10x GENOMICS

User Guide | CG000424 | Rev C

Chromium Next GEM Single Cell 5' HT Reagent Kits v2 (Dual Index)

with Feature Barcode technology for Cell Surface Protein and Immune Receptor Mapping

For use with:

Chromium Next GEM Single Cell 5' HT Kit v2
48 rxns PN-1000356 | 8 rxns PN-1000374

Chromium Next GEM Chip N Single Cell Kit* *80 rxns PN-1000357 | 16 rxns PN-1000375*
*(*Included with Chromium Next GEM Single Cell 5' HT Kit v2; 16 rxn kit can also be ordered separately)*

5' Feature Barcode Kit *16 rxns PN-1000256*

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*

Dual Index Kit TN Set A *96 rxns PN-1000250*

Notices

Document Number

CG000424 | Rev C

Legal Notices

Instrument & Licensed Software Updates Warranties

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

Support

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Document Revision Summary

Document Number

CG000424 | Rev C

Title

Chromium Next GEM Single Cell 5' HT Reagent Kits v2
(Dual Index) with Feature Barcode technology for Cell Surface Protein & Immune Receptor Mapping

Revision

Rev B to Rev C

Revision Date

April 20, 2022

Specific Changes

- Updated 16 rxn chip kit to include one Partitioning Oil tube (PN-220088) and one Recovery Agent tube (PN-2000434)
- Updated Tips & Best Practices to include reference to Best Practices to Minimize Chromium Next GEM Chip Clogs and Wetting Failure (CG000479)
- Updated the annealing temperature to 62°C in step d of V(D)J Amplification 2
- Updated step 6.3c to specify supernatant removal

General Changes

Updated for general minor consistency of language and terms throughout.

Table of Contents

Table of Contents

Table of Contents

Introduction

Chromium Next GEM Single Cell 5' HT Reagent Kits v2

Refer to SDS for handling and disposal information

Chromium Next GEM Single Cell 5' HT Kit v2, 48 rxns PN-1000356

Chromium Next GEM
Single Cell 5' HT GEM Kit v2
48 rxns, PN-1000359
Store at -20°C

	#	PN
RT Reagent B	3	2000435
RT Enzyme C	3	2000436
Poly-dT RT Primer	3	2000437
Reducing Agent B	3	2000087
Cleanup Buffer	3	2000438
cDNA Primers	3	2000439
Amp Mix	3	2000440

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Library Construction Kit
48 rxns, PN-1000352
Store at -20°C

	#	PN
Fragmentation Enzyme	3	2000090
Fragmentation Buffer	3	2000091
Ligation Buffer	3	2000092
DNA Ligase	3	220110
Adaptor Oligos	3	2000094
Amp Mix	3	2000047

10x GENOMICS

Chromium Next GEM
Single Cell 5' HT Gel Bead Kit v2
48 rxns, PN-1000358
Store at -80°C

	#	PN
Single Cell 5' HT Gel Beads v2	3	2000444

10x GENOMICS

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

	#	PN
Dynabeads MyOne SILANE	6	2000048

Chromium Next GEM Chip N Single Cell Kit, 80 rxns PN-1000357

Chromium
Partitioning Oil
Store at ambient temperature

	#	PN
Partitioning Oil	5	220088

Chromium
Recovery Agent
Store at ambient temperature

	#	PN
Recovery Agent	5	2000434

Chromium Next GEM
Chip N & Gaskets
Store at ambient temperature

	#	PN
Chromium Next GEM Chip N	5	2000418
Chip Gasket, HT, 5-pack	1	3000614

10x GENOMICS

Chromium Next GEM Single Cell 5' HT Kit v2, 8 rxns PN-1000374

Chromium Next GEM
Single Cell 5' HT GEM Kit v2
8 rxns, PN-1000377
Store at -20°C

	#	PN
RT Reagent B	1	2000165
RT Enzyme C	1	2000085
Poly-dT RT Primer	1	2000007
Reducing Agent B	1	2000087
Cleanup Buffer	2	2000088
cDNA Primers	1	2000089
Amp Mix	1	2000047

10x GENOMICS

Library Construction Kit
16 rxns, PN-1000190
Store at -20°C

	#	PN
Fragmentation Enzyme	1	2000090
Fragmentation Buffer	1	2000091
Ligation Buffer	1	2000092
DNA Ligase	1	220110
Adaptor Oligos	1	2000094
Amp Mix	1	2000047

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Chromium Next GEM
Single Cell 5' HT Gel Bead Kit v2
8 rxns, PN-1000376
Store at -80°C

	#	PN
Single Cell 5' HT Gel Beads v2 (8 rxns)	1	2000444

10x GENOMICS

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

	#	PN
Dynabeads MyOne SILANE	2	2000048

Depending on the experimental goals, additional library Construction Kits (PN-1000190) may be required. Refer to 10x Genomics support website for further guidance.

Chromium Next GEM Chip N Single Cell Kit, 16 rxns PN-1000375

Chromium
Partitioning Oil
Store at ambient temperature

	#	PN
Partitioning Oil	1	220088

Chromium
Recovery Agent
Store at ambient temperature

	#	PN
Recovery Agent	1	2000434

Chromium Next GEM
Chip N & Gaskets
Store at ambient temperature

	#	PN
Chromium Next GEM Chip N	1	2000418
Chip Gasket, HT, 2-pack	1	3000656

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5' Feature Barcode Kit, 16 rxns PN-1000256

Two 5' Feature Barcode 16 rxn kits are required for processing 16 samples.

5' Feature Barcode Kit
16 rxns, PN-1000256
Store at -20°C

	#	PN
Feature cDNA Primers 4	1	2000277
Amp Mix	1	2000047

10x GENOMICS

Dual Index Kit TT Set A
Store at -20°C

	#	PN
Dual Index Plate TT Set A	1	3000431

Dual Index Kit TN Set A, 96 rxns PN-1000250

Dual Index Kit TN Set A
Store at -20°C

	#	PN
Dual Index Plate TN Set A	1	3000510

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	Part Number (Item)
10x Vortex Adapter	120251	330002
10x Magnetic Separator HT	1000394	2000431
Chromium X Chip Holder	1000393	3000598

Recommended Thermal Cyclers

Thermal cyclers used must support uniform heating of 100 µl emulsion volumes.

Supplier	Description	Part Number
BioRad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module	1851197
Eppendorf	MasterCycler Pro (discontinued)	North America 950030010 International 6321 000.019
Thermo Fisher Scientific	Veriti 96-Well Thermal Cycler	4375786

Additional Kits, Reagents & Equipment

The items in the table below have been validated by 10x Genomics and are highly recommended for 10x Genomics workflows, training, and system operations. Substituting materials may adversely affect system performance. This list does not include standard laboratory equipment, such as water baths, centrifuges, vortex mixers, pH meters, freezers, etc.

Supplier	Description	Part Number (US)
Plastics		
Choose either Eppendorf, USA Scientific, or Thermo Fisher Scientific PCR 8-tube strips		
Eppendorf	PCR Tubes 0.2 ml 8-tube strips	951010022
	DNA LoBind Tubes, 1.5 ml	022431021
	DNA LoBind Tubes, 2.0 ml	022431048
USA Scientific	TempAssure PCR 8-tube strip *(alternate to Eppendorf or Thermo Fisher Scientific product)*	1402-4700
Thermo Fisher Scientific	MicroAmp 8-Tube Strip, 0.2 ml *(alternate to Eppendorf or USA Scientific product)*	N8010580
	MicroAmp 8 -Cap Strip, clear	N8010535
Kits & Reagents		
Thermo Fisher Scientific	Nuclease-free Water Low TE Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA)	AM9937 12090-015
Millipore Sigma	Ethanol, Pure (200 Proof, anhydrous)	E7023-500ML
Beckman Coulter	SPRIselect Reagent Kit	B23318
Bio-Rad	10% Tween 20	1662404
Ricca Chemical Company	Glycerin (glycerol), 50% (v/v) Aqueous Solution	3290-32
Qiagen	Qiagen Buffer EB	19086
Equipment		
VWR	Vortex Mixer Divided Polystyrene Reservoirs	10153-838 41428-958
Thermo Fisher Scientific	MYFUGE 12 Mini Centrifuge *(alternatively, use any equivalent mini centrifuge)*	C1012
	Isotemp Drybath Incubators (when using 48 rxn reagent kits) *(alternatively, use any equivalent drybath or waterbath)*	11-718-22Q
Eppendorf	Eppendorf ThermoMixer C (when using 8 rxn reagent kits)	5382000023
	Eppendorf SmartBlock 1.5 ml, Thermoblock for 24 reaction vessel *(alternatively, use a temperature-controlled Heat Block)*	5360000038

Supplier	Description	Part Number (US)
Quantification & Quality Control		
Choose either Bioanalyzer, TapeStation, Fragment Analyzer, Labchip, or Qubit based on availability & preference.		
Agilent	2100 Bioanalyzer Laptop Bundle (discontinued) (Replacement 2100 Bioanalyzer Instrument/2100 Expert Laptop Bundle)	G2943CA G2939BA/G2953CA
	High Sensitivity DNA Kit	5067-4626
	4200 TapeStation	G2991AA
	High Sensitivity D5000 ScreenTape/Reagents	5067-5584/ 5067-5585
Thermo Fisher Scientific	Qubit 4.0 Flourometer Qubit dsDNA HS Assay Kit	Q33226 Q32854
Advanced Analytical	Fragment Analyzer Automated CE System - 12 cap	FSv2-CE2F
	Fragment Analyzer Automated CE System - 48/96 cap	FSv2-CE10F
	High Sensitivity NGS Fragment Analysis Kit	DNF-474
PerkinElmer	LabChip GX Touch HT Nucleic Acid Analyzer DNA High Sensitivity Reagent Kit	CLS137031 CLS760672
KAPA Biosystems	KAPA Library Quantification Kit for Illumina Platforms	KK4824

Protocol Steps & Timing

	Steps	Timing	Stop & Store
3 h	**Cell Preparation and Labeling** Dependent on cell type and labeling protocol used	~1-2 h	
	Step 1 – GEM Generation & Barcoding		
	1.1 Prepare Reaction Mix	20 min	
	1.2 Load Chromium Next GEM Chip N	10 min	
	1.3 Run the Chromium X	18 min	
	1.4 Transfer GEMs	3 min	
	1.5 GEM-RT Incubation	55 min STOP	4°C ≤72 h or –20°C ≤1 week
	Step 2 – Post GEM RT Cleanup, cDNA Amplification & QC		
6 h	2.1 Post GEM-RT Cleanup – Dynabead	60 min	
	2.2 cDNA Amplification	50 min STOP	4°C ≤72 h or –20°C ≤1 week
	2.3 cDNA Cleanup		
	2.3A Pellet Cleanup	20 min STOP	4°C ≤72 h or –20°C ≤4 weeks
	2.3B Supernatant Cleanup	30 min STOP	4°C ≤72 h or –20°C ≤4 weeks
	2.4 cDNA Quantification & QC	50 min	
	Step 3 – V(D)J Amplification from cDNA		
	3.1 V(D)J Amplification 1	40 min STOP	4°C ≤72 h
	3.2 Post V(D)J Amplification 1 Double Sided Size Selection – SPRIselect	20 min STOP	4°C ≤72 h or –20°C ≤1 week
	3.3 V(D)J Amplification 2	40 min STOP	4°C ≤72 h
	3.4 Post V(D)J Amplification 2 Double Sided Size Selection – SPRIselect	30 min STOP	4°C ≤72 h or –20°C ≤1 week
	3.5 Post V(D)J Amplification QC & Quantification	50 min	
10 h plus* *Time dependent on Stop options used.	**Step 4 – V(D)J Library Construction**		
	4.1 Fragmentation, End Repair & A-tailing	45 min	
	4.2 Adaptor Ligation	25 min	
	4.3 Post Ligation Cleanup – SPRIselect	20 min	
	4.4 Sample Index PCR	40 min STOP	4°C ≤72 h
	4.5 Post Sample Index PCR Cleanup – SPRIselect	20 min STOP	4°C ≤72 h or –20°C long-term
	4.6 Post Library Construction QC	50 min	
	Step 5 – 5' Gene Expression (GEX) Library Construction		
	5.1 GEX Fragmentation, End Repair & A-tailing	45 min	
	5.2 GEX Post Fragmentation, End Repair & A-tailing Double Sided Size Selection – SPRIselect	30 min	
	5.3 GEX Adaptor Ligation	25 min	
	5.4 GEX Post Ligation Cleanup – SPRIselect	20 min	
	5.5 GEX Sample Index PCR	40 min STOP	4°C ≤72 h
	5.6 GEX Post Sample Index PCR Double Sided Cleanup – SPRIselect	30 min STOP	4°C ≤72 h or –20°C long-term
	5.7 GEX Post Library Construction QC	50 min	
	Step 6 – Cell Surface Protein/Immune Receptor Mapping Library Construction		
	6.1 Sample Index PCR	30 min	
	6.2 Post Sample Index PCR Size Selection – SPRIselect	20 min STOP	4°C ≤72 h or –20°C long-term
	6.3 Post Library Construction QC	50 min	

Stepwise Objectives

The Chromium Single Cell 5' v2 HT workflow with Feature Barcode technology offers a comprehensive, scalable approach to detect cell surface proteins and analyze antigen specificity along with the gene expression and immune repertoire information from the same single cell. This is accomplished by labeling cell surface proteins with antibodies or multimeric MHC peptide complexes, such as Dextramer reagents conjugated to a Feature Barcode oligonucleotide, followed by direct capture of the Feature Barcode by the Gel Bead primer. 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 2,000-20,000 individual cells per sample can be assessed.

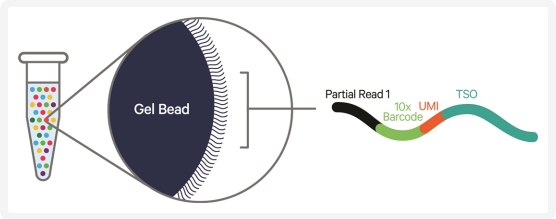
A pool of ~750,000 barcodes are sampled separately to index each cell's transcriptome and antigen specificity. It is done by partitioning thousands of cells into nanoliter-scale Gel Beads-in-emulsion (GEMs), where all generated cDNA share a common 10x Barcode. Libraries are generated and sequenced and 10x Barcodes are used to associate individual reads back to the individual partitions.

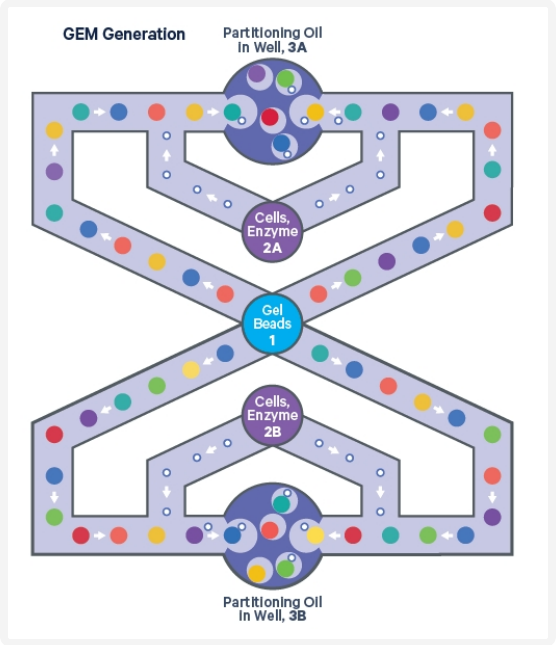
This document outlines the high throughput (HT) protocol to generate the following libraries:

- 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 polyadenylated mRNA
- Single Cell 5' Cell Surface Protein libraries (include immune receptor mapping when cells are also labeled with multimeric MHC peptide complexes, such as Dextramer reagents) from amplified DNA derived from Feature Barcode

Step 1: GEM Generation & Barcoding

GEMs are generated by combining barcoded Gel Beads, a Master Mix containing cell surface protein-labeledcells, and Partitioning Oil onto Chromium Next GEM Chip N. 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 remainder largely contain a single cell.



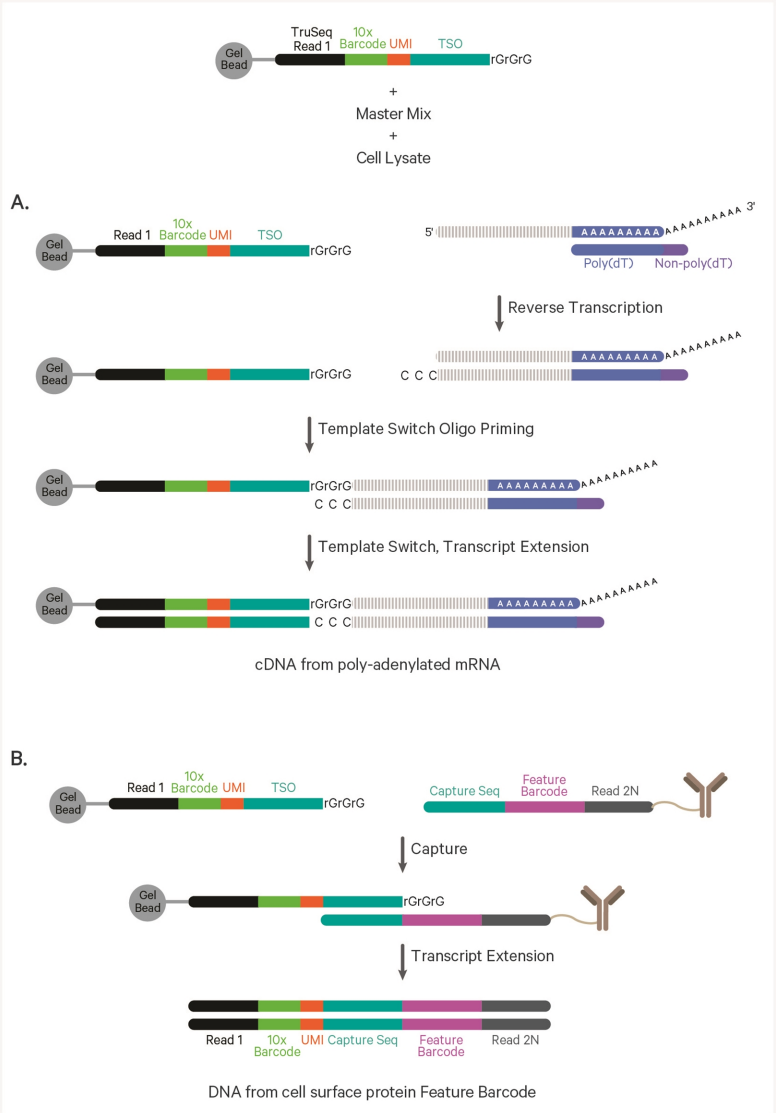


Immediately following GEM generation, the Gel Bead is dissolved and any copartitioned cell is lysed. Gel Bead primers containing an Illumina TruSeq Read 1 sequence (read 1 sequencing primer), a 16 nt 10x Barcode, a 10 nt unique molecular identifier (UMI), and 13 nt template switch oligo (TSO) are released and mixed with both the cell lysate and a Master Mix containing reverse transcription (RT) reagents and poly(dT) primers.

The cell lysate and the released Gel Bead primer incubated with the Master Mix containing RT reagents, produce 10x Barcoded, full-length cDNA from poly-adenylated mRNA.

Simultaneously in the same partition, the Gel Bead primer captures the cell surface protein Feature Barcode conjugated to the antibody or to antibody and antigen containing (i) a Nextera Read 2 (Read 2N), (ii) a 15 nt Feature Barcode, and (iii) Capture Sequence. Incubation of the GEMs with the Master Mix containing RT reagents, produces 10x Barcoded, DNA from the cell surface protein Feature Barcode.

Inside Individual GEMs



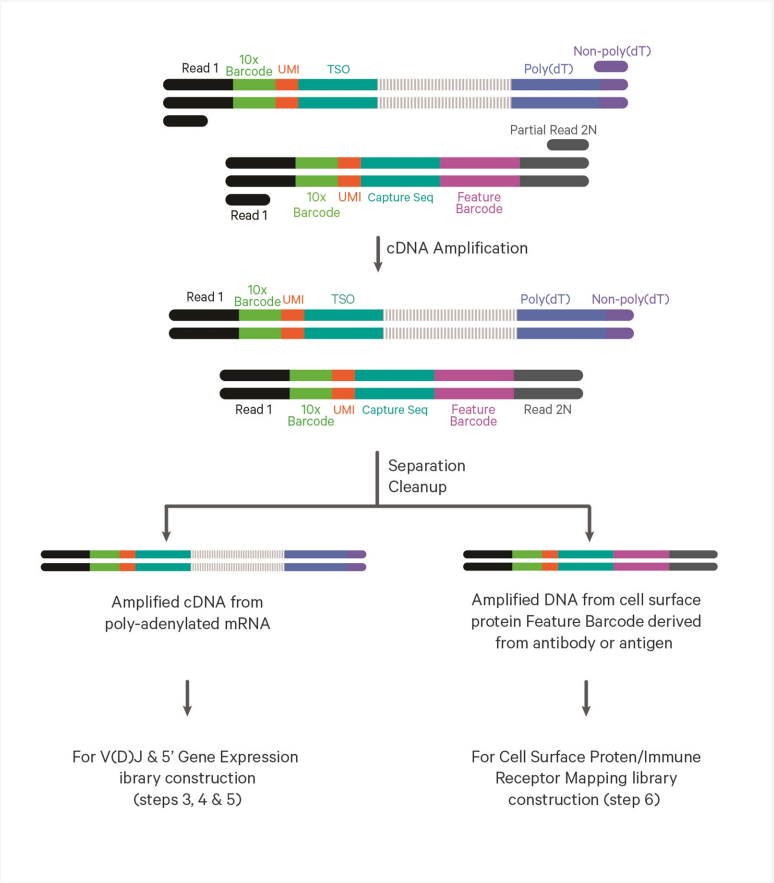
cDNA from poly-adenylated mRNA and DNA from cell surface protein Feature Barcode are generated simultaneously from the same single cell inside the GEM

Step 2: Post GEM-RT Cleanup & cDNA Amplification

GEMs are broken and pooled after GEM-RT reaction mixtures are recovered. Silane magnetic beads are used to purify the 10x Barcoded first-strand cDNA from polyadenylated mRNA and DNA from cell surface protein/antigen specificity Feature Barcode from the post GEM-RT reaction mixture, which includes leftover biochemical reagents and primers. 10x Barcoded, full-length cDNA from poly-adenylated mRNA and DNA from protein Feature Barcode are amplified. Amplification generates sufficient material to construct multiple libraries from the same cells, e.g. both T and/or B cell libraries (steps 3 and 4), 5' Gene Expression libraries (step 5), and Cell Surface Protein libraries (step 6).

The amplified cDNA from polyadenylated mRNA and the amplified DNA from cell surface protein Feature Barcode are separated by size selection for generating V(D)J and/or 5' Gene Expression libraries and Cell Surface Protein libraries, respectively.

Pooled cDNA Amplification



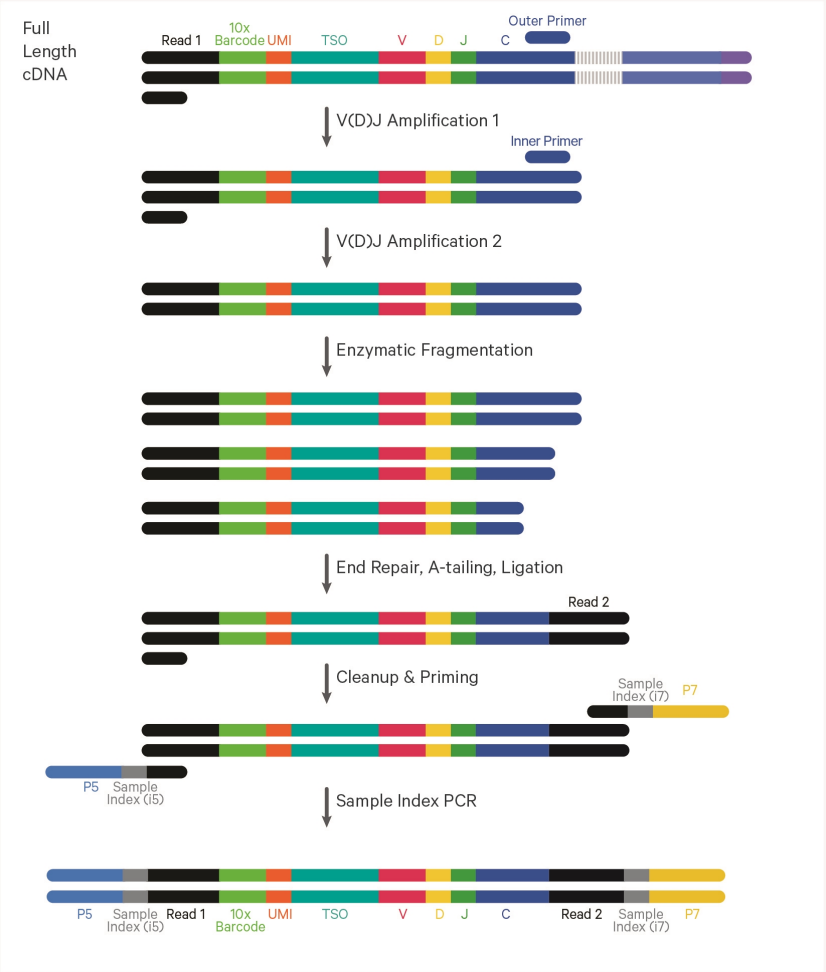
Step 3: V(D)J Amplification from cDNA

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 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 priming sites used in Illumina sequencing.

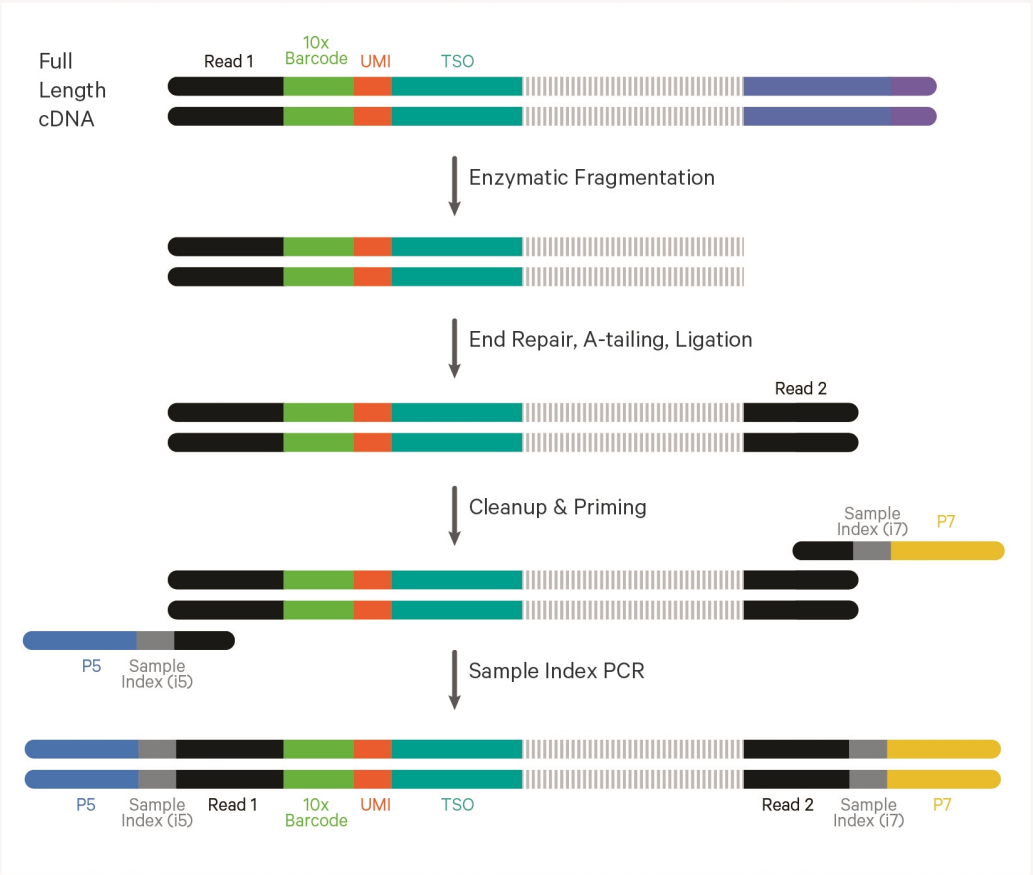
Pooled Amplified cDNA Processed in Bulk



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 priming sites used in Illumina sequencers.

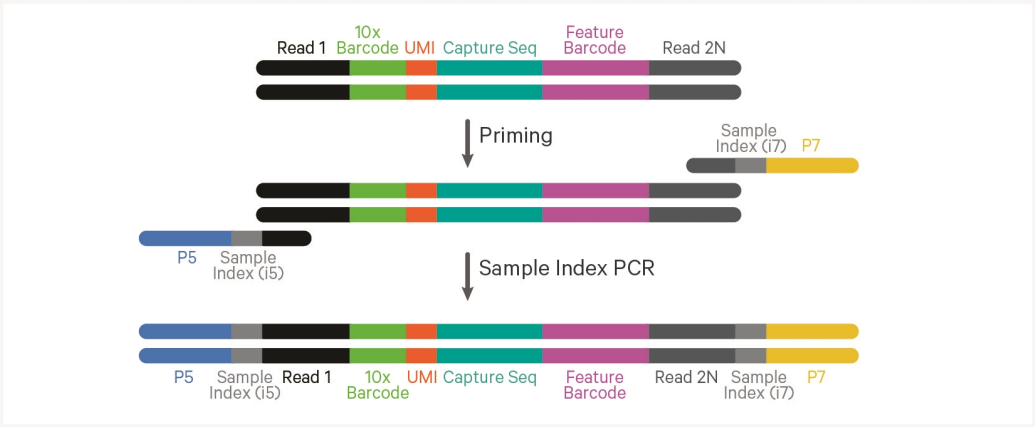
Pooled Amplified cDNA Processed in Bulk



Step 6: Cell Surface Protein/Immune Receptor Mapping Library Construction

Amplified DNA from the cell surface protein Feature Barcodes derived from the antibody or antibody and multimeric MHC peptide complexes, such as Dextramer reagents is used to construct the Cell Surface Protein library. A Cell Surface Protein library also detects antigen specificity if cells were labeled with both antibody and antigen. P5, P7, i5 and i7 sample indexes, and Nextera Read 2 (Read 2N primer sequence) are added via Sample Index PCR. The final libraries contain the P5 and P7 priming sites used in Illumina sequencers.

Pooled Amplified DNA Processed in Bulk



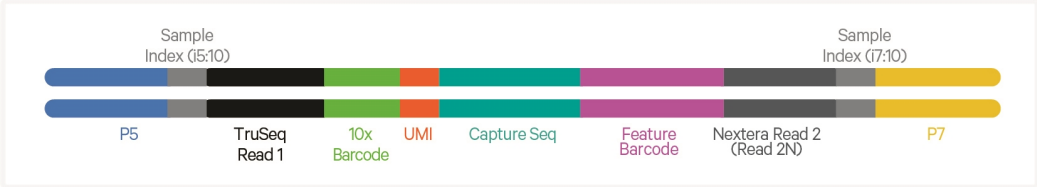
Step 7: Sequencing

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 step 7.

Chromium Single Cell V(D)J Dual Index Library

Chromium Single Cell 5' Gene Expression Dual Index Library

Chromium Single Cell 5' Cell Surface Protein Dual Index Library*



**Detects antigen specificity in cells labeled with antibodies and antigen*

See Appendix for Oligonucleotide Sequences on page 118

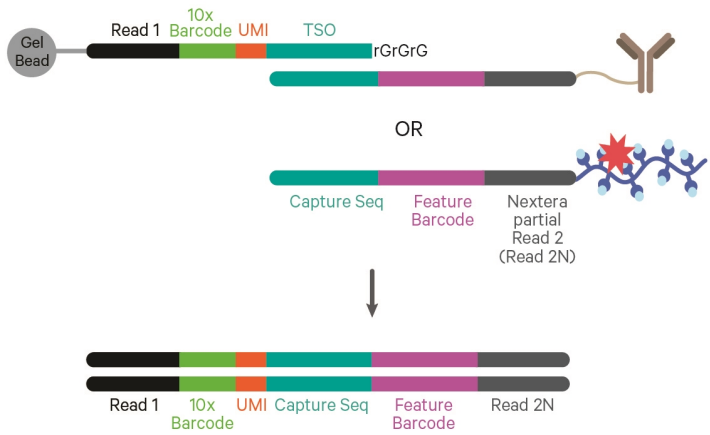
Cell Labeling Guidelines

Overview

Protein/s on the surface of a cell can be labeled with:

- a Feature Barcode oligonucleotide conjugated to a specific protein binding molecule, such as an antibody for detecting cell surface protein expression
- a Feature Barcode oligonucleotide conjugated to an MHC peptide, such as a dCODE Dextramer along with the Feature Barcode oligonucleotide conjugated antibody for mapping immune receptors

The Feature Barcode conjugated molecule bound to the cell surface protein can be directly captured by the Gel Bead inside a GEM during GEM generation and amplified (see Stepwise Objectives for assay scheme specifics). The amplified DNA generated from the Feature Barcode can be used for Cell Surface Protein Library Construction.



DNA from cell surface protein Feature Barcode

Demonstrated Protocols for cell labeling

- For antibody-oligonucleotide conjugation guidance and cell surface protein labeling protocol, consult Demonstrated Protocol Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (Document CG000149).
- Demonstrated Protocol Cell Labeling with Dextramer Reagents for Single Cell RNA Sequencing Protocols with Feature Barcode technology (Document CG000203).

Cell Surface Protein Library:

Amplified DNA from the cell surface protein Feature Barcode derived from the antibody or antibody and antigen is used to construct the Cell Surface Protein library. If cells were labeled with both antibody and antigen, the cell surface protein library will also map immune receptor.

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

Tips & Best Practices

TIPS

Icons

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

Next GEM High Throughput (HT) specific protocol step updates

Emulsion-safe Plastics

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

Cell Concentration

- Recommended starting point is to load ~3,000 cells per reaction, resulting in recovery of ~2,000 cells, and a multiplet rate of ~0.8%. The optimal input cell concentration is 700-1,200 cells/µl.
- The presence of dead cells and debris in the suspension may also reduce the recovery rate. Consult the 10x Genomics Single Cell Protocols Cell Preparation Guide and the Best Practices to Minimize Chromium Next GEM Chip Clogs and Wetting Failure (Documents CG00053 and CG000479 respectively) for more information on preparing cells.

Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~0.8%	~3,130	~2,000
~1.6%	~6,320	~4,000
~2.4%	~9,550	~6,000
~3.2%	~12,800	~8,000
~4.0%	~16,100	~10,000
~4.8%	~19,500	~12,000
~5.6%	~22,900	~14,000
~6.4%	~26,300	~16,000
~7.2%	~29,800	~18,000
~8.0%	~33,300	~20,000

General Reagent Handling

- Fully thaw and thoroughly mix reagents before use.
- Keep all enzymes and Master 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.
- Cover Partitioning Oil tubes and reservoirs 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

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

  OR

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

Pipette Calibration

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

Chromium Next GEM 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.
- Only even number of reactions can be run on the chip. Refer to 1.2 Load Chromium Next GEM Chip N on page 49 for specific instructions.
- Fill all unused paired input wells on a chip with an appropriate volume of 50% glycerol solution before loading the used wells.
- 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 either clogs or wetting failures.
- Minimize the distance that a loaded chip is moved to reach the Chromium X.
- 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.

Chromium X Chip Holders

- Chromium X Chip Holders encase Chromium Next GEM Chips used for the HT (high throughput) assay.
- 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 side of the holder together to unlock the lid and return the holder to a flat position.

Chromium Next GEM 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 guide on the holder is inserted into the chip. 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 into the wells.

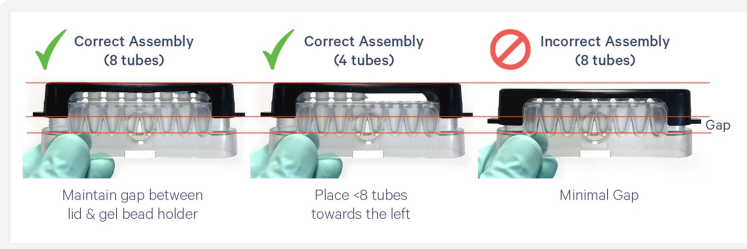
Chromium Next GEM Chip Loading

- 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 1.2 Load Chromium Next GEM Chip N on page 49 for specific instructions.

Gel Bead Handling

- Use one tube of Gel Beads per sample **pair**. 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**.

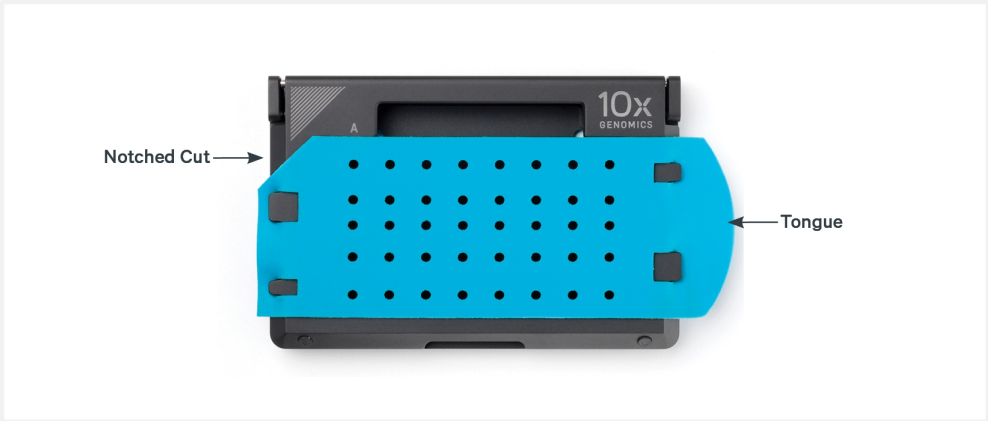
- Centrifuge the Gel Bead strip for **~5 sec** after removing from the holder. Confirm there are no bubbles at the bottom of tubes and the liquid levels look even. Place Gel Bead strip back in the holder and secure the holder lid.
- Ensure that the gel bead strip is positioned with one tube in the left-most position (do not center the strip if using fewer than 8 tubes). Gently depress the lid until light resistance is met. DO NOT attempt to further depress the lid, even if it may be angled with respect to the strip holder.



- 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 Gasket Attachment

- **Before reagents are loaded,** 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.
- Keep the assembled unit with the gasket attached until ready for dispensing reagents into the wells.
- After loading reagents, DO NOT press down on the top of the gasket. Keep the assembly horizontal to avoid wetting the gasket with Partitioning Oil.



10x Magnetic Separator HT

- 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.
- The 10x Magnetic Separator HT can accommodate four 8-Tube Strips
- If using MicroAmp 8-Tube Strips, use the high position (magnet•**High**) only throughout the protocol.

10x Magnetic Separator HT

High

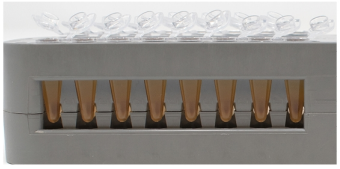
Low

Magnetic Bead Cleanup Steps

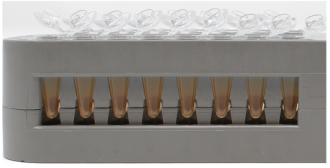
- During magnetic bead based cleanup steps that specify waiting “until the solution clears”, visually confirm clearing of solution before proceeding to the next step. See adjacent panel for an example.
- The time needed for the solution to clear may vary based on specific step, reagents, volume of reagents etc.
- When processing multiple tube strips, prevent bead over drying after ethanol removal by drying the beads only for the specified time. With the tube strips on the magnet, immediately add indicated buffer (EB or ES1) to all tube strips. Then, remove one tube strip at a time from the magnet and mix.

Visually Confirm Clearing of Magnetic Bead Solution

1. Magnetic beads mixed with reagent



2. Separation in progress



3. Separation continuing

4. Separation complete; solution clear

SPRIselect Cleanup & Size Selection

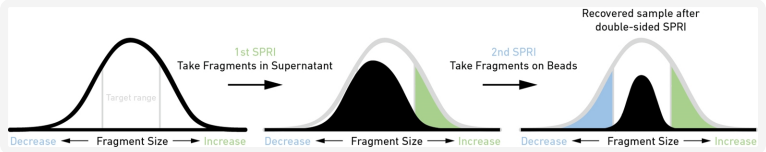
- After aspirating the desired volume of SPRIselect reagent, examine the pipette tips before dispensing to ensure the correct volume is transferred.
- Pipette mix thoroughly as insufficient mixing of sample and SPRIselect reagent will lead to inconsistent results. Use fresh preparations of 80% Ethanol.

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

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

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

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

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

Enzymatic Fragmentation

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

Sample Indices in Sample Index PCR

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

Index Hopping Mitigation

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

- Remove adapters during cleanup steps.
- Ensure no leftover primers and/or adapters 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 0.8X SPRI may be performed for the pooled libraries to remove any free adapters before sequencing.
- Hopped indices can be computationally removed from the data generated from single cell dual index libraries.

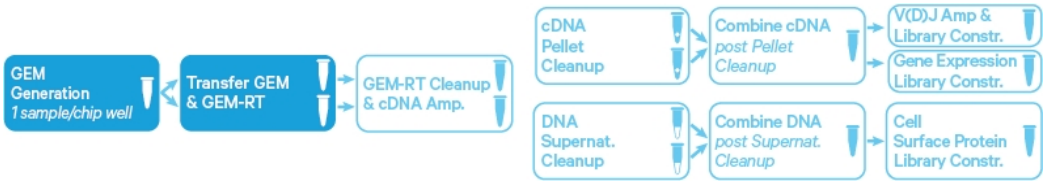
Step 1:

GEM Generation and Barcoding

1

1.0 Get Started

Overview



Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐	**Single Cell 5' HT v2 Gel Beads**	2000444	Equilibrate to room temperature 30 min before loading the chip.	−80°C
☐ ●	**RT Reagent B**	2000435/ 2000165	Vortex, verify no precipitate, centrifuge briefly.	−20°C
☐ ●	**Poly-dT RT Primer**	2000437/ 2000007	Vortex, verify no precipitate, centrifuge briefly.	−20°C
☐ ○	**Reducing Agent B**	2000087	Vortex, verify no precipitate, centrifuge briefly.	−20°C
Place on Ice				
☐ ●	**RT Enzyme C**	2000436/ 2000085	Centrifuge briefly before adding to the mix.	−20°C
☐ ⚠	**Labeled Cell Suspension**	—	Refer to Demonstrated Protocols for Cell Surface Protein Labeling (CG000149) and Cell Labeling with Dextramers (CG000203)	—
Obtain				
☐ ●	**Partitioning Oil**	220088	—	Ambient
☐	**Chromium Next GEM Chip N**	2000418	See Tips & Best Practices.	Ambient
☐	**10x Gasket**	3000614/ 3000656	See Tips & Best Practices.	Ambient
☐	**10x Vortex Adapter**	330002	See Tips & Best Practices.	Ambient

Action	Item	10x PN	Preparation & Handling	Storage
☐	**50% glycerol solution** *If using < 16 reactions*	—	See Tips & Best Practices.	—
☐	**Chromium X Chip Holder**	3000598	See Tips & Best Practices.	Ambient

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**	**2X* + 10% (µl)**	**8X + 10% (µl)**	**16X +10% (µl)**
●	**RT Reagent B**	2000435/ 2000165	82.5	330.0	660.0
●	**Poly-dT RT Primer**	2000437/ 2000007	32.1	128.7	257.4
○	**Reducing Agent B**	2000087	8.6	34.3	68.6
●	**RT Enzyme C**	2000436/ 2000085	36.3	145.4	290.8
	Total	–	**159.5**	**638.4**	**1276.8**

**Volume for 2 rxns; only even number of rxns can be run on the chip*

a. Add **72.6** Master Mix into each tube of a PCR 8-tube strip on ice.

Assemble Chromium Next GEM Chip N

TIPS

See Tips & Best Practices on page 29 for chip handling instructions.

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

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

c. Open the chip holder.

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

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

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

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

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

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

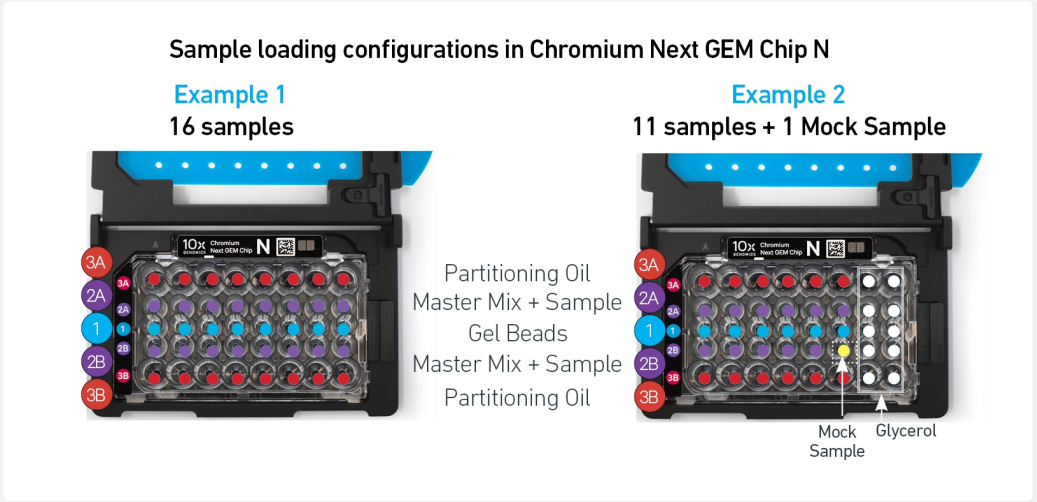
For GEM generation, load the indicated reagents only in the specified rows, starting from row labeled 1, followed by rows labeled 2A & 2B and 3A & 3B.

Sample Loading Guidelines

Read these guidelines before loading Chromium Next GEM Chip N

- Up to 16 independent samples can be run on the chip.
- Only even number of reactions can be run on the chip.
- Corresponding wells in rows 2A & 2B of the chip should both be either loaded with independent samples/mock-sample or with glycerol.
- **For even number of samples**:
  Load independent samples (Master Mix + Cells) in pairs in rows 2A & 2B. See Example 1 below.
- **For odd number of samples** :
  Load the unpaired sample in a well in row 2A and a mock-sample (Master Mix + Water) in the corresponding well in row 2B. Additionally, add Partitioning Oil to the corresponding well in row 3B.
  See Example 2 below.
- Follow the step-by-step chip loading instructions provided in step 1.2.

Sample loading configuration examples



Cell Suspension Volume Calculator

Volume of Cell Suspension Stock per reaction (µl) | Volume of Nuclease-free Water per reaction (µl)

DO NOT add nuclease-free water directly to single cell suspension. Add nuclease-free water to the Master Mix. Refer to step 1.2.

Cell Stock Concentration (Cells/µl)	2000	4000	6000	8000	10000	12000	14000	16000	18000	20000
100	31.3 46.1	63.2 14.2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
200	15.6 61.8	31.6 45.8	47.8 29.6	64.1 13.3	n/a	n/a	n/a	n/a	n/a	n/a
300	10.4 67.0	21.1 56.3	31.8 45.6	42.7 34.7	53.8 23.6	64.9 12.5	76.2 1.2	n/a	n/a	n/a
400	7.8 69.6	15.8 61.6	23.9 53.5	32.1 45.3	40.3 37.1	48.7 28.7	57.2 20.2	65.8 11.6	74.4 3.0	n/a
500	6.3 71.1	12.6 64.8	19.1 58.3	25.6 51.8	32.3 45.1	39.0 38.4	45.7 31.7	52.6 24.8	59.6 17.8	66.6 10.8
600	5.2 72.2	10.5 66.9	15.9 61.5	21.4 56.0	26.9 50.5	32.5 44.9	38.1 39.3	43.8 33.6	49.6 27.8	55.5 21.9
700	4.5 72.9	9.0 68.4	13.6 63.8	18.3 59.1	23.0 54.4	27.8 49.6	32.7 44.7	37.6 39.8	42.5 34.9	47.6 29.8
800	3.9 73.5	7.9 69.5	11.9 65.5	16.0 61.4	20.2 57.2	24.4 53.0	28.6 48.8	32.9 44.5	37.2 40.2	41.6 35.8
900	3.5 73.9	7.0 70.4	10.6 66.8	14.2 63.2	17.9 59.5	21.6 55.8	25.4 52.0	29.2 48.2	33.1 44.3	37.0 40.4
1000	3.1 74.3	6.3 71.1	9.6 67.8	12.8 64.6	16.1 61.3	19.5 57.9	22.9 54.5	26.3 51.1	29.8 47.6	33.3 44.1
1100	2.8 74.6	5.7 71.7	8.7 68.7	11.7 65.7	14.7 62.7	17.7 59.7	20.8 56.6	23.9 53.5	27.1 50.3	30.3 47.1
1200	2.6 74.8	5.3 72.1	8.0 69.4	10.7 66.7	13.4 64.0	16.2 61.2	19.1 58.3	21.9 55.5	24.8 52.6	27.7 49.7
1300	2.4 75.0	4.9 72.5	7.3 70.1	9.9 67.5	12.4 65.0	15.0 62.4	17.6 59.8	20.2 57.2	22.9 54.5	25.6 51.8
1400	2.2 75.2	4.5 72.9	6.8 70.6	9.2 68.2	11.5 65.9	13.9 63.5	16.3 61.1	18.8 58.6	21.3 56.1	23.8 53.6
1500	2.1 75.3	4.2 73.2	6.4 71.0	8.5 68.9	10.8 66.6	13.0 64.4	15.2 62.2	17.5 59.9	19.9 57.5	22.2 55.2
1600	2.0 75.4	4.0 73.4	6.0 71.4	8.0 69.4	10.1 67.3	12.2 65.2	14.3 63.1	16.4 61.0	18.6 58.8	20.8 56.6
1700	1.8 75.6	3.7 73.7	5.6 71.8	7.5 69.9	9.5 67.9	11.5 65.9	13.5 63.9	15.5 61.9	17.5 59.9	19.6 57.8
1800	1.7 75.7	3.5 73.9	5.3 72.1	7.1 70.3	9.0 68.4	10.8 66.6	12.7 64.7	14.6 62.8	16.5 60.9	18.5 58.9
1900	1.6 75.8	3.3 74.1	5.0 72.4	6.7 70.7	8.5 68.9	10.3 67.1	12.0 65.4	13.8 63.6	15.7 61.7	17.5 59.9
2000	1.6 75.8	3.2 74.2	4.8 72.6	6.4 71.0	8.1 69.3	9.7 67.7	11.4 66.0	13.2 64.2	14.9 62.5	16.6 60.8

Grey boxes: Exceeds allowable volume

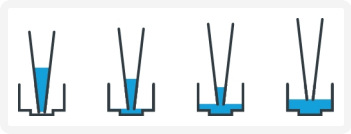
Blue boxes: Optimal range of cell stock concentration to maximize the likelihood of achieving the desired cell recovery target

Yellow boxes: Indicate a low transfer volume that may result in higher cell load variability

1.2 HT Load Chromium Next GEM Chip N

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.
- 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.



a. Add 50% glycerol solution to each unused well

(if loading less than 16 samples/chip)

- **130 µl** in each unused well in row labeled 1
- **140 µl** in each unused well in rows labeled 2A & 2B
- **140 µl** in each unused well in rows labeled 3A & 3B

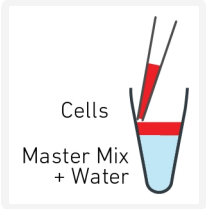
DO NOT use any substitute for 50% glycerol solution.
For odd number of samples, if a sample is loaded in a well in row 2A, load a mock-sample (Master Mix + 86.4 µl water) and NOT glycerol in the corresponding well in row 2B.

b. Prepare Gel Beads

- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**.
- Remove the Gel Bead strip from the holder and centrifuge it 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.

c. Prepare Master Mix + Cell suspension

- Refer to the Cell Suspension Volume Calculator Table.
- Add the appropriate volume of nuclease-free water to Master Mix. Pipette mix 5X.
- Gently pipette mix the cell suspension and add corresponding volume of single cell suspension to Master Mix.
  Total of 150 µl in each tube.



d. Load Row Labeled 1

- Puncture the foil seal of the Gel Bead tubes. Slowly aspirate **130 µl** Gel Beads.
- Dispense into the wells in **row labeled 1** without introducing bubbles.
- Wait **30 sec**.

e. Load Rows Labeled 2A, 2B

ONLY even number of reactions should be run on the chip. See Sample Loading Guidelines for more information and examples.

- Up to 16 independent samples can be run on the chip. Sample inputs for 2A should be equal to 2B (e.g. if processing only 8 samples, run 4 in 2A and 4 in 2B)
- **First, process up to 8 samples:** Gently pipette mix the Master Mix + Cell Suspension (prepared at step 1.2c) using a multichannel pipette. Using the same pipette tips, dispense **140 µl** Master Mix + Cell Suspension into the bottom center of wells in **row labeled 2A**.
- **Next, process up to 8 additional samples:** Gently pipette mix the Master Mix + Cell Suspension (prepared at step 1.2c) using a multichannel pipette. Using the same pipette tips, dispense **140 µl** Master Mix + Cell Suspension into the bottom center of wells in **row labeled 2B**.
- Wait **30 sec**.

f. Load Rows Labeled 3A, 3B

- Dispense **140 µl** Partitioning Oil into the wells in **rows labeled 3A & 3B** from a reagent reservoir.

Failure to add Partitioning Oil to the rows labeled 3A and 3B will prevent GEM generation and can damage Chromium X.

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.

*Run the chip in Chromium X **immediately** after loading the Partitioning Oil. ONLY even number of reactions should be run on the chip. See* *Sample Loading Guidelines on page 47* *for more information & examples.*

1.3 HT Run Chromium X

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

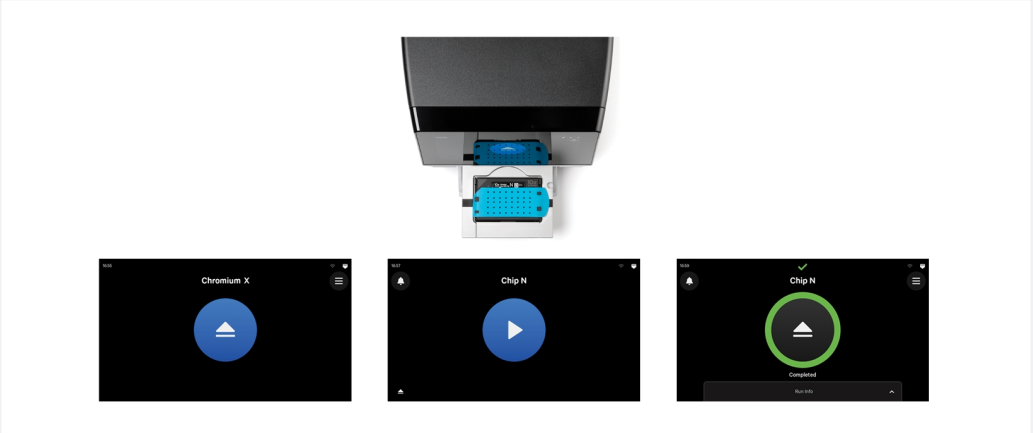
a. Press the eject button on the Chromium X to eject the tray.
If the eject button is not touched within **1 min**, tray will close automatically. System requires a few seconds before the tray can be ejected again.

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. Press the play button.

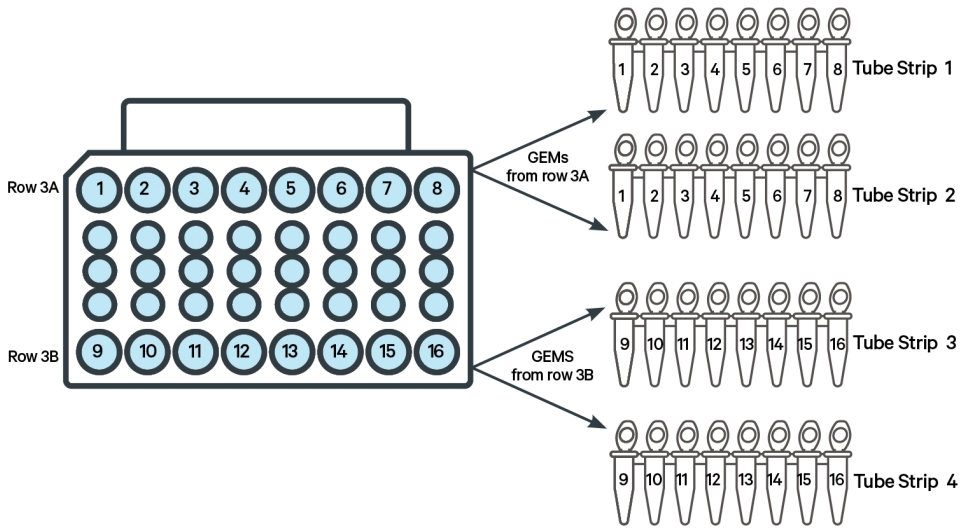
d. At completion of the run **(~18 min)**, Chromium X will chime. **Immediately** proceed to the next step.

Chromium X



GEM Transfer Overview

For a sample loaded in a well in either row 2A or 2B of the chip, GEMs are retrieved from the corresponding well in row 3A and 3B and transferred to two tubes. The example below shows transfer of GEMs generated from 16 samples. GEMs from the chip are transferred to four tube strips, where the GEMs generated from each sample are transferred to 2 corresponding tubes in the indicated tube strips.



1.4 HT Transfer GEMs

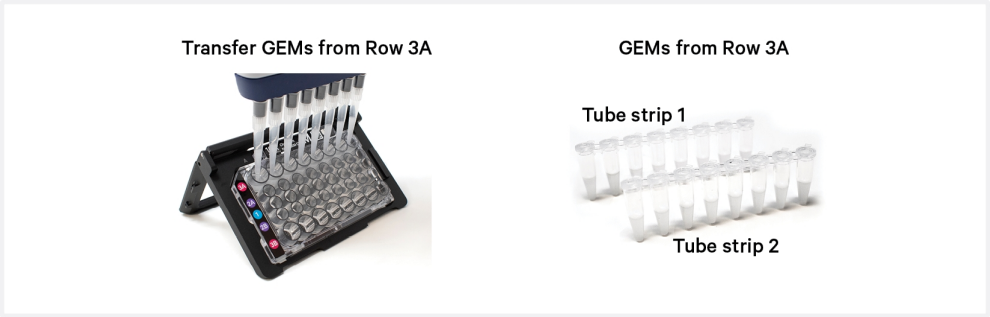
a. Label four tube strips and place on ice.

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

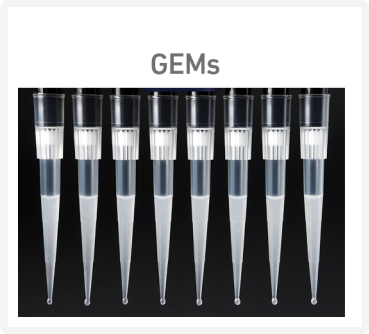
c. Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees.

d. Check the volume in rows labeled 1, 2A, and 2B. Abnormally high volume relative to other wells indicates a clog. Significant volume of non-sample fluid is expected in rows 2A and 2B after a successful run and does not indicate a sample clog.

e. Retrieve GEMs from row labeled 3A: Slowly aspirate **90 µl** GEMs from the lowest points of the recovery wells in the top row labeled 3A without creating a seal between the tips and the bottom of the wells.



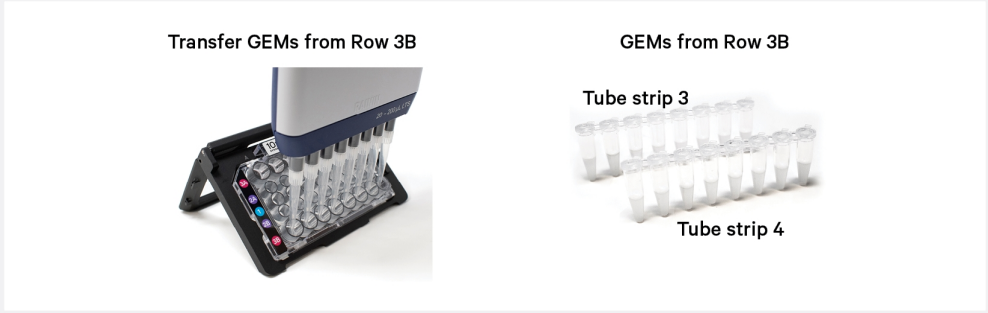
f. Withdraw pipette tips from the wells. GEMs should appear opaque and uniform across all channels. Excess Partitioning Oil (clear) in the pipette tips indicates a potential clog.



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

h. Using the same pipette tips, slowly aspirate remaining **90 µl** GEMs from the wells in the top row labeled 3A and dispense in second tube strip as described above.

i. Retrieve from row labeled 3B: Slowly aspirate **90 µl** GEMs from the lowest points of the recovery wells in the bottom row labeled 3B without creating a seal between the tips and the bottom of the wells.



j. Repeat steps f and g, dispensing GEMs into third tube strip on ice with the pipette tips against the sidewalls of the tubes.

k. Using the same pipette tips, slowly aspirate remaining **90 µl** GEMs from the wells in the bottom row labeled 3B and dispense in fourth tube strip as described above.

l. If multiple chips are run back-to-back, cap/cover the GEM-containing tube strips and place on ice for no more than **1 h**.

1.5 GEM-RT Incubation

Use a thermal cycler that can accommodate at least 100 µl volume. 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
53°C	125 µl	~55 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	53°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.

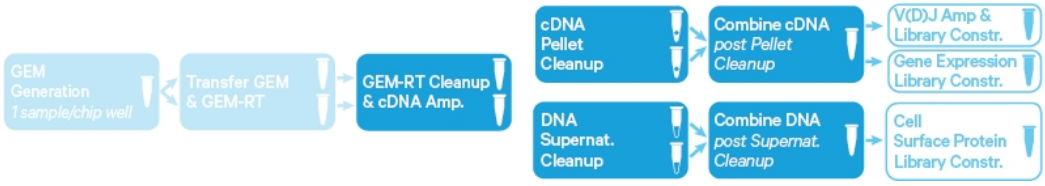
Step 2:

Post GEM–RT Cleanup & cDNA Amplification

2

2.0 Get Started

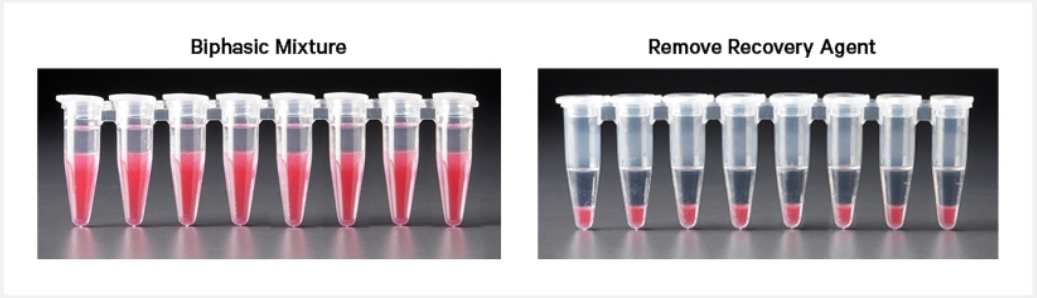
Overview



Action		Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
☐	○	**Reducing Agent B**	2000087	Thaw, vortex, verify no precipitate, centrifuge.	−20°C
☐	●	**Feature cDNA Primers 4** *Verify name & PN*	2000277	Thaw, vortex, centrifuge briefly.	−20°C
☐		**Dynabeads MyOne SILANE**	2000048	Vortex thoroughly (≥30 sec) **immediately** before adding to the mix.	4°C
☐		**Beckman Coulter SPRIselect Reagent**	—	Manufacturer's recommendations.	—
☐		**Agilent Bioanalyzer High Sensitivity Kit** *If used for QC & quantification*	—	Manufacturer's recommendations.	—
☐		**Agilent TapeStation ScreenTape & Reagents** *If used for QC & quantification*	—	Manufacturer's recommendations.	—
☐		**Qubit dsDNA HS Assay Kit** *If used for QC & quantification*	—	Manufacturer's recommendations.	—
☐		**DNA High Sensitivity Reagent Kit** *If used for QC*	—	Manufacturer's recommendations.	—

Action	Item	10x PN	Preparation & Handling	Storage
Place on Ice				
☐ ◯	**Amp Mix** *Retrieve from Single Cell 5' HT GEM Kit*	2000440/ 2000047	Vortex. Ensure all liquid is at the bottom of the tube.	−20°C
Thaw at 65°C				
☐ ◯	**Cleanup Buffer**	2000088/ 2000438	Thaw for 10 min at 65° C either in a water or bead bath, mixing every 5 min (for 16 rxn kit) or at max speed on a thermomixer (for 8 rxn kit). Verify no visible crystals. Cool to room temperature.	−20°C
Obtain				
☐ ◯	**Recovery Agent**	2000434	—	Ambient
☐	**Qiagen Buffer EB**	—	Manufacturer's recommendations.	—
☐	**Bio-Rad 10% Tween 20**	—	Manufacturer's recommendations.	—
☐	**10x Magnetic Separator HT**	2000431	—	Ambient
☐	**Prepare 80% Ethanol** *Prepare 60 ml for 16 reactions.*	—	—	—

2.1 Post GEM-RT Cleanup – Dynabeads



a. Add **125 µl** Recovery Agent to each sample at room temperature. 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.

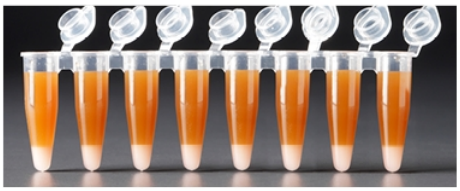
Before using Dynabeads MyOne SILANE to prepare the Dynabeads Cleanup Mix:

- 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	2X + 10% (µl)	16X + 10% (µl)	32X + 10% (µl)
●	**Cleanup Buffer**	2000088/ 2000438	400.4	3203.2	6406.4
	Dynabeads MyOne SILANE	2000048	17.6	140.8	281.6
○	**Reducing Agent B**	2000087	11	88	176
	Nuclease-free Water	—	11	88	176
	Total		**440**	**3520**	**7040**

d. Vortex and add **200 µl** to each tube. Pipette mix 10x (pipette set to 200 µl).

Add Dynabeads Cleanup Mix



e. Incubate **10 min** at **room temperature**. 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)	40X (µl)
	Buffer EB	—	98	3920
	10% Tween 20	—	1	40
○	**Reducing Agent B**	2000087	1	40
	Total		**100**	**4000**

TIPS

g. At the end of 10 min incubation, place the tube strips in slots 1-4 of a 10x Magnetic Separator HT• **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 magnet• **Low.**

n. Remove remaining ethanol. Air dry for **1 min**.

o. To avoid over drying the pellet, **immediately** add **36 µl** Elution Solution I to the tube strips, cap the tubes, remove all tube strips from the magnet, and centrifuge briefly.

p. Pipette mix (pipette set to 30 µl) without introducing bubbles.

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

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

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

2.2 cDNA Amplification

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

cDNA Amplification Reaction Mix *Add reagents in the order listed*		**PN**	**2X*+ 10% (µl)**	**16X + 10% (µl)**	**32X + 10% (µl)**
○	**Amp Mix** *Retrieve from Single Cell 5' HT GEM kit*	2000047/ 2000440	110	880	1760
●	**Feature cDNA Primers 4**	2000277	33	264	528
	Total		**143**	**1144**	**2288**

** 2X =1 sample; Two independent cDNA amp reactions are required for each sample in a single GEM well.*

b. Add **65 µl** cDNA Amplification Reaction Mix to **35 µl** sample.

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

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

Lid Temperature	**Reaction Volume**	**Run Time**
105°C	100 µl	~30-45 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

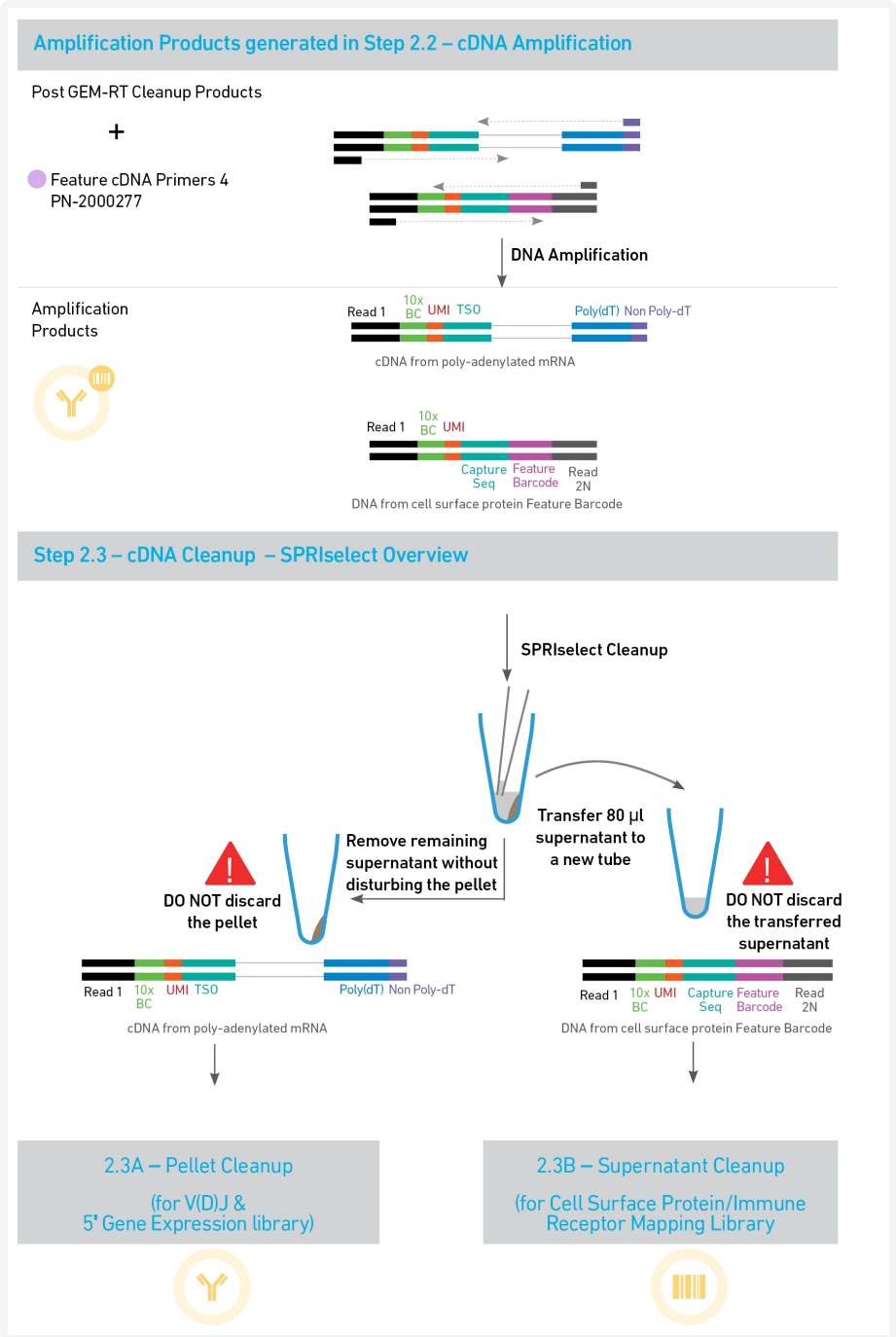
Recommended starting point for cycle number optimization. The optimal cycle number is a trade-off between generating sufficient final mass for libraries & minimizing PCR amplification artifacts. Select PCR cycles based on the target recovery per GEM well and not on the estimated number of cells in the two tubes containing the split sample. The cDNA cycles should be reduced if sampling large numbers of cells.

Targeted Cell Recovery (per GEM well)	**Total Cycles** *Low RNA Cells (primary cells)*	**Total Cycles** *High RNA Cells (cell lines)*
≤4,000	16	14
4,000-12,000	14	12
>12,000	13	11

STOP

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

Step Overview (steps 2.2 & 2.3)



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 **80 µl** supernatant in a new tube strip without disturbing the pellet. Maintain at **room temperature**. DO NOT discard the transferred supernatant (cleanup for Cell Surface Protein/Immune Receptor Mapping library construction; 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 and 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.

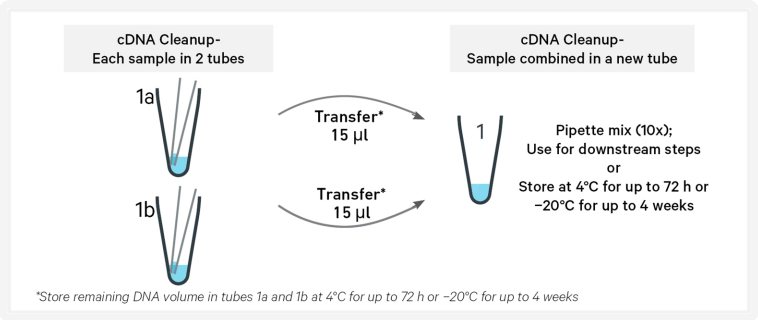
vi. Remove from the magnet. Add **46 µl** Buffer EB. Pipette mix 15x (pipette set to 35 µl).

vii. Incubate 2 min at room temperature.

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

ix. Transfer **45 µl** sample to a new tube strip.

*For each sample, the amplified cDNA will be in two corresponding tubes. Transfer **15 µl** cDNA from each of the two tubes to a third tube; pipette mix. Use for downstream steps. Store remaining cDNA in tubes at **4°C** for up to **72 h** or **−20°C** for up to **4 weeks** for additional libraries.*



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 steps 3-5 for V(D)J & 5' Gene Expression Library Construction.

2.3B Transferred Supernatant Cleanup for Cell Surface Protein/Immune Receptor Mapping

i. Vortex to resuspend the SPRIselect reagent. Add **70 µl** SPRIselect reagent (2.0X) to **80 µl** of the transferred supernatant and pipette mix 15x (pipette

set to 130 µl).

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

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

iv. Remove supernatant.

v. Add **300 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.

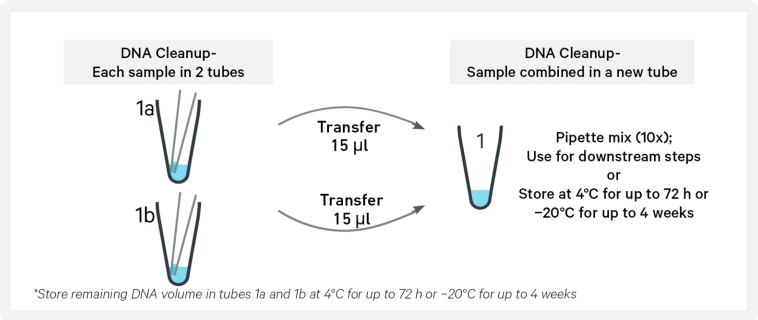
x. Remove from the magnet. Add **46 µl** Buffer EB. Pipette mix 15x (pipette set to 35 µl).

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

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

xiii. Transfer **45 µl** sample to a new tube strip.

*For each sample, the amplified DNA will be in two corresponding tubes. Transfer **15 µl** DNA from each of the two sample tubes to a third tube; pipette mix. Use for downstream steps. Store remaining DNA in tubes at **4°C** for up to **72 h** or **−20°C** for up to **4 weeks** for additional libraries.*



STOP

xiv. Store at **4°C** for up to **72 h** or at **−20°C** for up to **4 weeks**, or proceed directly to step 6 for Cell Surface Protein/immune Receptor Mapping.

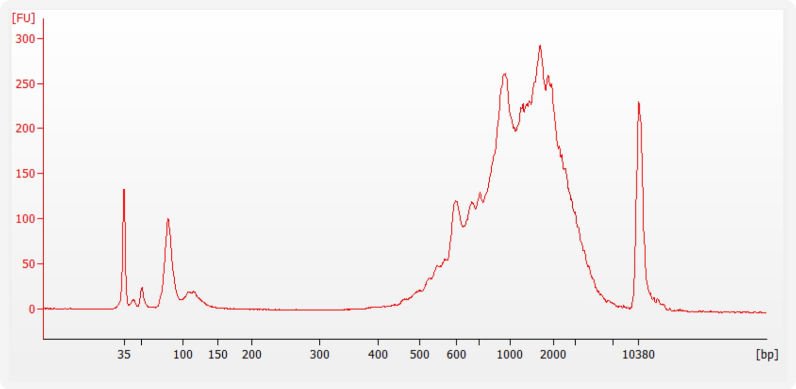
2.4 Post cDNA Amplification QC & Quantification

a. Run **1 µl** undiluted sample from Pellet Cleanup (step 2.3A-x) on an Agilent Bioanalyzer High Sensitivity chip. DO NOT run sample from 2.3B Transferred Supernatant Cleanup step.

Run 1 µl undiluted product for input cells with low RNA content (<1 pg total RNA/cell), and 1 µl of 1:10 diluted product for input cells with high RNA content.

b. If proceeding to 5' GEX Library Construction (step 5), determine the cDNA yield for each sample.

Representative Trace for PBMCs



See example calculation in the following page.

Alternate Quantification Methods

Agilent TapeStation

LabChip

Qubit Fluoromter and Qubit dsDNA HS Assay Kit

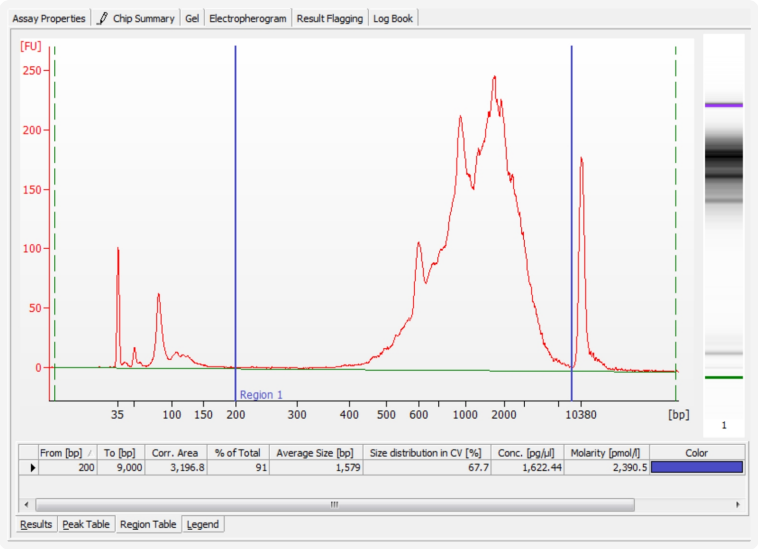
See Appendix for

- Agilent TapeStation Traces on page 116
- LabChip Traces on page 117

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 cDNA concentration [pg/µl] reported via Agilent 2100 Expert Software by dilution factor and divide by 1000 to obtain the total cDNA yield in ng/µl.

Example Calculation of cDNA Total Yield

Concentration: **1622.44 pg/µl** Dilution Factor: **1**

Total cDNA Yield

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

$$= \frac{\text{1622.44 (pg/µl) x 1}}{\text{1000 (pg/ng)}} = \mathbf{1.6\ ng/µl}$$

Carry forward only 20 µl Sample for 5' GEX Library Construction

= 1.6 ng/µl x 20 µl = **32 ng**

Refer to step 5.5 (5' GEX Library Construction) for appropriate number of Sample Index PCR cycles based on carry forward cDNA yield/input cDNA.

Step 3:

V(D)J Amplification from cDNA

3

3.0 Get Started

Overview

Action	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.	—
☐	**Qubit dsDNA HS Assay Kit** *If used for quantification*	—	Manufacturer's recommendations.	—
☐	**Agilent Bioanalyzer High Sensitivity Kit** *If used for QC and quantification*	—	Manufacturer's recommendations.	—

Action	Item	10x PN	Preparation & Handling	Storage
☐	**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*	2000047	Vortex, centrifuge briefly.	−20°C
Obtain				
☐	**Qiagen Buffer EB**	—	Manufacturer's recommendations.	Ambient
☐	**10x Magnetic Separator HT**	2000431	See Tips & Best Practices.	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 step x) 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)**	**8X + 10% (µl)**	**16X + 10% (µl)**
○	**Amp Mix**	2000047	50	440	880
● ●	**T Cell Mix 1 v2** **or** **B Cell Mix 1 v2**	Human 2000242/ Mouse 2000256 or Human 2000254/ Mouse 2000258	48	422.4	844.8
	Total		**98**	**862.4**	**1724.8**

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

STOP

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

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

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

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

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

DO NOT discard supernatant.

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

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

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

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

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

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

j. Remove the ethanol.

k. **Repeat** steps i and j for a total of 2 washes.

l. Centrifuge briefly. Place on the magnet•**Low**

m. Remove remaining ethanol wash. DO NOT over-dry beads to ensure maximum elution efficiency.

n. Remove from the magnet. Add **36 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.

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.

3.3 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)**	**8X + 10% (µl)**	**16X + 10% (µl)**
○	Amp Mix	2000047	50	440	880
●	T Cell Mix 2 v2	Human 2000246/ Mouse 2000257	15	132	264
	or	or			
●	B Cell Mix 2 v2	Human 2000255/ Mouse 2000259			
	Total		65	572	1144

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.

n. Remove from the magnet. Add **46 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 **45 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.

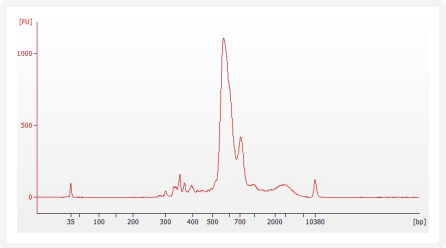
3.5 Post V(DJ) Amplification QC & Quantification

a. Run **1 µl** sample **1:5 dilution** (Dilution Factor 5) on an Agilent Bioanalyzer High Sensitivity chip.

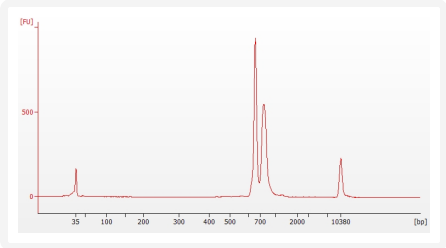
Samples of RNA-rich cells may require additional dilution in nuclease-free water. The number of distinct peaks may vary. Higher molecular weight product (2,000-9,000 bp) may be present. This does not affect sequencing.

Representative Traces

PBMCs amplified for TCR



PBMCs amplified for BCR



See example calculation in the following page.

Alternate Quantification Methods

Agilent TapeStation

LabChip

Qubit Fluorometer and Qubit dsDNA HS Assay Kit

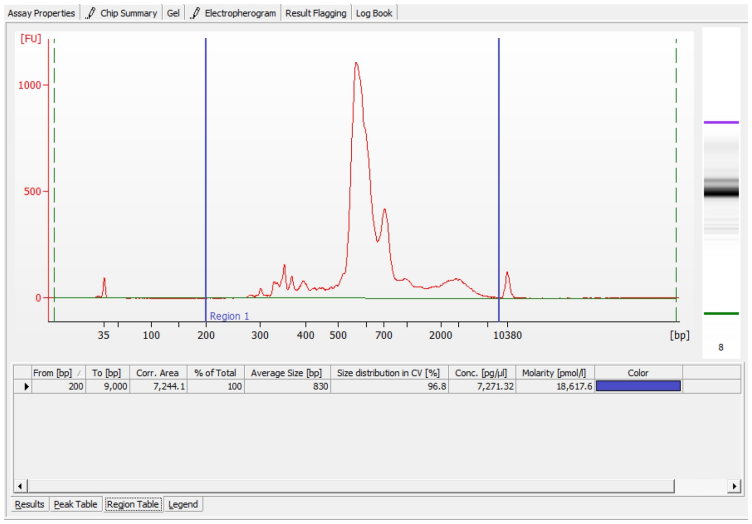
See Appendix for

- Agilent TapeStation Traces on page 116
- LabChip Traces on page 117

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.

Example Calculation of V(D)J Amplified Product Concentration

Concentration: **7271.32 pg/µl** Dilution Factor: **5**

V(D)J Amplified Product Concentration

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

$$= \frac{\text{7271.32 (pg/µl) x 5}}{\text{1000 (pg/ng)}} = \mathbf{36.35\ ng/µl}$$

Carry forward only 10 µl Sample for V(D)J Library Construction

= 36.35 ng/µl x 10 µl = **363.5 ng**

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

Step 4:

V(D)J Library Construction

4

4.0 Get Started

Overview

Action		Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
☐	●	**Fragmentation Buffer**	2000091	Thaw, vortex, verify no precipitate, centrifuge briefly	−20°C
☐	●	**Adaptor Oligos**	2000094	Thaw, vortex, centrifuge briefly.	−20°C
☐	●	**Ligation Buffer**	2000092	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 and quantification*	—	Manufacturer's recommendations.	—
☐		**Agilent TapeStation ScreenTape & Reagents** *If used for QC and quantification*	—	Manufacturer's recommendations.	—
☐		**Qubit dsDNA HS Assay Kit** *If used for quantification*	—	Manufacturer's recommendations.	—
Place on Ice					
☐	●	**Fragmentation Enzyme** *Ensure that Fragmentation Buffer*	2000090	Centrifuge briefly.	−20°C

Action		Item	10x PN	Preparation & Handling	Storage
		and Fragmentation Enzyme from the same kit are used together. Lots are matched for optimal performance			
☐	●	**DNA Ligase**	220110	Centrifuge briefly.	−20°C
☐	○	**Library Amp Mix/Amp Mix** *Retrieve from Library Construction Kit*	2000531/2000047	Vortex, centrifuge briefly.	−20°C
Obtain					
☐		**Qiagen Buffer EB**	—	Manufacturer's recommendations.	Ambient
☐		**10x Magnetic Separator HT**	2000431	See Tips & Best Practices.	Ambient
☐		**Prepare 80% Ethanol** *Prepare 15 ml for 8 reactions*	—	Prepare fresh.	Ambient

4.1 Fragmentation, End Repair & A-tailing

a. Dispense **10 µl** sample in a tube strip **on ice**

b. 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

c. Vortex Fragmentation Buffer. Verify there is no precipitate.

d. Prepare Fragmentation Mix on ice. Pipette mix and centrifuge briefly.

Fragmentation Mix *Add reagents in the order listed*	PN	1X (µl)	8X + 10% (µl)	16X + 10% (µl)
EB Buffer	-	25	220	440
● **Fragmentation Buffer**	2000091	5	44	88
● **Fragmentation Enzyme**	2000090	10	88	176
Total		**40**	**352**	**704**

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)	8X + 10% (µl)	16X + 10% (µl)
Ligation Buffer	2000092	20	176	352
DNA Ligase	220110	10	88	176
Adaptor Oligos	2000094	20	176	352
Total		**50**	**440**	**880**

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	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 **31 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** Amp Mix/Amp Mix (PN-2000531/2000047) 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 total cycles should be optimized based on 10 µl carry forward V(D)J amplified product concentration calculated during Post V(D)J Amplification QC & Quantification (step 3.5).

Recommended Cycle Numbers

V(D)J Amplified Product Input	Total Cycles
<25 ng	9
25-150 ng	8
151-500 ng	7
501-1,000 ng	6
>1000 ng	5

STOP

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

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 **36 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.

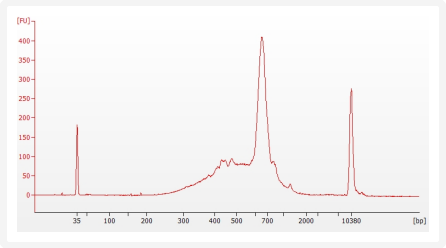
4.6 Post Library Construction QC

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

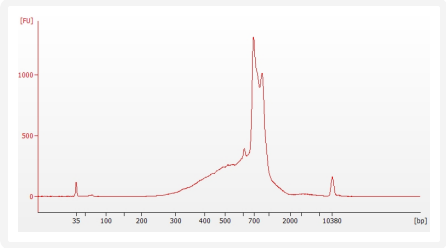
b. Determine the average fragment size from the Bioanalyzer trace. This will be used as the insert size for library quantification.

Representative Traces

PBMCs amplified for TCR



PBMCs amplified for BCR



Alternate QC Method

Agilent TapeStation

LabChip

See Appendix for:

- Post Library Construction Quantification on page 115
- Agilent TapeStation Traces on page 116
- LabChip Traces on page 117

Step 5:

5' Gene Expression Library Construction

5

5.0 Get Started

Overview

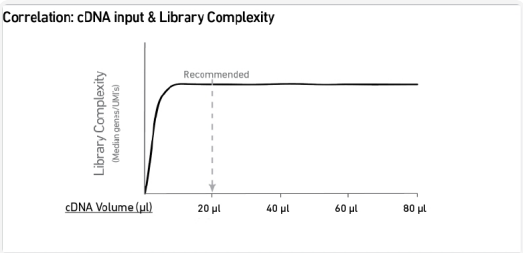
Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐ ●	**Fragmentation Buffer**	2000091	Thaw, vortex, verify no precipitate, centrifuge.	−20°C
☐ ●	**Adaptor Oligos**	2000094	Thaw, vortex, centrifuge briefly.	−20°C
☐ ●	**Ligation Buffer**	2000092	Thaw, vortex thoroughly (≥30 sec) immediately before adding to the mix.	−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 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	Centrifuge briefly.	−20°C

Action	Item	10x PN	Preparation & Handling	Storage
☐	**DNA Ligase**	220110	Centrifuge briefly.	−20°C
☐	**Amp Mix** *Retrieve from Library Construction Kit.*	2000047	Centrifuge briefly.	−20°C
☐	**KAPA Library Quantification Kit for Illumina Platforms**	—	Manufacturer's recommendations.	—
Obtain				
☐	**Qiagen Buffer EB**	—	Manufacturer's recommendations.	Ambient
☐	**10x Magnetic Separator HT**	2000431	See Tips & Best Practices.	Ambient
☐	**Prepare 80% Ethanol** *Prepare 20 ml for 8 reactions.*	—	Prepare fresh.	Ambient

Step Overview (Step 5.1d)

Correlation between input & library complexity

A Single Cell 5' Gene Expression library is generated using a fixed volume (20 µl) of the total cDNA obtained at step2.3A-ix. The complexity of this library will be comparable to one generated using a higher volume of the cDNA. The remaining proportion of the cDNA may be stored at **4°C** for up to **72 h** or at **−20°C** for longer-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 (20 µl) 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	562.5 ng	20 µl	125 ng	12
	High	4275 ng	20 µl	950 ng	9
Low RNA Content	Low	2.25 ng	20 µl	0.5 ng	16
	High	450 ng	20 µl	100 ng	13

5.1 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 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. Pipette mix and centrifuge briefly.

Fragmentation Mix *Add reagents in the order listed*	PN	1X (µl)	8X + 10% (µl)	16X + 10% (µl)
Buffer EB	-	15	132	264
● **Fragmentation Buffer**	2000091	5	44	88
● **Fragmentation Enzyme**	2000090	10	88	176
Total		**30**	**264**	**528**

d. Transfer **ONLY 20 µl** purified cDNA sample from Pellet Cleanup (step 2.3A-x) to a tube strip.

- Note that only **20 µl** cDNA sample is sufficient for generating 5' 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 5' Gene Expression libraries.

e. Add **30 µl** Fragmentation Mix into each tube containing **20 µl** sample.

f. Pipette mix 15x (pipette set to 35 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 **(0.6X)** reagent 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. 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. Remove remaining ethanol pipetting slowly. DO NOT over dry to ensure maximum elution efficiency.

m. Remove from the magnet. Add **51 µl** Buffer EB to each sample. Pipette mix 15x.

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

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

p. Transfer **50 µl** sample to a new tube strip pipetting slowly.

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)**	**8X + 10% (µl)**	**16X + 10% (µl)**
Ligation Buffer	2000092	20	176	352
DNA Ligase	220110	10	88	176
Adaptor Oligos	2000094	20	176	352
Total		**50**	**440**	**880**

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	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 **31 µl** Buffer EB. Pipette mix 15x.

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

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

m. Transfer **30 µl** sample to a new tube strip.

5.5 GEX Sample Index PCR

a. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x sample index name (PN-3000431 Dual Index Plate TT Set A well ID) used.

b. Add **50 µl** Amp Mix (PN-2000047) 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	~25-40 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	54°C	00:00:30
4	72°C	00:00:20
5	Go to step 2, see below for # of cycles	
6	72°C	00:01:00
7	4°C	Hold

The total cycles should be optimized based on 20 µl carry forward cDNA yield/input calculated during Post cDNA Amplification QC & Quantification (step 2.4).

Recommended Cycle Numbers

cDNA Input	Total Cycles
0.25-25 ng	14-16
25-150 ng	12-14
150-500 ng	10-12
500-1,000 ng	8-10
1,000-1,500 ng	6-8
>1500 ng	5

STOP

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

5.6 GEX 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**. Remove remaining ethanol.

m. Remove from the magnet. Add **36 µl** Buffer EB. Pipette mix 15x.

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

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

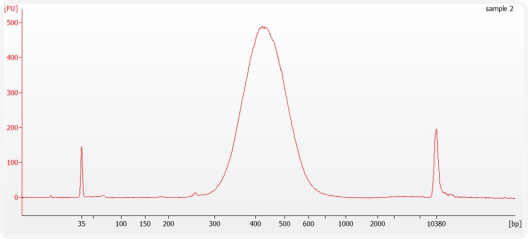
p. Transfer **35 µl** to a new tube strip.

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

5.7 Post Library Construction QC

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

Representative Trace



Determine the average fragment size from the Bioanalyzer trace. This will be used as the insert size for library quantification.

Alternate QC Method

Agilent TapeStation

LabChip

See Appendix for:

- Post Library Construction Quantification on page 115
- Agilent TapeStation Traces on page 116
- LabChip Traces on page 117

Step 6:

Cell Surface Protein/Immune Receptor Mapping Library Construction

6

6.0 Get Started

Overview

Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐ ⚠	**Dual Index Plate TN Set A** *Verify name & PN. Use indicated plate only*	3000510	—	−20°C
☐	**Beckman Coulter SPRIselect Reagent**	—	Manufacturer's recommendations.	—
☐	**Agilent Bioanalyzer High Sensitivity Kit** *If used for QC*	—	Manufacturer's recommendations.	—
☐	**Agilent TapeStation ScreenTape & Reagents** *If used for QC*	—	Manufacturer's recommendations.	—
Place on Ice				
☐ ○	**Amp Mix** *Retrieve from 5' Feature Barcode Kit*	2000047	Centrifuge briefly.	−20°C
☐	**KAPA Library Quantification Kit for Illumina Platforms**	—	Manufacturer's recommendations.	—
Obtain				
☐	**Qiagen Buffer EB**	—	Manufacturer's recommendations.	Ambient
☐	**10x Magnetic Separator HT**	2000431	See Tips & Best Practices.	Ambient
☐	**Prepare 80% Ethanol** *Prepare 20 ml for 8 reactions.*	—	Prepare fresh.	Ambient

6.1 Sample Index PCR

a. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x sample index name (PN-3000510 Dual Index Plate TN Set A well ID) used.

b. Prepare Sample Index PCR Mix.

Sample Index PCR Mix *Add reagents in the order listed*		**PN**	**1X (µl)**	**8X + 10% (µl)**	**16X + 10% (µl)**
◯	**Amp Mix** *Retrieve from 5' Feature Barcode Kit*	2000047	50	440	880
	Nuclease-free Water	—	20	176	352
	Total		**70**	**616**	**1232**

c. Transfer **ONLY 10 µl** from the Transferred Supernatant Cleanup (step 2.3B-xiv) to a new tube strip.

- Note that only **10 µl** sample transfer is sufficient for generating Cell Surface Protein library.
- The remaining sample can be stored at **4°C** for up to **72 h** or at **−20°C** for up to **4 weeks**, for generating additional libraries.

d. Add **70 µl** Sample Index PCR Mix to **10 µl** Transferred Supernatant Cleanup sample.

e. Add **20 µl** of an individual Dual Index TN Set A to each sample and record the well ID used. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.

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

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25-40 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	54°C	00:00:30
4	72°C	00:00:20
5	Go to step 2, repeat 7X for a total of 8 cycles*	
6	72°C	00:01:00
7	4°C	Hold

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

6.2 Post Sample Index PCR Size Selection – SPRIselect

a. Vortex to resuspend the SPRIselect reagent. Add **120 µl** SPRIselect Reagent **(1.2X)** to each sample. Pipette mix 15x (pipette set to 150 µl).

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

c. Place the magnet•**High** until the solution clears. Remove the supernatant.

d. Add **300 µl** 80% ethanol to the pellet. Wait 30 sec.

e. Remove the ethanol.

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

g. Remove the ethanol.

h. Centrifuge briefly. Place on the magnet•**Low**.

i. Remove remaining ethanol. Air dry for **1 min**.

j. Remove from the magnet. Add **36 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.

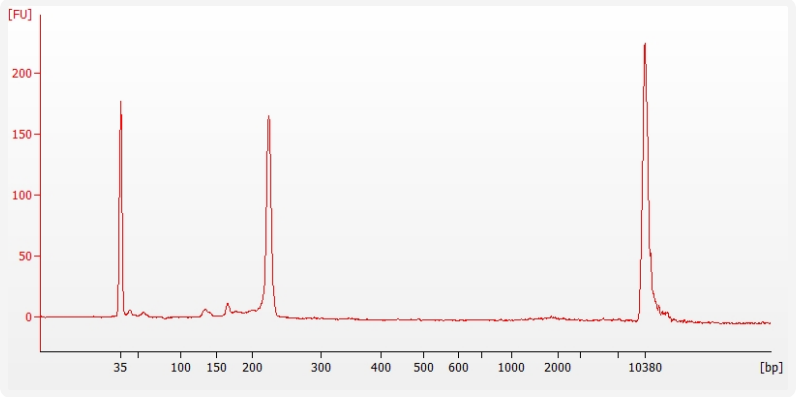
STOP

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

6.3 Post Library Construction QC

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

Representative Trace



Determine the average fragment size from the Bioanalyzer trace. This will be used as the insert size for library quantification.

Alternate QC Method

- Agilent TapeStation
- LabChip

See Appendix for:

- Post Library Construction Quantification on page 115
- Agilent TapeStation Traces on page 116
- LabChip Traces on page 117

Step 7:

Sequencing

7

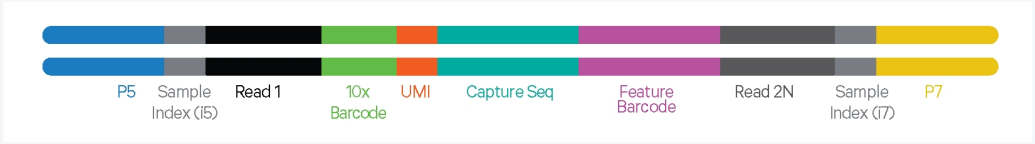
Sequencing Libraries

Chromium Single Cell V(D)J, 5' Gene Expression, and Cell Surface Protein Dual Index libraries comprise standard Illumina paired-end constructs which begin with P5 and end with P7. These libraries include 16 bp 10x Barcodes encoded at the start of TruSeq Read 1. Sample index sequences are incorporated as the i5 and i7 index read for V(D)J and 5' Gene Expression libraries; as i5 and i7 index read N for Cell Surface Protein library. TruSeq Read 1, TruSeq Read 2, and Nextera Read 2 (Read 2N) are all standard Illumina sequencing primer sites. TruSeq Read 1 and TruSeq Read 2 are used in paired-end sequencing of V(D)J and 5' Gene Expression libraries. TruSeq Read 1 and Nextera Read 2 (Read 2N) are used for paired-end sequencing of Cell Surface Protein library. Sequencing these libraries produce a standard Illumina BCL data output folder.

Chromium Single Cell V(D)J Dual Index Library

Chromium Single Cell 5' Gene Expression Dual Index Library

Chromium Single Cell 5' Cell Surface Protein Dual Index Library



Illumina Sequencer Compatibility

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

- MiSeq
- NextSeq 500/550

- NextSeq 1000/2000
- HiSeq 2500 (Rapid Run)
- HiSeq 3000/4000
- NovaSeq

Sample Indices

Each sample index in the Dual Index Kit TT Set A (PN-1000215) or Dual Index Kit TN Set A (PN-1000250) is 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 TT Set A plate well ID, SI-TT-) is needed in the sample sheet used for generating FASTQs. 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 Sequencing Depth & Run Parameters

Parameter	Description
Sequencing Depth	Minimum 5,000 read pairs/cell for V(D)J Dual Index library Minimum 20,000 read pairs/cell for 5' Gene Expression Dual Index library Minimum 5,000 read pairs/cell for 5' Cell Surface Protein Dual Index library
Sequencing Type	Paired-end, dual indexing
Sequencing Read	Recommended Number of Cycles
Read 1 i7 Index i5 Index Read 2	26 cycles 10 cycles 10 cycles 90 cycles

Library Loading

Once quantified and normalized, the 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.

Library Loading

Instrument	Loading Concentration (pM)	PhiX (%)
MiSeq	10	1
NextSeq 500	1.5	1
HiSeq 2500 (RR)	10	1
HiSeq 4000	180	1
NovaSeq	150*/300	1
NextSeq 2000	650	1

** Use 150 pM loading concentration for Illumina XP workflow.*

Library Pooling

V(D)J, 5' Gene Expression, and Cell Surface Protein libraries maybe pooled for sequencing, taking into account the differences in cell number and per-cell read depth requirements between each library. Samples utilizing the same sample index should not be pooled together or run on the same flow cell lane, as this would not enable correct sample demultiplexing.

Library Pooling Example

Libraries	Sequencing Depth (read pairs per cell)	Library Pooling Ratio
Example 1		
V(D)J library	5,000	1
5' Gene Expression library	20,000	4
Cell Surface Protein library	5,000	1
Example 2		
V(D)J library	5,000	1
5' Gene Expression library	50,000	10
Cell Surface Protein library	5,000	1

Data Analysis and Visualization

Sequencing data may be analyzed using Cell Ranger or 10x Genomics Cloud Analysis 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: 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 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

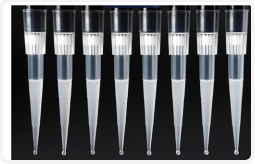
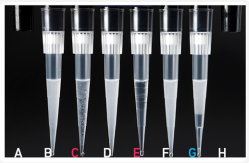
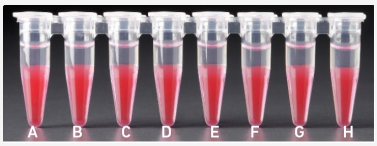
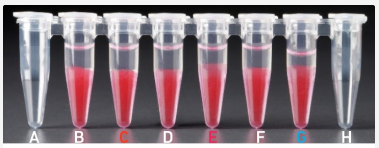
Loupe Browser is an interactive data visualization tool that requires no prior programming knowledge.

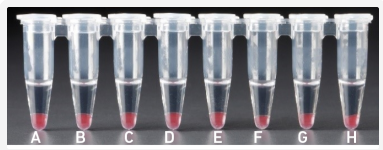
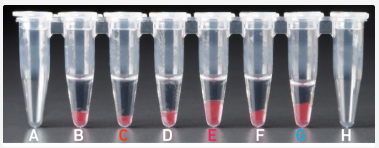
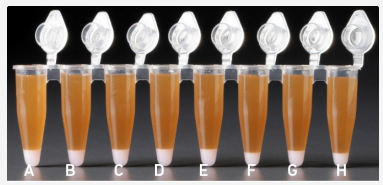
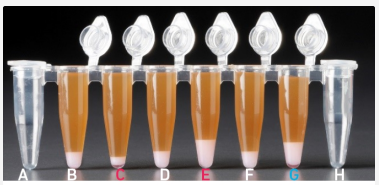
- Input: .cloupe
- Output: Data visualization, including t-SNE and UMAP projections, custom clusters, differentially expressed genes
- Operating System: MacOS, Windows

Troubleshooting

8

GEMs

Step	Normal	Reagent Clogs & Wetting Failures
1.4d **After Chip N is removed from Chromium X and the wells are exposed**	 All 16 recovery wells (rows 3A, 3B) (rows 3A, 3B) are similar in volume and opacity.	 Recovery well G indicates a reagent clog. Recovery well C and E indicate a wetting failure. Wells A, H, I & P contain 50% Glycerol Solution. Rest of the recovery wells have normal GEM generation.
1.4f **Transfer GEMs**	 All liquid levels are similar in volume and opacity without air trapped in the pipette tips.	 Pipette tips C and E indicate a wetting failure. Pipette tip C contains partially emulsified GEMs. Emulsion is absent in pipette tip E. Pipette tip G indicates a reagent clog.
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).

Step	Normal	Reagent Clogs & Wetting Failures
2.1 b 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).
2.1 d 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.

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

9

Post Library Construction Quantification

a. Thaw KAPA Library Quantification Kit for Illumina Platforms.

b. Dilute **2 µl** sample with deionized water to appropriate dilutions that fall within the linear detection range of the KAPA Library Quantification Kit for Illumina Platforms. (For more accurate quantification, make the dilution(s) in duplicate).

c. Make enough Quantification Master Mix for the DNA dilutions per sample and the DNA Standards (plus 10% excess) using the guidance for 1 reaction volume below.

Quantification Master Mix	1X (µl)
SYBR Fast Master Mix + Primer	12
Water	4
Total	**16**

d. Dispense **16 µl** Quantification Master Mix for sample dilutions and DNA Standards into a 96 well PCR plate.

e. Add **4 µl** sample dilutions and **4 µl** DNA Standards to appropriate wells. Centrifuge briefly.

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

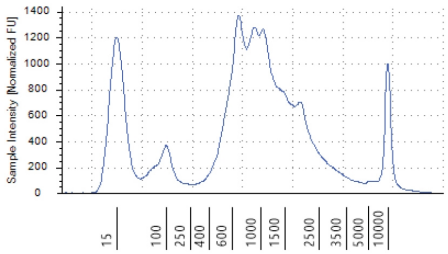
Step	Temperature	Run Time
1	95°C	00:03:00
2	95°C	00:00:05
3	67°C Read Signal	00:00:30
4	Go to Step 2, 29X (Total 30 cycles)	

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

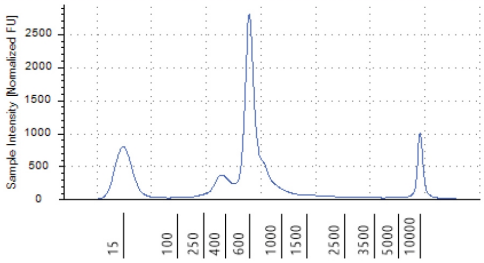
Agilent TapeStation Traces

Agilent TapeStation High Sensitivity D5000 ScreenTape was used. Protocol steps correspond to the steps in this user guide.

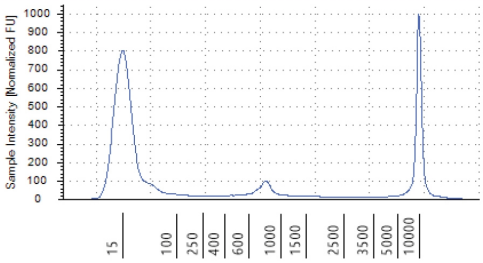
Protocol Step 2.4 – cDNA QC & Quantification



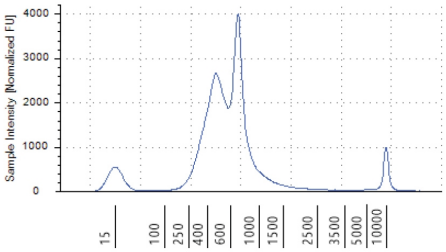
Protocol Step 3.5 – Post TCR Amplification QC



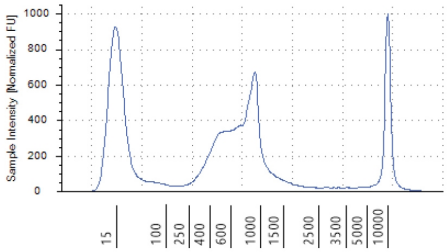
Protocol Step 3.5 – Post BCR Amplification QC



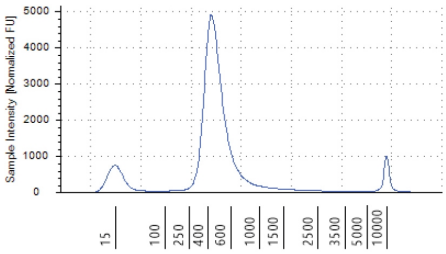
Protocol Step 4.6 – Post Library Construction QC
(PBMCs amplified for TCR)



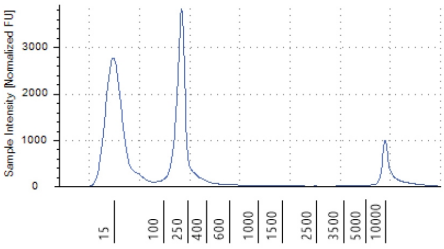
Protocol Step 4.6 – Post Library Construction QC
(PBMCs amplified for BCR)



Protocol Step 5.7 – GEX Post Library Construction QC



Protocol Step 6.3 – Post Library Construction QC

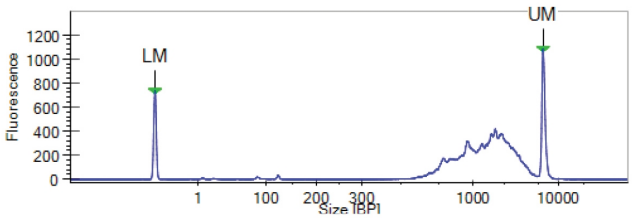


All traces are representative

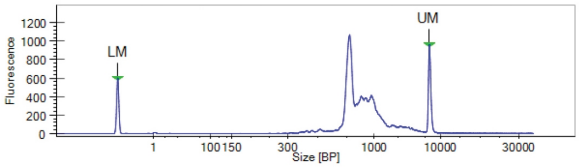
LabChip Traces

DNA High Sensitivity Reagent Kit was used. Protocol steps correspond to the steps in this user guide.

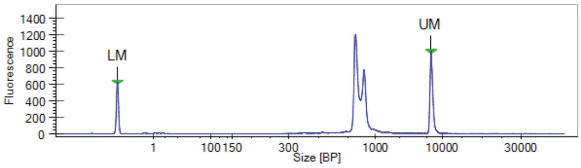
Protocol Step 2.4 – cDNA QC & Quantification



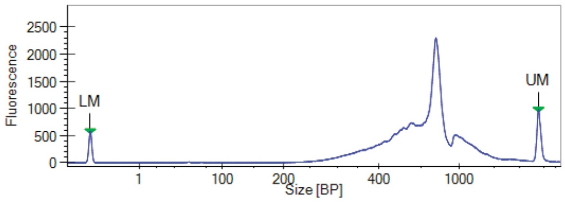
Protocol Step 3.5 – Post TCR Amplification QC



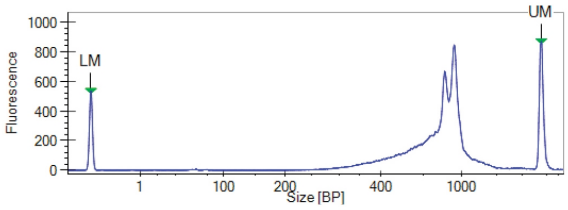
Protocol Step 3.5 – Post BCR Amplification QC



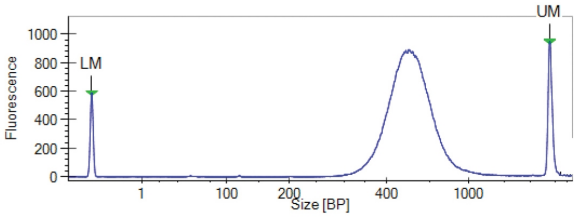
Protocol Step 4.6 – Post Library Construction QC
(PBMCs amplified for TCR)



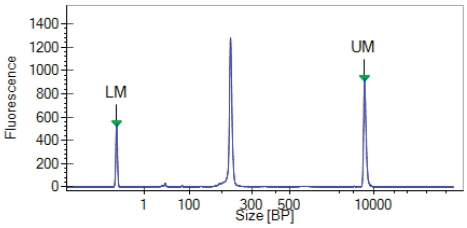
Protocol Step 4.6 – Post Library Construction QC
(PBMCs amplified for BCR)



Protocol Step 5.7 – GEX Post Library Construction QC



Protocol Step 6.3 – Post Library Construction QC



All traces are representative

Oligonucleotide Sequences

Protocol Step 1.5 – GEM-RT Incubation

Gel Bead Primer

5'-CTACACGACGCTCTTCCGATCT-N16-N10-TTTCTTATATrGrGrG-3'

Poly-dT RT Primer PN-2000437/2000007

5'-AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'

Feature Barcode

3'-AAAGAATATACCC-N9-N15-N10-GACAGAGAATATGTGTAGAGGC-5'

GEM-RT Products

cDNA from poly-adenylated mRNA

3'-GATGTGCTGCGAGAAGGCTAGA-N16-N10-AAAGAATATACCC-cDNA_Insert-NVTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-CATGAGACGCAACTATGGTGACGAA-5'

DNA from cell surface protein Feature Barcode

5'-CTACACGACGCTCTTCCGATCT-N16-N10-TTTCTTATATGGG-N9-N15-N10-CTGTCTCTTATACACATCTCCG-3'
3'-GATGTGCTGCGAGAAGGCTAGA-N16-N10-AAAGAATATACCC-N9-N15-N10-GACAGAGAATATGTGTAGAGGC-5'

Protocol Step 2.2 – cDNA Amplification

Feature cDNA Primers 4 -2000277

Amplifies cDNA

Forward Primer: Partial Read 1
5'-CTACACGACGCTCTTCCGATCT-3'

Reverse Primer: Non-poly(dT)
5'-AAGCAGTGGTATCAACGCAGAG-3'

Amplifies DNA from cell surface protein Feature Barcode

Forward Primer: Partial Read 1
5'-CTACACGACGCTCTTCCGATCT-3'

Reverse Primer: Read 2N
5'-CTCGTGGGCTCGGAGATGTG-3'

Amplified Products

Amplified cDNA from poly-adenylated mRNA

5'-CTACACGACGCTCTTCCGATCT-N16-N10-TTTCTTATATGGG-cDNA_Insert-GTACTCTGCGTTGATACCACTGCTT-3'
3'-GATGTGCTGCGAGAAGGCTAGA-N16-N10-AAAGAATATACCC-cDNA_Insert-CATGAGACGCAACTATGGTGACGAA-5'

Amplified DNA from cell surface protein Feature Barcode

5'-CTACACGACGCTCTTCCGATCT-N16-N10-TTTCTTATATGGG-N9-N15-N10-CTGTCTCTTATACACATCTCCGAGCCCACGAG-3'
3'-GATGTGCTGCGAGAAGGCTAGA-N16-N10-AAAGAATATACCC-N9-N15-N10-GACAGAGAATATGTGTAGAGGCTCGGGTGCTC-5'

Protocol Step 3.1 – V(D)J Amplification 1				
Human T Cell Mix 1 v2 PN-2000242	Forward Primer: 5'-GATCTACACTCTTTCCCTACACGACGC-3'	PCR Primer	Reverse Outer Primers: 5'-TGAAGGCGTTTGCACATGCA-3' 5'-TCAGGCAGTATCTGGAGTCATTGAG-3	Outer Primer
Human B Cell Mix 1 v2 PN-2000254	Forward Primer: 5'-GATCTACACTCTTTCCCTACACGACGC-3'	PCR Primer	Reverse Outer Primers: 5'-CAGGGCACAGTCACATCCT-3' 5'-TGCTGGACCACGCATTTGTA-3' 5'-GGTTTTTGTTGTCGACCCAGTCT-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: 5'-GATCTACACTCTTTCCCTACACGACGC-3'	PCR Primer	Reverse Outer Primers: 5'-CTGGTTGCTCCAGGCAATGG-3' 5'-TGTAGGCCTGAGGGTCCGT-3'	Outer Primer
Mouse B Cell Mix 1 v2 PN-2000258	Forward Primer: 5'-GATCTACACTCTTTCCCTACACGACGC-3'	PCR Primer	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: 5'-GATCTACACTCTTTCCCTACACGACGC-3'	PCR Primer	Reverse Inner Primers: 5'-AGTCTCTCAGCTGGTACACG-3' 5'-TCTGATGGCTCAAACACAGC-3'	Inner Primer
Human B Cell Mix 2 v2 PN-2000255	Forward Primer: 5'-GATCTACACTCTTTCCCTACACGACGC-3'	PCR Primer	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: 5'-GATCTACACTCTTTCCCTACACGACGC-3'	PCR Primer	Reverse Inner Primers: 5'-AGTCAAAGTCGGTGAACAGGCA-3' 5'-GGCCAAGCACACGAGGGTA-3'	Inner Primer
Mouse B Cell Mix 2 v2 PN-2000259	Forward Primer: 5'-GATCTACACTCTTTCCCTACACGACGC-3'	PCR Primer	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

V(D)J Amplification Product

5'-GATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N10-TTTCTTATATGGG-cDNA_Insert-Inner_Primer-3'
3'-CTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N10-AAAGAATATACCC-cDNA_Insert-Inner_Primer-5'

Protocol Step 4.2 – Adaptor Ligation (for V(D)J Library Construction)

Adaptor (Read 2) PN-220026

5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'
3'-TCTAGCCTTCTCG-5'

Ligation Product

5'-GATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N10-TTTCTTATATGGG-cDNA_Insert-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'
3'-CTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N10-AAAGAATATACCC-cDNA_Insert-TCTAGCCTTCTCG-5'

Protocol Step 4.4 – Sample Index PCR (for V(D)J Library Construction)

Dual Indexing

Dual Index Kit TT Set A PN-1000215

Forward Primer:

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTC-3'

Reverse Primer:

5'-CAAGCAGAAGACGGCATACGAGAT-N10-GTGACTGGAGTTCAGACGTGT-3'

Sample Index PCR Product

5'-AATGATACGGCGACCACCGAGATCTACAC-N10 -ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N10-TTTCTTATATGGG-Insert-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'
3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N10-AAAGAATATACCC-Insert-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'

Protocol Step 5.3 –GEX Adaptor Ligation (for 5' Gene Expression (GEX) Library Construction)

Adaptor (Read 2) PN-220026

5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'
3'-TCTAGCCTTCTCG-5'

Ligation Product

5'-CTACACGACGCTCTTCCGATCT-N16-N10-TTTCTTATATGGG-cDNA_Insert-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'
3'-GATGTGCTGCGAGAAGGCTAGA-N16-N10-AAAGAATATACCC-cDNA_Insert-TCTAGCCTTCTCG-5'

Protocol Step 5.5 – Sample Index PCR (for 5' Gene Expression (GEX) Library Construction)

Dual Indexing

Dual Index Kit
TT Set A
PN-1000215

Forward Primer:

5'-AATGATACGGCGACCACCGAGATCT-N10-ACACTCTTTCCCTACACGACGCTC-3'

Reverse Primer:

5'-CAAGCAGAAGACGGCATACGAGAT-N10-GTGACTGGAGTTCAGACGTGT-3'

Sample Index PCR Product

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N10-TTTCTTATATGGG-cDNA_Insert-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'
3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N10-AAAGAATATACCC-cDNA_Insert-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'

Protocol Step 6.1 – Sample Index PCR (for Cell Surface Protein/Immune Receptor Mapping Library Construction)

Dual Indexing

Dual Index Kit
TN Set A
PN-1000250

Forward Primer:

5'-AATGATACGGCGACCACCGAGATCT-N10-ACACTCTTTCCCTACACGACGCTC-3'

Reverse Primer:

5'-CAAGCAGAAGACGGCATACGAGAT-N10-GTCTCGTGGGCTCGG-3'

Sample Index PCR Product

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N10-TTTCTTATATGGG-N9-N15-N10-CTGTCTCTTATACACATCTCCGAGCCCACGAGAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'
3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N10-AAAGAATATACCC-N9-N15-N10-GACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'