer Mix B**	2001145	2.2	9.7	19.4
○	**Reducing Agent B**	2000087	2.0	8.8	17.6
●	**RT Enzyme E**	2001105/2001146	7.0	30.8	61.6
	Total	–	**27.5**	**121.0**	**242.0**

b. Add **27.5 µl** Master Mix into each tube of a PCR 8-tube strip on ice.

Assemble GEM-X Chip

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

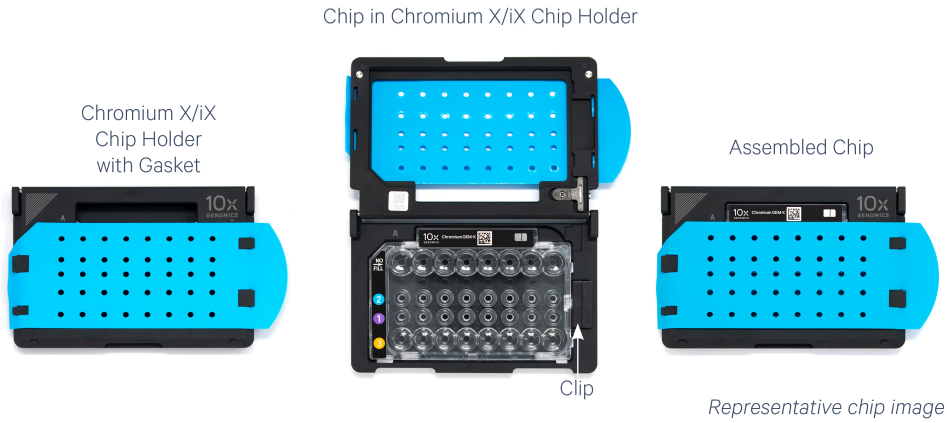


See Tips & Best Practices on page 24 for chip handling instructions.

- 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' Chip is shown on the next page.
- 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.



Representative chip image

Cell Suspension Volume Calculator Table

For step 1.2 of this protocol.

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

Cell Recovery Target – 500-10,000

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	6000	7000	8000	9000	10000
100	7.3	14.5	29.0								
	30.3	23.0	8.5								
200	3.6	7.3	14.5	21.8	29.0	36.3					
	33.9	30.3	23.0	15.8	8.5	1.3					
300	2.4	4.8	9.7	14.5	19.3	24.2	29.0	33.8			
	35.1	32.7	27.8	23.0	18.2	13.3	8.5	3.7			
400	1.8	3.6	7.3	10.9	14.5	18.1	21.8	25.4	29.0	32.6	36.3
	35.7	33.9	30.3	26.6	23.0	19.4	15.8	12.1	8.5	4.9	1.3
500	1.5	2.9	5.8	8.7	11.6	14.5	17.4	20.3	23.2	26.1	29.0
	36.1	34.6	31.7	28.8	25.9	23.0	20.1	17.2	14.3	11.4	8.5
600	1.2	2.4	4.8	7.3	9.7	12.1	14.5	16.9	19.3	21.8	24.2
	36.3	35.1	32.7	30.3	27.8	25.4	23.0	20.6	18.2	15.8	13.3
700	1.0	2.1	4.1	6.2	8.3	10.4	12.4	14.5	16.6	18.6	20.7
	36.5	35.4	33.4	31.3	29.2	27.1	25.1	23.0	20.9	18.9	16.8
800	0.9	1.8	3.6	5.4	7.3	9.1	10.9	12.7	14.5	16.3	18.1
	36.6	35.7	33.9	32.1	30.3	28.4	26.6	24.8	23.0	21.2	19.4
900	0.8	1.6	3.2	4.8	6.4	8.1	9.7	11.3	12.9	14.5	16.1
	36.7	35.9	34.3	32.7	31.1	29.4	27.8	26.2	24.6	23.0	21.4
1000	0.7	1.5	2.9	4.4	5.8	7.3	8.7	10.2	11.6	13.1	14.5
	36.8	36.1	34.6	33.2	31.7	30.3	28.8	27.4	25.9	24.5	23.0
1100	0.7	1.3	2.6	4.0	5.3	6.6	7.9	9.2	10.5	11.9	13.2
	36.8	36.2	34.9	33.5	32.2	30.9	29.6	28.3	27.0	25.6	24.3
1200	0.6	1.2	2.4	3.6	4.8	6.0	7.3	8.5	9.7	10.9	12.1
	36.9	36.3	35.1	33.9	32.7	31.5	30.3	29.0	27.8	26.6	25.4
1300	0.6	1.1	2.2	3.3	4.5	5.6	6.7	7.8	8.9	10.0	11.2
	36.9	36.4	35.3	34.2	33.0	31.9	30.8	29.7	28.6	27.5	26.3
1400	0.5	1.0	2.1	3.1	4.1	5.2	6.2	7.3	8.3	9.3	10.4
	37.0	36.5	35.4	34.4	33.4	32.3	31.3	30.3	29.2	28.2	27.1
1500	0.5	1.0	1.9	2.9	3.9	4.8	5.8	6.8	7.7	8.7	9.7
	37.0	36.5	35.6	34.6	33.6	32.7	31.7	30.7	29.8	28.8	27.8
1600	0.5	0.9	1.8	2.7	3.6	4.5	5.4	6.3	7.3	8.2	9.1
	37.0	36.6	35.7	34.8	33.9	33.0	32.1	31.2	30.3	29.3	28.4
1700	0.4	0.9	1.7	2.6	3.4	4.3	5.1	6.0	6.8	7.7	8.5
	37.1	36.6	35.8	34.9	34.1	33.2	32.4	31.5	30.7	29.8	29.0
1800	0.4	0.8	1.6	2.4	3.2	4.0	4.8	5.6	6.4	7.3	8.1
	37.1	36.7	35.9	35.1	34.3	33.5	32.7	31.9	31.1	30.3	29.4
1900	0.4	0.8	1.5	2.3	3.1	3.8	4.6	5.3	6.1	6.9	7.6
	37.1	36.7	36.0	35.2	34.4	33.7	32.9	32.2	31.4	30.6	29.9
2000	0.4	0.7	1.5	2.2	2.9	3.6	4.4	5.1	5.8	6.5	7.3
	37.1	36.8	36.1	35.3	34.6	33.9	33.2	32.4	31.7	31.0	30.3

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 (500-10,000 cells)

Cell Recovery Target – 10,000-20,000

Volume of Cell Suspension Stock per reaction (µl) | Volume of PBS per reaction (µl)

Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery										
	10000	11000	12000	13000	14000	15000	16000	17000	18000	19000	20000
100											
200											
300											
400	36.3										
	1.3										
500	29.0	31.9	34.8								
	8.5	5.6	2.7								
600	24.2	26.6	29.0	31.4	33.8	36.3					
	13.3	10.9	8.5	6.1	3.7	1.3					
700	20.7	22.8	24.9	26.9	29.0	31.1	33.1	35.2	37.3		
	16.8	14.7	12.6	10.6	8.5	6.4	4.4	2.3	0.2		
800	18.1	19.9	21.8	23.6	25.4	27.2	29.0	30.8	32.6	34.4	36.3
	19.4	17.6	15.8	13.9	12.1	10.3	8.5	6.7	4.9	3.1	1.3
900	16.1	17.7	19.3	20.9	22.6	24.2	25.8	27.4	29.0	30.6	32.2
	21.4	19.8	18.2	16.6	14.9	13.3	11.7	10.1	8.5	6.9	5.3
1000	14.5	16.0	17.4	18.9	20.3	21.8	23.2	24.7	26.1	27.6	29.0
	23.0	21.6	20.1	18.7	17.2	15.8	14.3	12.9	11.4	10.0	8.5
1100	13.2	14.5	15.8	17.1	18.5	19.8	21.1	22.4	23.7	25.0	26.4
	24.3	23.0	21.7	20.4	19.0	17.7	16.4	15.1	13.8	12.5	11.1
1200	12.1	13.3	14.5	15.7	16.9	18.1	19.3	20.5	21.8	23.0	24.2
	25.4	24.2	23.0	21.8	20.6	19.4	18.2	17.0	15.8	14.5	13.3
1300	11.2	12.3	13.4	14.5	15.6	16.7	17.8	19.0	20.1	21.2	22.3
	26.3	25.2	24.1	23.0	21.9	20.8	19.7	18.5	17.4	16.3	15.2
1400	10.4	11.4	12.4	13.5	14.5	15.5	16.6	17.6	18.6	19.7	20.7
	27.1	26.1	25.1	24.0	23.0	22.0	20.9	19.9	18.9	17.8	16.8
1500	9.7	10.6	11.6	12.6	13.5	14.5	15.5	16.4	17.4	18.4	19.3
	27.8	26.9	25.9	24.9	24.0	23.0	22.0	21.1	20.1	19.1	18.2
1600	9.1	10.0	10.9	11.8	12.7	13.6	14.5	15.4	16.3	17.2	18.1
	28.4	27.5	26.6	25.7	24.8	23.9	23.0	22.1	21.2	20.3	19.4
1700	8.5	9.4	10.2	11.1	11.9	12.8	13.6	14.5	15.4	16.2	17.1
	29.0	28.1	27.3	26.4	25.6	24.7	23.9	23.0	22.1	21.3	20.4
1800	8.1	8.9	9.7	10.5	11.3	12.1	12.9	13.7	14.5	15.3	16.1
	29.4	28.6	27.8	27.0	26.2	25.4	24.6	23.8	23.0	22.2	21.4
1900	7.6	8.4	9.2	9.9	10.7	11.4	12.2	13.0	13.7	14.5	15.3
	29.9	29.1	28.3	27.6	26.8	26.1	25.3	24.5	23.8	23.0	22.2
2000	7.3	8.0	8.7	9.4	10.2	10.9	11.6	12.3	13.1	13.8	14.5
	30.3	29.5	28.8	28.1	27.4	26.6	25.9	25.2	24.5	23.7	23.0

Volumes that would exceed the allowable PBS volume in each reaction

Optimal range of cell stock concentration to maximize the likelihood of achieving the desired cell recovery target (10,000-20,000 cells)

1.2 Load GEM-X Chip

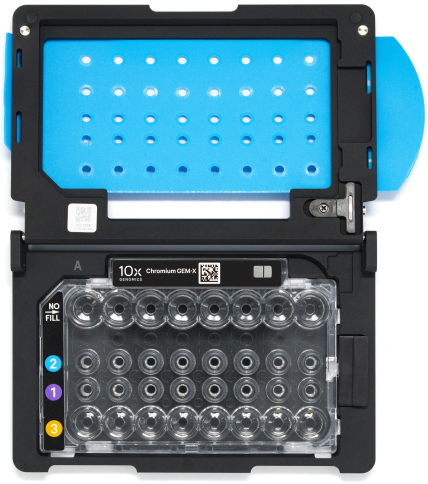
TIPS

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

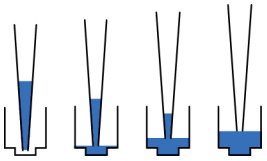
Color Legend
2: Gel beads
1: Sample
3: Oil

The Chromium X/iX Chip Holder, Chip Gasket X/iX, and GEM-X chip images shown below are representative and do not show the specific color & label. Chip holder and gasket should be black and blue in color, respectively. Refer to Assemble GEM-X Chip on page 37 for details.

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



Pipette technique

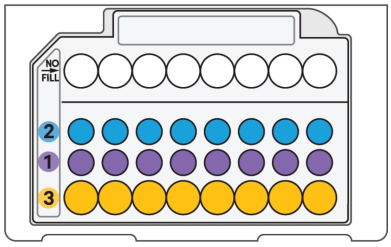


a. If loading less than 8 samples/chip, add 50% glycerol solution to each unused well in row 1, 2, and 3

- **60 µl** in each unused well in row labeled 1
- **60 µl** in each unused well in row labeled 2
- **250 µl** in each unused well in row labeled 3

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

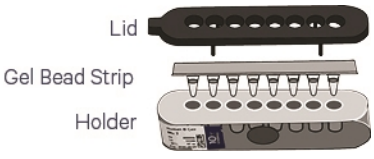
Glycerol
in GEM-X 5' Chip



b. Prepare Gel Beads

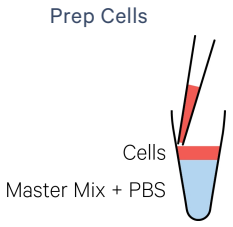
- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**.
- Centrifuge the Gel Bead strip for **~5 sec**.
- Confirm there are no bubbles at the bottom of the tubes & the liquid levels are even.
- Place the Gel Bead strip back in the holder. Secure the holder lid.

Prep Gel Beads



c. Prepare Master Mix + Cell suspension

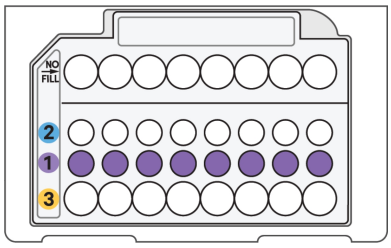
- Refer to the Cell Suspension Volume Calculator Table on page 39.
- Add the appropriate volume of PBS to Master Mix.
- Add corresponding volume of single cell suspension into the Master Mix. *Total of 65 µl in each tube.*
  Gently pipette mix the cell suspension before adding to the Master Mix.



d. Load Row Labeled 1

- Gently pipette mix the Master Mix + Cell Suspension.
- Using the same pipette tip, dispense **60 µl** Master Mix + Cell Suspension into the bottom center of each well in **row labeled 1** without introducing bubbles.
- Wait **30 sec**.

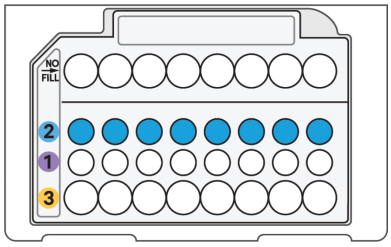
60 µl Master Mix + Cell Suspension in GEM-X 5' Chip



e. Load Row Labeled 2

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

60 µl Gel Beads in GEM-X 5' Chip

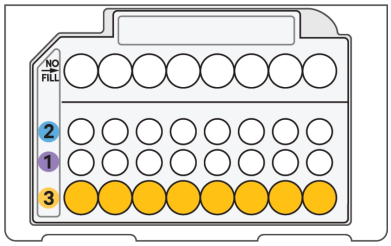


f. Load Row Labeled 3

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

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

250 µl Partitioning Oil B in GEM-X 5' Chip

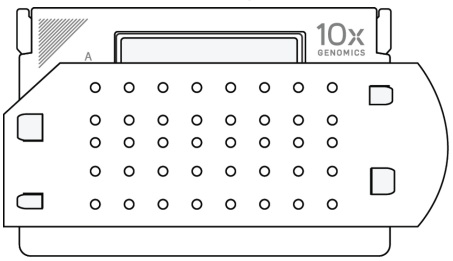


g. Prepare for Run

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

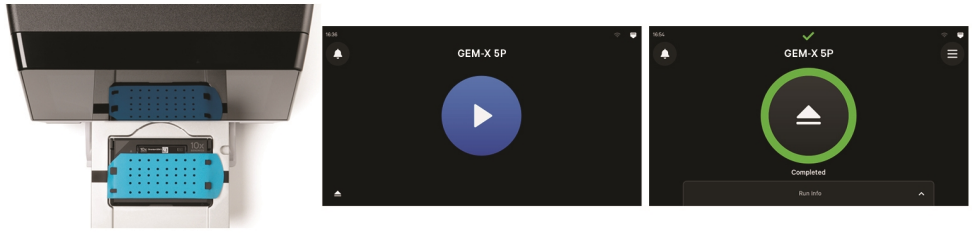
GEM-X 5' Chip, closed



1.3 Run the Chromium X/iX

Firmware Version 2.0.0 or higher is required in the Chromium X/iX used for this protocol.

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



a. Press the eject button on the Chromium X 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 program on the screen. Press the play button.

d. At completion of the run **(~6 min)**, Chromium X/iX 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 Chromium X/iX and remove the chip.

c. Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees.

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

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

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

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

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

Representative chip image

1.5 GEM-RT Incubation

Use a thermal cycler that can accommodate at least 100 µl volume. A volume corresponding to the maximum allowable amount, for a given supported thermal cycler, is the preferred setting. For example a volume of 125 µl is the preferred setting on Bio-Rad C1000 Touch. In alternate thermal cyclers, use highest reaction volume setting.

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

Lid Temperature	Reaction Volume	Run Time
48°C	125 µl	~55 min
Step	**Temperature**	**Time *hh:mm:ss***
1	48°C	00:45:00
2	85°C	00:05:00
3	4°C	Hold

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

Step 2:

Post GEM–RT Cleanup & cDNA Amplification

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				

Item		10x PN	Preparation & Handling	Storage
☐ ◯	**Recovery Agent**	220016	—	Ambient
☐	**10x Magnetic Separator B**	2001212	—	Ambient
☐	**Qiagen Buffer EB**	—	Manufacturer's recommendations.	—
☐	**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**.

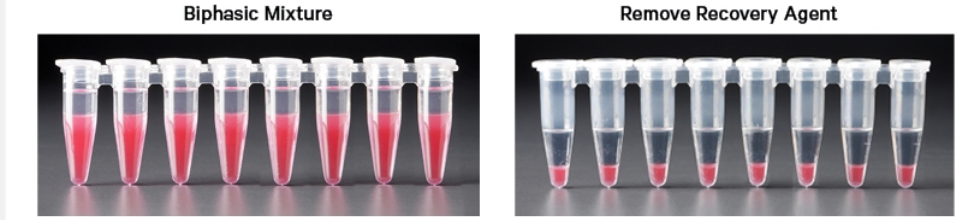
The resulting biphasic mixture contains Recovery Agent/Partitioning Oil (pink) and aqueous phase (clear), with no persisting emulsion (opaque).

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:

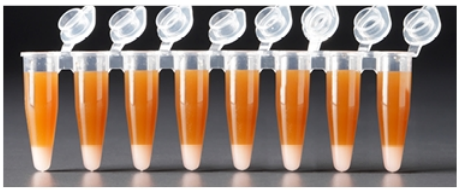
Vortex the Dynabeads thoroughly (**≥30 sec**) **immediately** before adding to the mix.

- 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)	4X + 10% (µl)	8X + 10% (µl)
○	**Cleanup Buffer**	2000088	182	801	1602
	Dynabeads MyOne SILANE	2000048	8	35	70
○	**Reducing Agent B**	2000087	5	22	44
	Nuclease-free Water	—	5	22	44
	Total		**200**	**880**	**1760**

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.

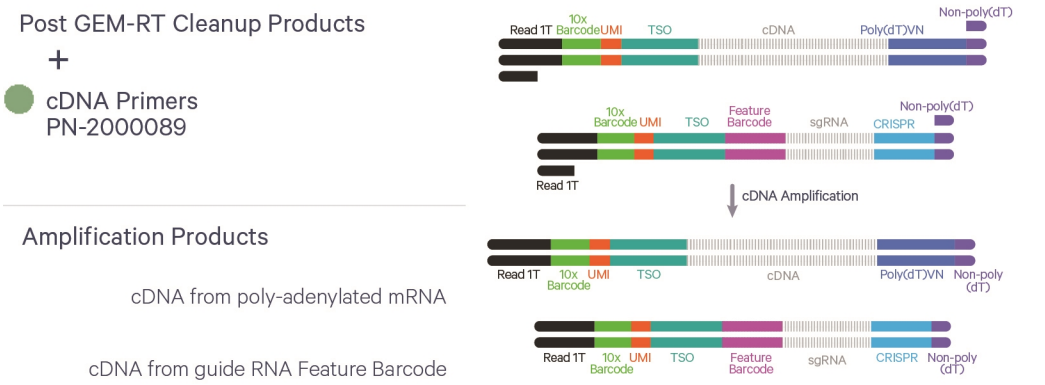
q. Incubate **1 min** at **room temperature**.

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

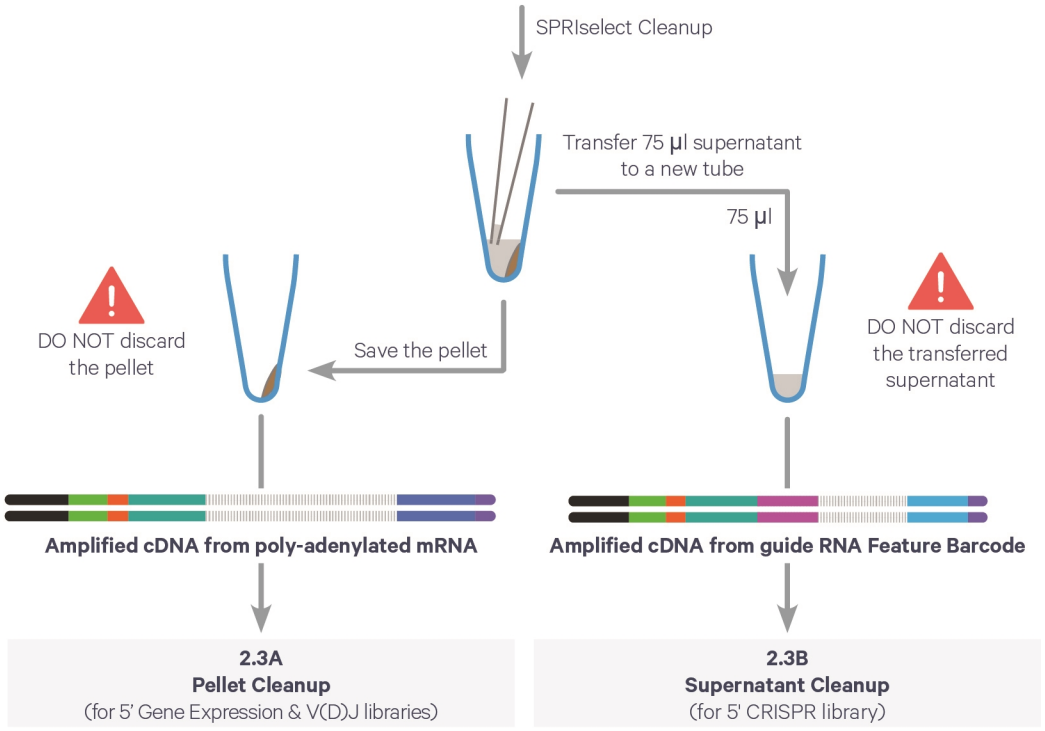
s. Transfer **35 µl** sample to a new tube strip.

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)	4X + 10% (µl)	8X + 10% (µl)
○	**Amp Mix** DO NOT use the Library Amp Mix PN-2000531 (if provided)	2000047/ 2000103	50	220	440
●	**cDNA Primers**	2000089	15	66	132
	Total		**65**	**286**	**572**

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.

Version Specific

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	Total Cycles
<500	17
501–2,000	15

Targeted Cell Recovery	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.

STOP

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.

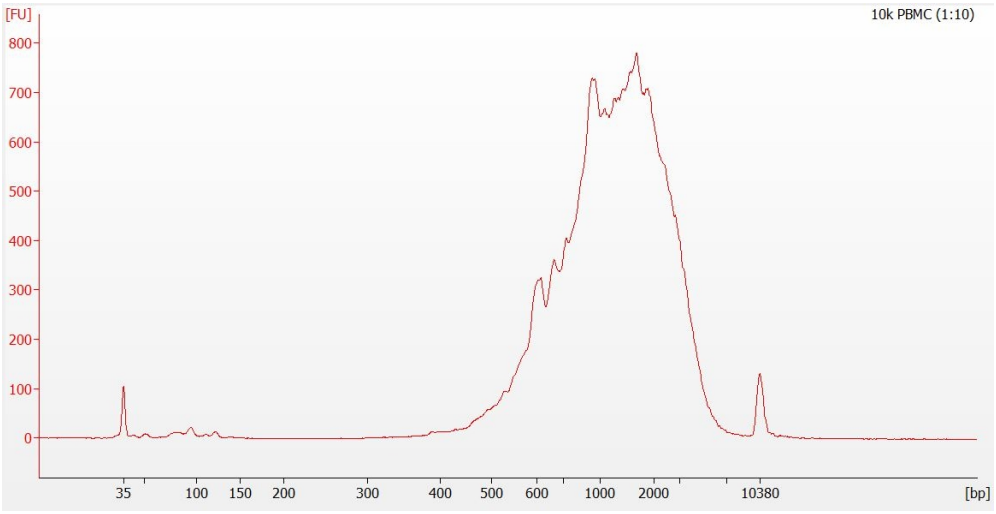
xiv. Store at **4°C** for up to **72 h** or at **−20°C** for up to **4 weeks**, or proceed directly to CRISPR Screening Library Construction.

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

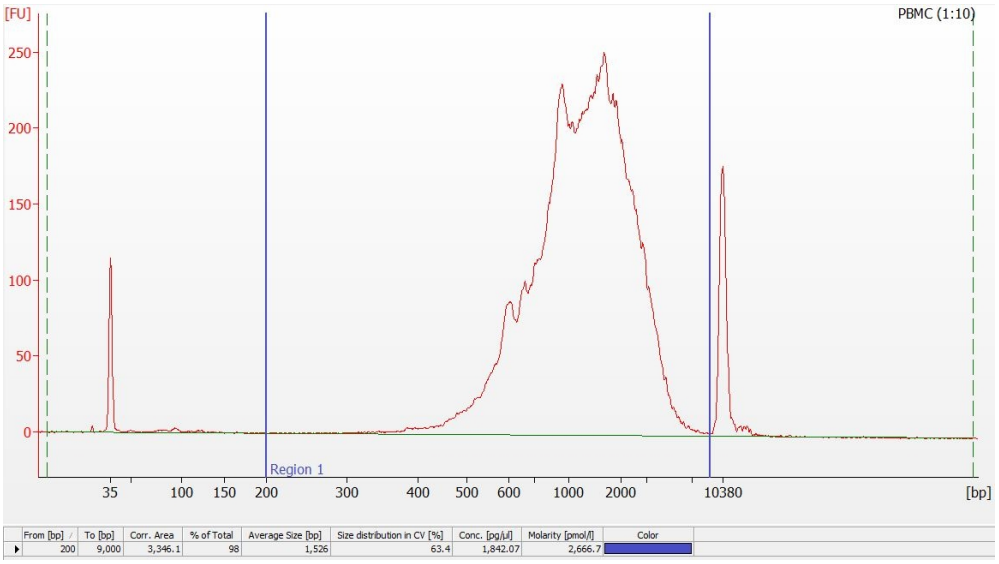
LabChip

See Appendix on page 109 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

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

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

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

Step 3:

V(D)J Amplification from cDNA

3

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	2000047	Vortex, centrifuge briefly.	−20°C

Item		10x PN	Preparation & Handling	Storage
	Mix (PN 2000531) at this step to avoid a significant decrease in assay performance			
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)	4X + 10% (µl)	8X + 10% (µl)
○	**Amp Mix**	2000047	50	220	440
●	**T Cell Mix 1 v2**	Human 2000242/ Mouse 2000256	48	211.2	422.4
	or	or			
●	**B Cell Mix 1 v2**	Human 2000254/ Mouse 2000258			
	Total		**98**	**431.2**	**862.4**

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

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)	4X + 10% (µl)	8X + 10% (µl)
○	Amp Mix	2000047	50	220	440
●	T Cell Mix 2 v2	Human 2000246/ Mouse 2000257	15	66	132
	or	or			
●	B Cell Mix 2 v2	Human 2000255/ Mouse 2000259			
	Total		65	286	572

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.

Lid Temperature	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

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.

GEM-X

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.

STOP

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

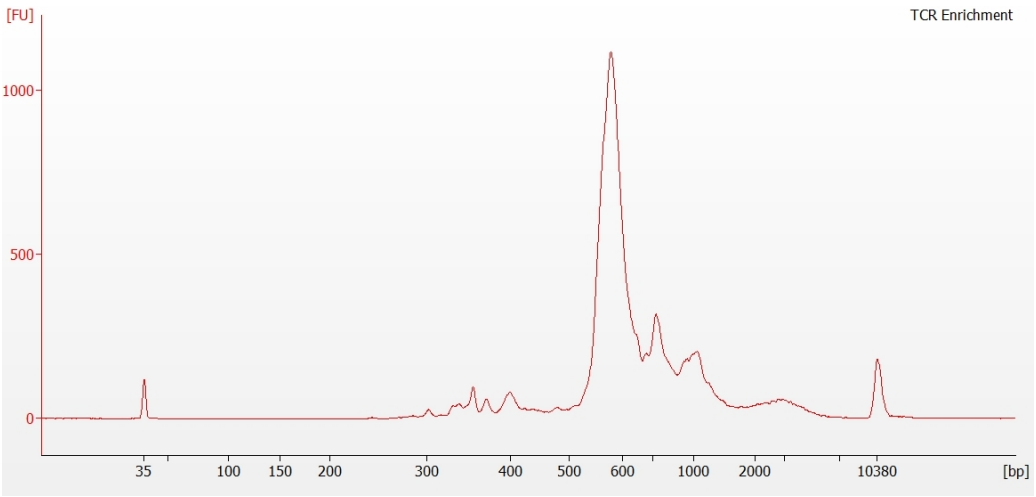
Sample Dilution is dependent on the sample type.

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

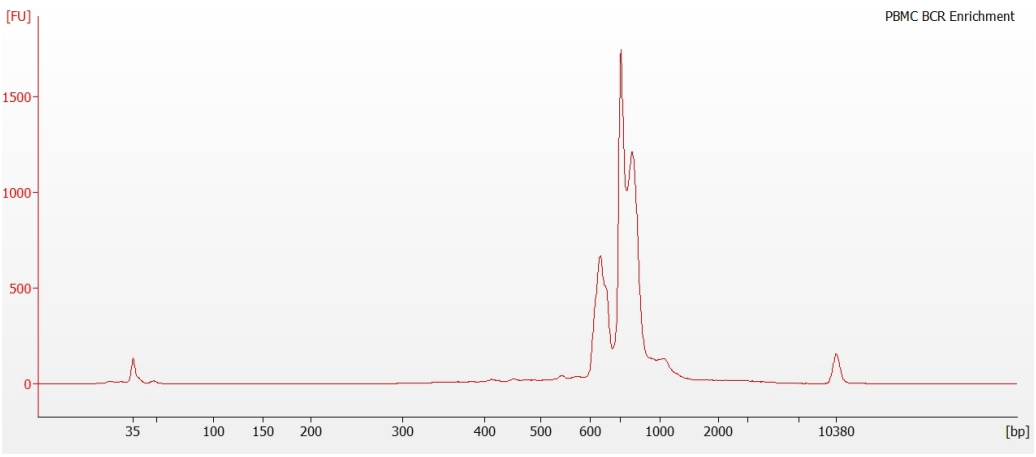
Samples of RNA-rich cells may require additional dilution in nuclease-free water. The number of distinct peaks may vary. Higher molecular weight product (2,000-9,000 bp) may be present. This does not affect sequencing.

Representative Traces

PBMCs amplified for TCR



PBMCs amplified for BCR



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

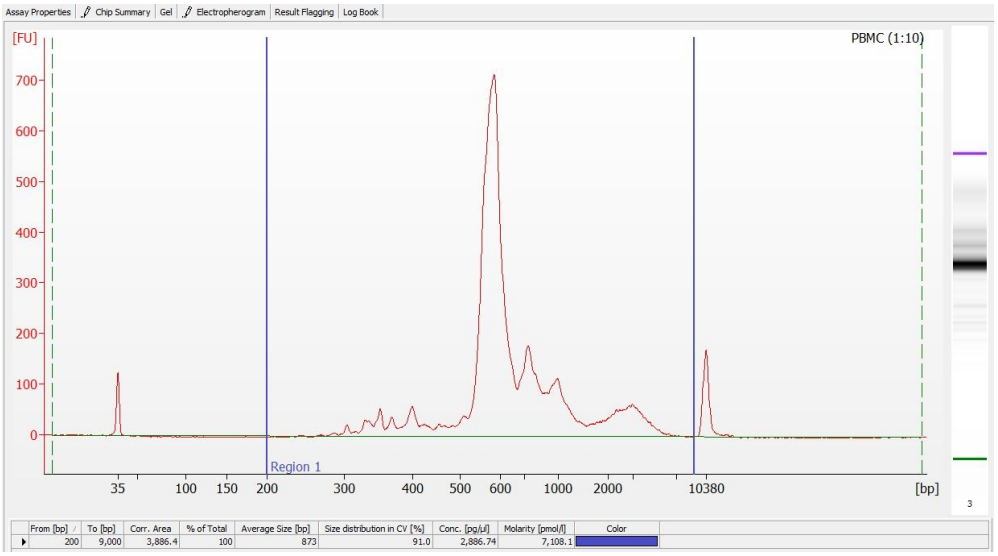
Alternate QC Methods:

- Agilent TapeStation
- LabChip
- See Appendix on page 109 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.

Step 4:

V(D)J Library Construction

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	—	Manufacturer's recommendations.	—

Item		10x PN	Preparation & Handling	Storage
	Illumina Platforms			
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

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)	4X + 10% (µl)	8X + 10% (µl)
Buffer EB	-	25	110	220
Fragmentation Buffer	2000091	5	22	44
Fragmentation Enzyme	2000090/ 2000104	10	44	88
Total		**40**	**176**	**352**

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)	4X + 10% (µl)	8X + 10% (µl)
Ligation Mix	2001109	40	176	352
DNA Ligase	220110/220131	10	44	88
Total		**50**	**220**	**440**

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

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

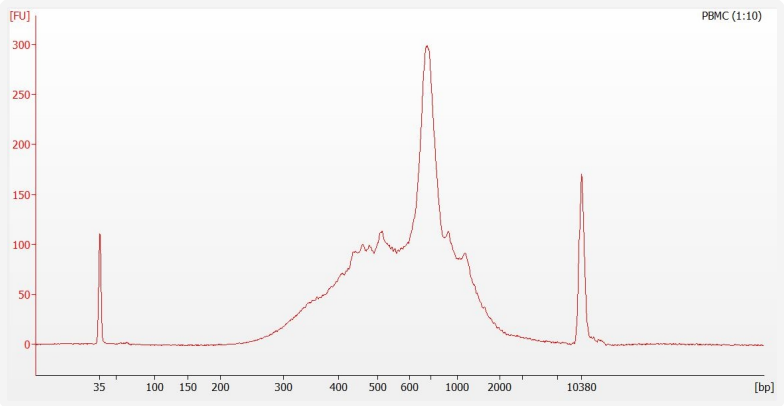
Use Agilent Bioanalyzer, Perkin Elmer LabChip, or Agilent Tapestation for QC.

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

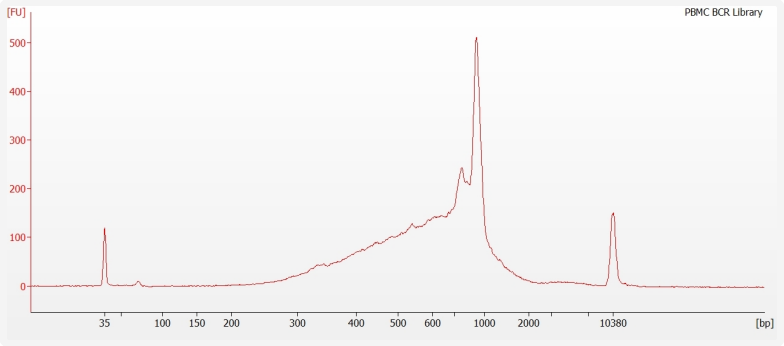
b. Select the region between 200-2,000 bp to determine average size of the library. This will be used as the insert size for library quantification.

Representative Traces

PBMCs amplified for TCR



PBMCs amplified for BCR



- See Appendix on page 109 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 111

Step 5:

5' Gene Expression Library Construction

5.0 Get Started

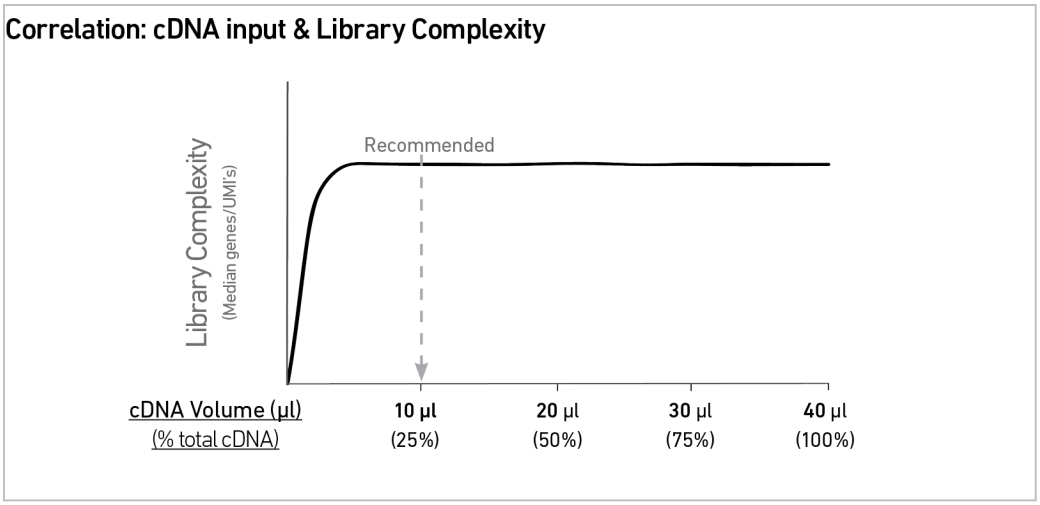
		Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
☐	●	**Fragmentation Buffer**	2000091	Thaw, vortex, verify no precipitate, centrifuge.	−20°C
☐	●	**Ligation Mix**	2001109	Thaw, vortex, verify no precipitate, centrifuge.	−20°C
☐	⚠	**Dual Index Plate TT Set A** *Verify name & 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
☐	○	**Library Amp Mix or Amp Mix**	2000531 or 2000047/2000103	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

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).

Example: Library Construction Input Mass & SI PCR Cycles

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

Example: Library Construction Input Mass & SI PCR Cycles (continued)

Cell	Targeted Cell Recovery	Total cDNA Yield (ng)	cDNA Input into Fragmentation		SI PCR Cycle Number
			Volume (µl)	Mass (ng)	
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)	4X + 10% (µl)	8X + 10% (µl)
	Buffer EB	-	25	110	220
●	**Fragmentation Buffer**	2000091	5	22	44
●	**Fragmentation Enzyme**	2000090/ 2000104	10	44	88
	Total		**40**	**176**	**352**

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)	4X + 10% (µl)	8X + 10% (µl)
Ligation Mix	2001109	40	176	352
DNA Ligase	220110/220131	10	44	88
Total		**50**	**220**	**440**

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** 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

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

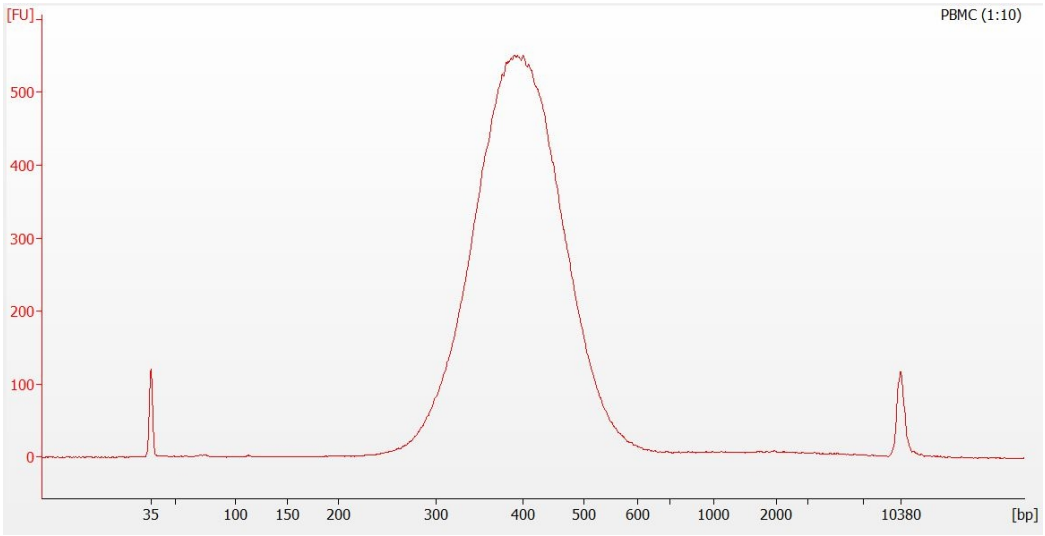
Library QC

Use Agilent Bioanalyzer, Perkin Elmer LabChip, or Agilent Tapestation 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 109 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 111

Step 6:

CRISPR Screening Library Construction

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.

STOP

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)	4X + 10% (µl)	8X + 10% (µl)
○ **Amp Mix**	2000047	50	220	440
● **Feature SI Primers 4**	2000592	45	198	396
Total		**95**	**418**	**836**

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.

STOP

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

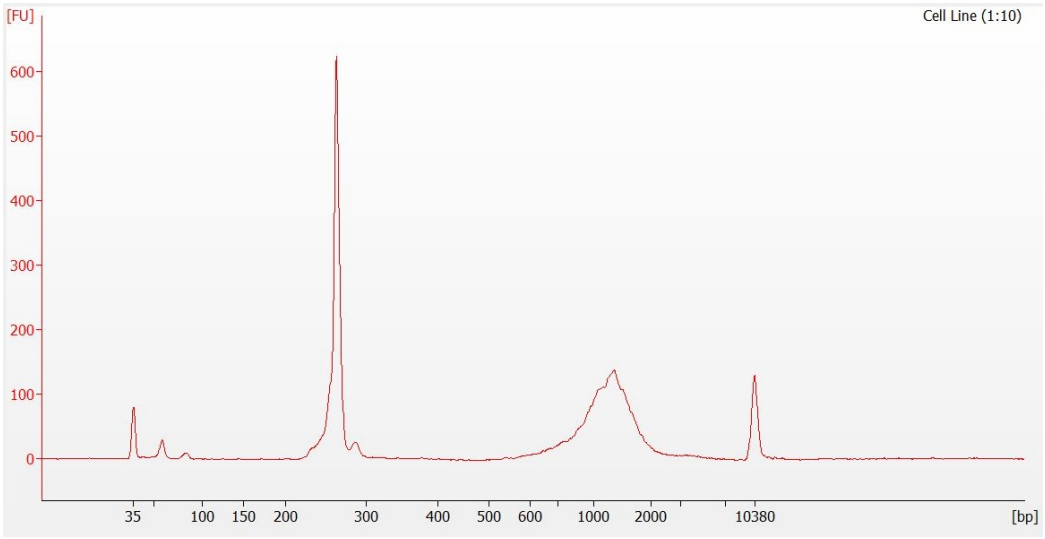
Library QC

Use Agilent Bioanalyzer, Perkin Elmer LabChip, or Agilent Tapestation 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 109 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 111

Step 7:

Sequencing

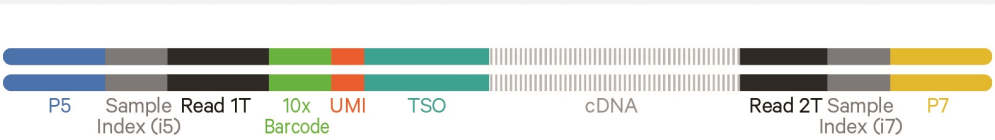
Sequencing Libraries

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

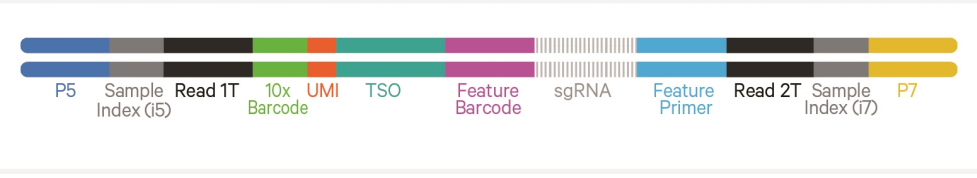
Chromium Single Cell 5' Gene Expression Library



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.

Illumina Sequencer Compatibility

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

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

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

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 32).

Library Sequencing Depth & Run Parameters

GEM-X

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	28 cycles
i7 Index	10 cycles
i5 Index	10 cycles
Read 2	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

**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

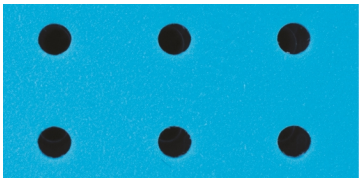
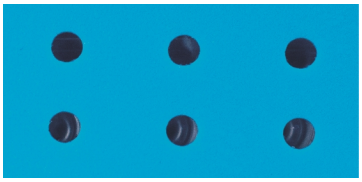
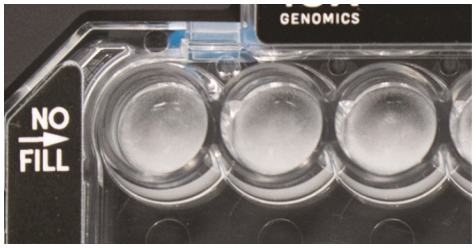
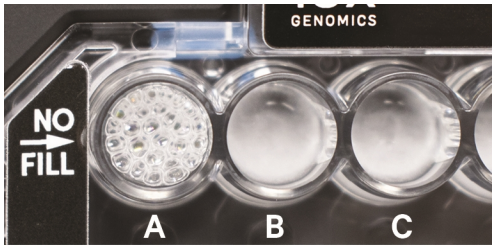
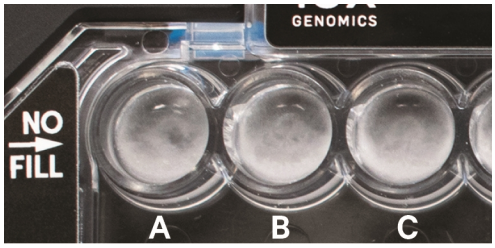
Loupe Browser

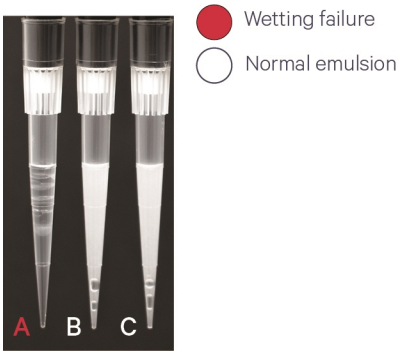
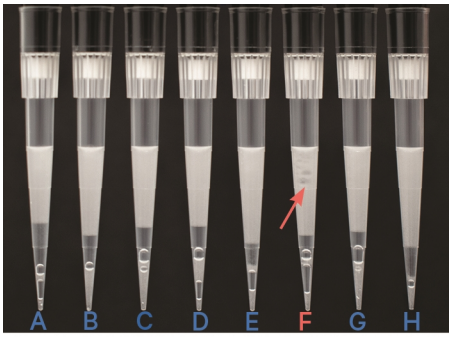
Loupe Browser is an interactive data visualization application that requires no prior programming knowledge.

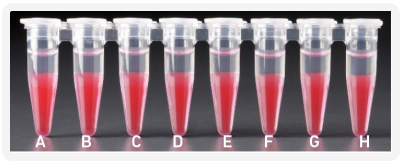
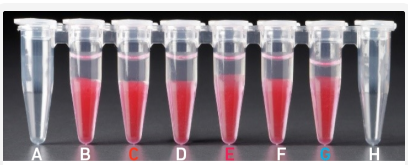
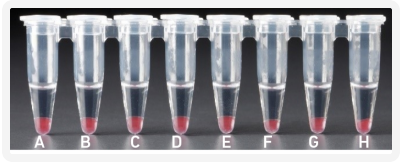
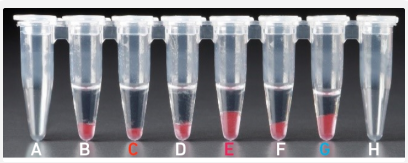
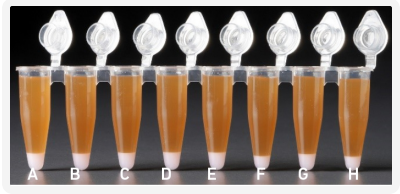
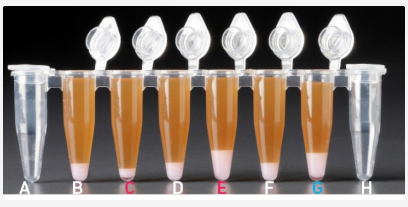
- Input: .cloupe, .vloupe
- Output: Data visualization, including t-SNE and UMAP projections, custom clusters, differentially expressed genes
- Operating System: MacOS, Windows

Troubleshooting

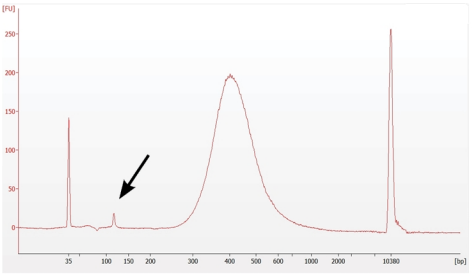
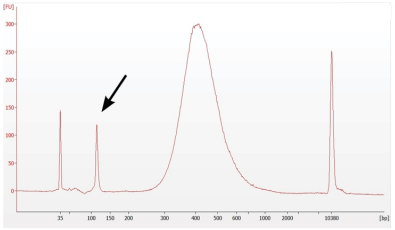
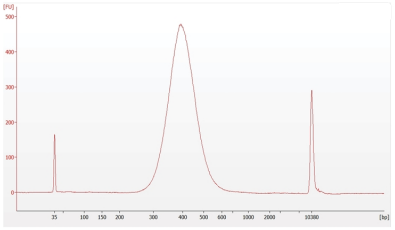
GEM Generation & Barcoding

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 rows of Chromium X/iX gasket will not align with the GEM-X chip. This is normal and will not impact the assay.*
1.4d **After Chip is removed from the instrument and the wells are exposed.**	 All recovery wells are similar in volume and opacity.	 Recovery well A indicates a wetting failure. Not all wetting failures may present themselves with excess bubbles (foam).  Recovery wells A-C show normal emulsions. Not all normal emulsions may have small (in size and number) bubbles in the recovery wells. **Inspecting emulsions in pipette tips is recommended for diagnosing emulsion failures.**

STEP	Normal	Impacted
1.4e **Transfer GEMs from Chip**		
	All liquid levels are similar in volume and opacity without air trapped in the pipette tips.	Pipette tip A shows wetting failure. Pipette tips B-C show uniform emulsions & slightly low volumes. (~95 µl) Most wetting failures will not impact emulsion volumes of other sample run on the chip. Occasionally, wetting failures may impact the emulsion volumes recovered from other lanes. This is expected and does not indicate an emulsion failure if the samples are uniform in volume and opacity (B-C).
		
		In pipette tips A-E & G-H, a portion of the emulsion displays separate layers of oil, which indicates clog. Pipette tip F indicates a clog (separate oil layers) and wetting failure (non-uniform emulsion).
	Emulsion failures are not expected to be observed across the entire chip. The above images are for illustrative purposes to show a range in emulsion failures. A clog is not expected to impact other lanes on the chip.	

STEP	Normal	Impacted
2.1a **After transfer of the GEMs + Recovery Agent**	 All liquid levels are similar in the aqueous sample volume (clear) and Recovery Agent/ Partitioning Oil (pink).	 Tube G indicates a reagent clog has occurred. There is a decreased volume of aqueous layer (clear). Tube C and E indicate a wetting failure has occurred. There is an abnormal volume of Recovery Agent/Partitioning Oil (pink).
2.1b **After aspiration of Recovery Agent/ Partitioning Oil**	 All liquid volumes are similar in the aqueous sample volume (clear) and residual Recovery Agent/Partitioning Oil (pink).	 Tube G indicates a reagent clog has occurred. There is a decreased volume of aqueous layer (clear). There is also a greater residual volume of Recovery Agent/ Partitioning Oil (pink). Tube C and E indicate a wetting failure has occurred. There is an abnormal residual volume of Recovery Agent/Partitioning Oil (pink).
2.1d **After addition of Dynabeads Cleanup Mix**	 All liquid volumes are similar after addition of the Dynabeads Cleanup Mix.	 Tube G indicates a reagent clog has occurred. There is an abnormal ratio of Dynabeads Cleanup Mix (brown) to Recovery Agent/Partitioning Oil (appears white). Tube C and E indicate a wetting failure has occurred. There is an abnormal ratio of Dynabeads Cleanup Mix (brown) to Recovery Agent/Partitioning Oil (appears white).

If a channel clogs or wetting failure occurs during GEM generation, it is recommended that the sample be remade. If any of the listed issues occur, take a picture and send it to support@10xgenomics.com for further assistance. Replacement reagents and chips may be provided for properly documented clogs or wetting failures if they are associated with runs of unexpired reagents and chips, and are reported within 30 days of the expiration date. Consult the Best Practices to Minimize Chromium Next GEM Chip Clogs and Wetting Failures (Technical Note CG000479) for more information.

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

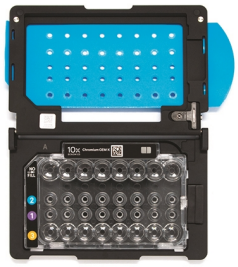
Appendix

GEM-X Chip Loading Overview

This section provides a quick overview to the GEM-X chip loading and does not include detailed instructions. Refer to Load GEM-X Chip on page 41 for details. The Chromium X/iX Chip Holder and X/iX Chip Gasket images shown here are representative. Refer to Assemble GEM-X Chip on page 37 for details.

Steps

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



Color Legend

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

a. Add 50% glycerol solution to each unused well

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

b. Prepare Gel Beads

- Vortex for 30 sec
- Centrifuge for 5 sec

c. Prepare GEM Master Mix + Sample

d. Load Row Labeled 1

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

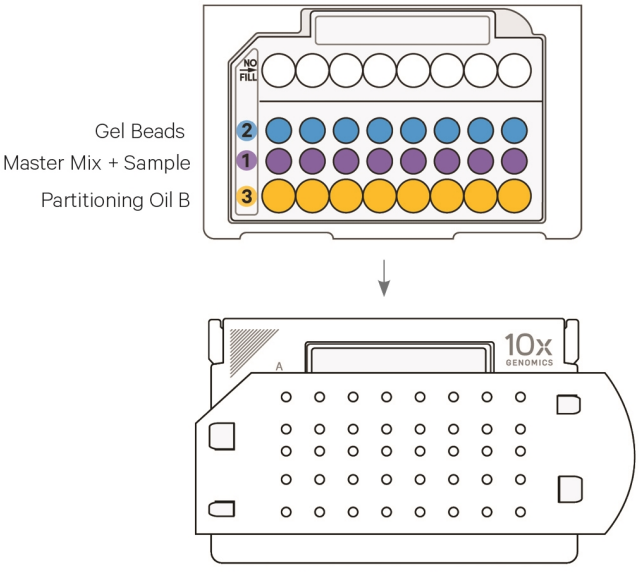
e. Load Row Labeled 2

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

f. Load Row Labeled 3

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

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)	

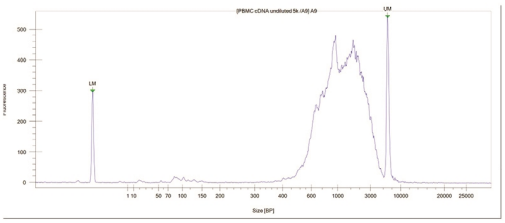
g. Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on insert size derived from the Bioanalyzer/TapeStation trace.

LabChip Traces

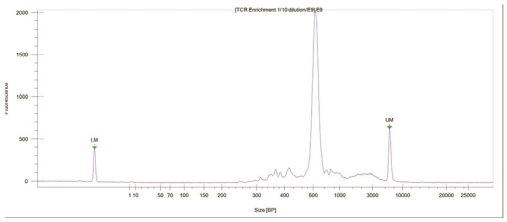
LabChip Traces

DNA High Sensitivity Reagent Kit was used.

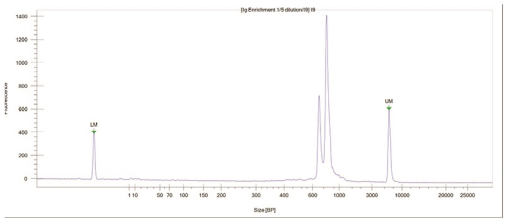
Post cDNA Amplification QC & Quantification



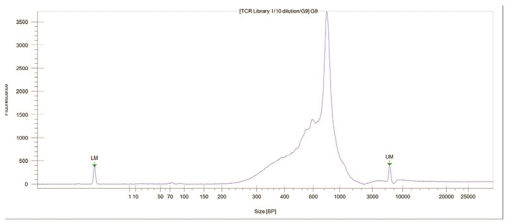
Post TCR Amplification QC



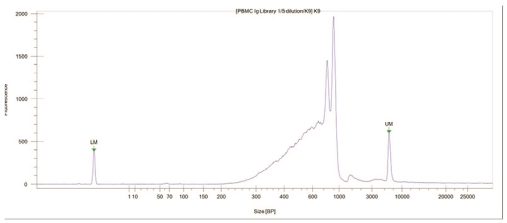
Post BCR Amplification QC



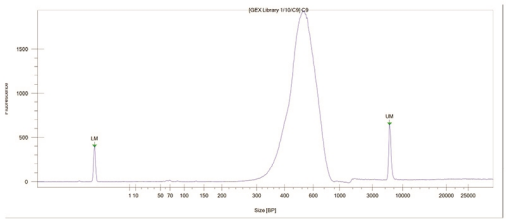
Post Library Construction QC (PBMCs amplified for TCR)



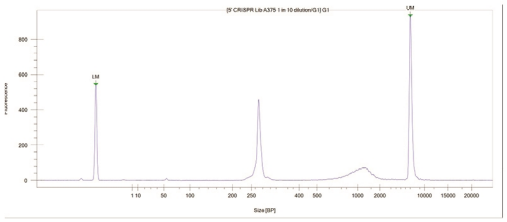
Post Library Construction QC (PBMCs amplified for BCR)



GEX- Post Library Construction QC



CRISPR Screening - Post Library Construction QC



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

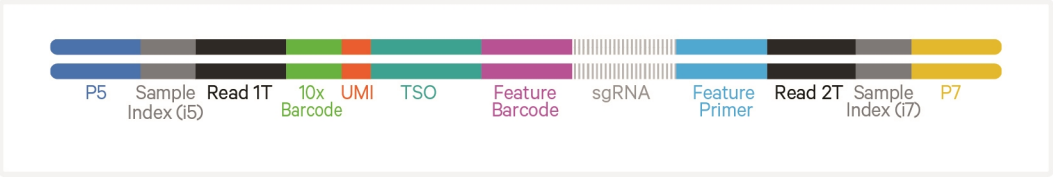
Sample Index PCR Product

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'