10x GENOMICS

User Guide | CG000420 | Rev C

Chromium Next GEM Single Cell 3' HT Reagent Kits v3.1 (Dual Index)

with Feature Barcode technology for Cell Surface Protein & Cell Multiplexing

For use with:

Chromium Next GEM Single Cell 3' HT Kit v3.1
48 rxns PN-1000348 | 8 rxns PN-1000370

Chromium Next GEM Chip M Single Cell Kit*
80 rxns PN-1000349 | 16 rxns PN-1000371
*(*Included with Chromium Next GEM Single Cell 3' HT Kit v3.1; 16 rxn kit can also be ordered separately)*

3' Feature Barcode Kit *16 rxns PN-1000262*

3' CellPlex Kit Set A *48 rxns PN-1000261*

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

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

Dual Index Kit NN Set A *96 rxns PN-1000243*

Notices

Document Number

CG000420 | 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.

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Document Revision Summary

Document Number

CG000420 | Rev C

Title

Chromium Next GEM Single Cell 3' HT Reagent Kits v3.1
(Dual Index) with Feature Barcode technology for Cell Surface Protein & Cell Multiplexing

Revision

Rev B to Rev C

Revision Date

April 18, 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 step 4.2c to specify supernatant removal

General Changes

Updated for general minor consistency of language and terms throughout.

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Introduction

Chromium Next GEM Single Cell 3' HT Reagent Kits v3.1

Refer to SDS for handling and disposal information

Chromium Next GEM Single Cell 3' HT Kit v3.1, 48 rxns PN-1000348

Chromium Next GEM
Single Cell 3' HT GEM Kit v3.1
48 rxns, PN-1000351
Store at -20°C

	#	PN
RT Reagent B	3	2000435
RT Enzyme C	3	2000436
Template Switch Oligo	6	3000228
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

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Chromium Next GEM
Single Cell 3' HT Gel Bead Kit v3.1
48 rxns, PN-1000350
Store at -80°C

	#	PN
Single Cell 3' HT Gel Beads v3.1	3	2000443

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

	#	PN
Dynabeads MyOne SILANE	6	2000048

Chromium Next GEM Chip M Single Cell Kit, 80 rxns PN-1000349

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 M & Gaskets
Store at ambient temperature

	#	PN
Chromium Next GEM Chip M	5	2000417
Chip Gasket, HT, 5-pack	1	3000614

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Chromium Next GEM Single Cell 3' HT Kit v3.1, 8 rxns PN-1000370

Chromium Next GEM
Single Cell 3' HT GEM Kit v3.1
8 rxns, PN-1000373
Store at -20°C

		#	PN
●	RT Reagent B	1	2000165
●	RT Enzyme C	1	2000085
●	Template Switch Oligo	1	3000228
○	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

10x GENOMICS

Chromium Next GEM
Single Cell 3' HT Gel Bead Kit v3.1
8 rxns, PN-1000372
Store at -80°C

	#	PN
Single Cell 3' HT Gel Beads v3.1 (8 rxns)	1	2000443

10x GENOMICS

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

	#	PN
Dynabeads MyOne SILANE	2	2000048

Chromium Next GEM Chip M Single Cell Kit, 16 rxns PN-1000371

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 M & Gaskets
Store at ambient temperature

	#	PN
Chromium Next GEM Chip M	1	2000417
Chip Gasket, HT, 2-pack	1	3000656

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3' Feature Barcode Kit, 16 rxns PN-1000262

Two 3' Feature Barcode 16 rxn kits are required for processing 16 samples.

3' Feature Barcode Kit
16 rxns, PN-1000262
Store at -20°C

	#	PN
Feature cDNA Primers 1	1	2000096
Feature cDNA Primers 2	1	2000097
Feature cDNA Primers 3	1	2000289
Feature SI Primers 3	1	2000263
Amp Mix	3	2000047

10x GENOMICS

3' CellPlex Kit Set A, 48 rxns PN-1000261

3' CellPlex Kit Set A
48 rxns, PN-1000261
Store at -20°C

	#	PN
Cell Multiplexing Oligo 301	1	2000307
Cell Multiplexing Oligo 302	1	2000308
Cell Multiplexing Oligo 303	1	2000309
Cell Multiplexing Oligo 304	1	2000310
Cell Multiplexing Oligo 305	1	2000311
Cell Multiplexing Oligo 306	1	2000312
Cell Multiplexing Oligo 307	1	2000313
Cell Multiplexing Oligo 308	1	2000314
Cell Multiplexing Oligo 309	1	2000315
Cell Multiplexing Oligo 310	1	2000316
Cell Multiplexing Oligo 311	1	2000317
Cell Multiplexing Oligo 312	1	2000318

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

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

	#	PN
Dual Index Plate TT Set A	1	3000431

Dual Index Kit NN Set A, 96 rxns PN-1000243

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

	#	PN
Dual Index Plate NN Set A	1	3000482

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

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

	#	PN
Dual Index Plate NT Set A	1	3000483

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 D1000 ScreenTape/Reagents	5067-5592/ 5067-5593
	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

Day	Steps		Timing	Stop & Store
1 h	**Cell Preparation & Labeling**			
	Dependent on Cell Type		~1-2 h	
	Step 1 – GEM Generation & Barcoding			
4h	1.1	Prepare Reaction Mix	20 min	
	1.2	Load Chromium Next GEM Chip M	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			
	2.1	Post GEM RT-Cleanup – Dynabead	60 min	
	2.2	cDNA Amplification	40 min	STOP 4°C ≤72 h or –20°C ≤1 week
6h	2.3	cDNA Cleanup – SPRIselect		
		2.3A Pellet Cleanup	20 min	STOP 4°C ≤72 h or –20°C ≤4 weeks
		2.3B Transferred Supernatant Cleanup	30 min	STOP 4°C ≤72 h or –20°C ≤4 weeks
	2.4	cDNA QC & Quantification	50 min	
	Step 3 – 3' Gene Expression Library Construction			
8h plus	3.1	Fragmentation, End Repair & A-tailing	50 min	
	3.2	Post Fragmentation, End Repair & A-tailing Double Sided Size Selection – SPRIselect	30 min	
	3.3	Adaptor Ligation	25 min	
	3.4	Post Ligation Cleanup- SPRIselect	20 min	
	3.5	Sample Index PCR	40 min	STOP 4°C ≤72 h
	3.6	Post Sample Index PCR Double Sided Size Selection- SPRIselect	30 min	STOP 4°C ≤72 h or -20°C long term
	3.6	Post Library Construction QC	50 min	
	Step 4 – Cell Surface Protein Library Construction			
	4.1	Sample Index PCR	40 min	
	4.2	Post Sample Index PCR Size Selection- SPRIselect	20 min	STOP 4°C ≤72 h or -20°C long term
	4.3	Post Library Construction QC	50 min	
	Step 5 – Cell Multiplexing Library Construction			
	4.1	Sample Index PCR	15 min	
	4.2	Post Sample Index PCR Size Selection- SPRIselect	20 min	STOP 4°C ≤72 h or -20°C long term
	4.3	Post Library Construction QC	50 min	

Stepwise Objectives

The Chromium Single Cell Gene Expression HT upgrades short read sequencers to deliver a scalable microfluidic platform for assessing Cell Surface Proteins and cell multiplexing combined with 3' digital gene expression by profiling 2,000-60,000 individual cells per sample. A pool of ~3,500,000 10x Barcodes are sampled separately to index each cell's transcriptome and cell surface protein along with cell multiplexing. 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. Dual indexed libraries are generated and sequenced from the cDNA, and 10x Barcodes are used to associate individual reads back to the individual partitions.

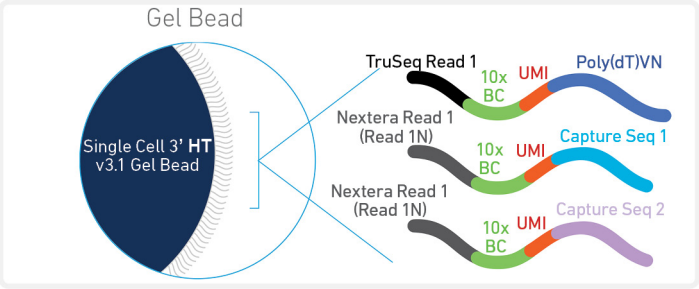
This document outlines the high throughput (HT) protocol for generating Single Cell 3' Gene Expression, Cell Surface Protein, and Cell Multiplexing dual index libraries from single cells.

Single Cell 3' HT v3.1 Gel Beads

In addition to the poly(dT) primer that enables the production of barcoded, full-length cDNA from poly-adenylated mRNA, the Single Cell 3' HT v3.1 Gel Beads also include two additional primer sequences (Capture Sequence 1 and Capture Sequence 2) that enable capture and priming of Feature Barcode technology compatible targets or analytes of interest.

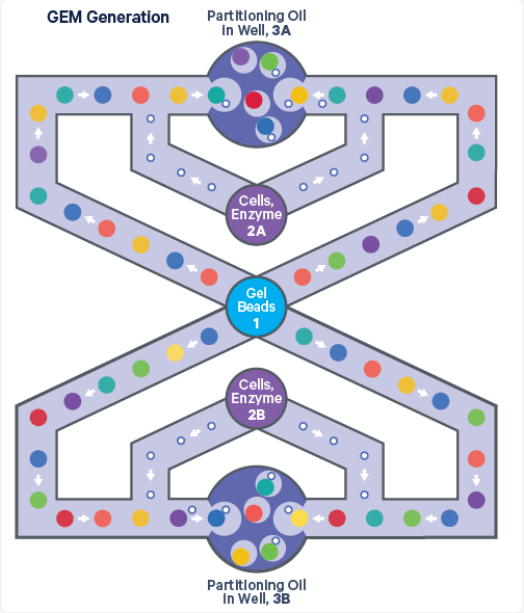
The poly(dT) primers along with one of the capture sequence primers are used in this protocol for generating Single Cell 3' Gene Expression and Cell Surface Protein/Cell Multiplexing libraries.

Gel Bead Primers



Step 1: GEM Generation & Barcoding

GEMs are generated by combining barcoded Gel Beads, a Master Mix containing cell surface protein labeled and Cell Multiplexing Oligo labeled cells, and Partitioning Oil onto Chromium Next GEM Chip M. 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.



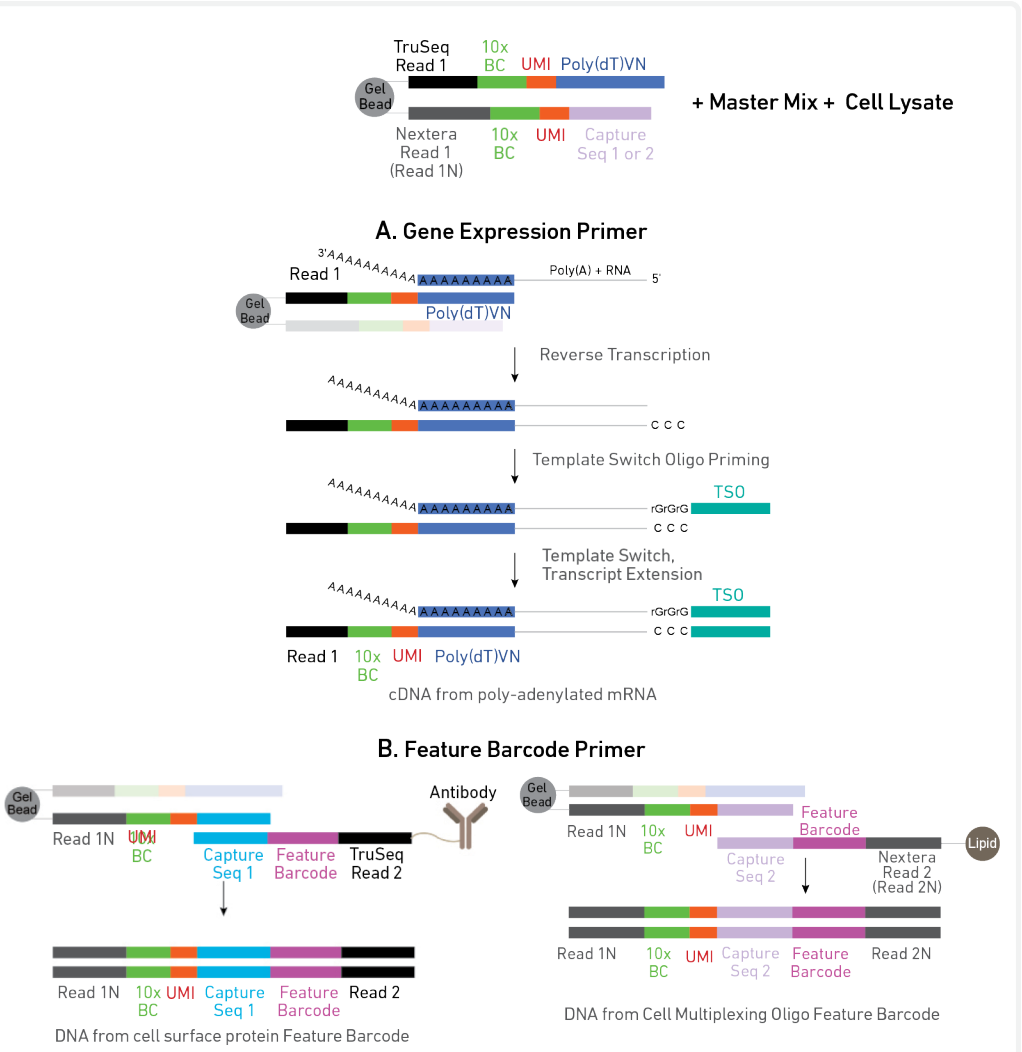
Immediately following GEM generation, the Gel Bead is dissolved, the three types of primers are released, and any co-partitioned cell is lysed. The poly (dT) and one of the capture sequence primers in the gel bead are engaged simultaneously in two different reactions inside individual GEMs (primer with only one of the Capture Sequences is illustrated in the example).

A. Primers containing:	B. Primers containing:
• an Illumina TruSeq Read 1 (read 1 sequencing primer)	• an Illumina Nextera Read 1 (Read 1N; read 1 sequencing primer)
• 16 nt 10x Barcode	• 16 nt 10x Barcode
• 12 nt unique molecular identifier (UMI)	• 12 nt unique molecular identifier (UMI)
• 30 nt poly(dT) sequence	• Capture Sequence 1 or 2

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

and barcoded DNA from the cell surface protein and cell multiplexing Feature Barcode oligonucleotides from reagents in (B).

Inside Individual GEMs

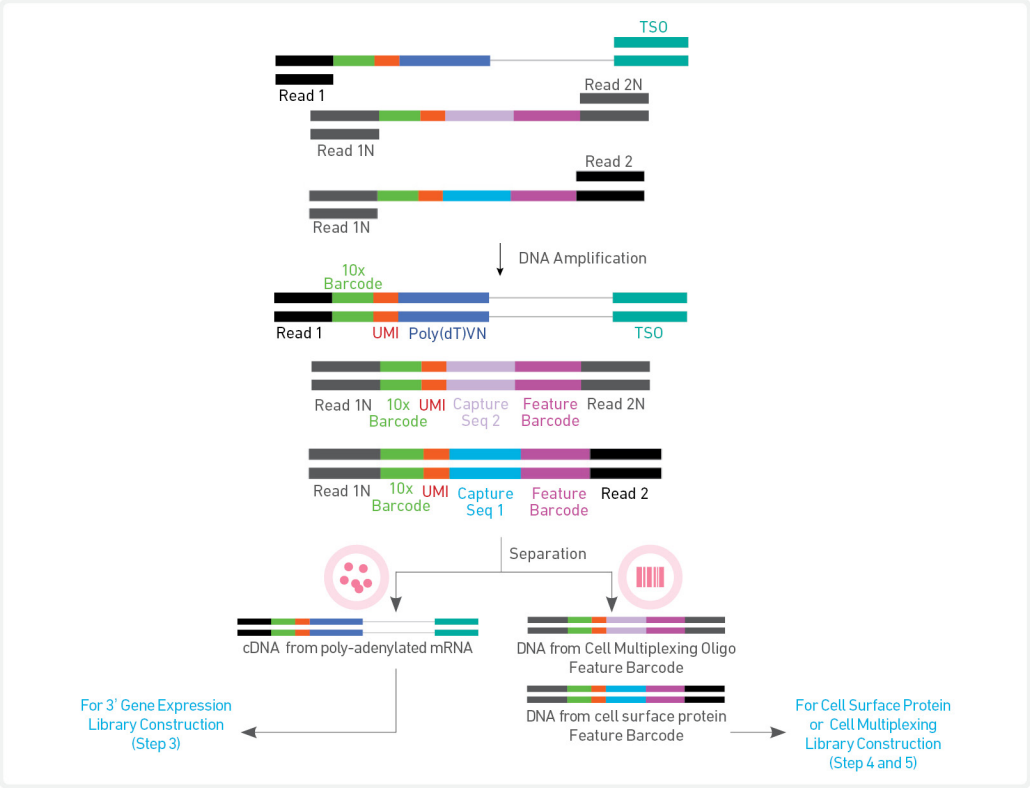


cDNA from poly-adenylated mRNA and DNA from cell surface protein and Cell Multiplexing Oligo Feature Barcode are generated simultaneously from the same single cell inside the GEM

Step 2: Post GEM-RT Cleanup & cDNA Amplification

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

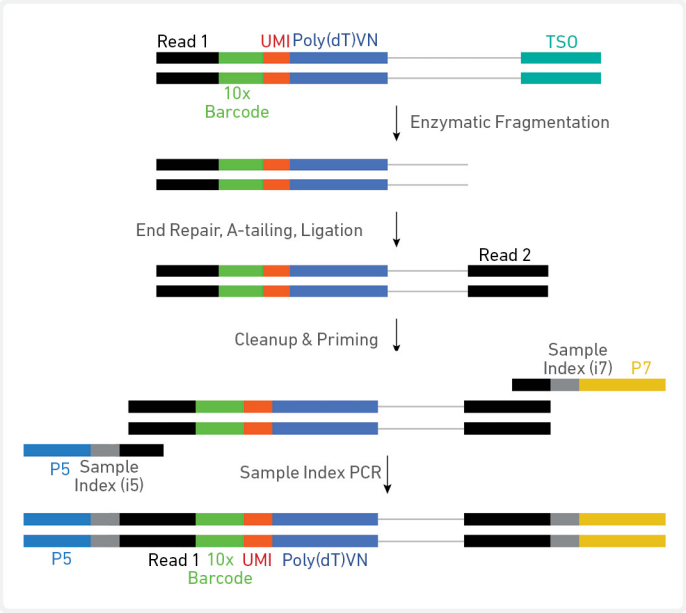
Pooled cDNA Amplification



Step 3: 3' Gene Expression Library Construction

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

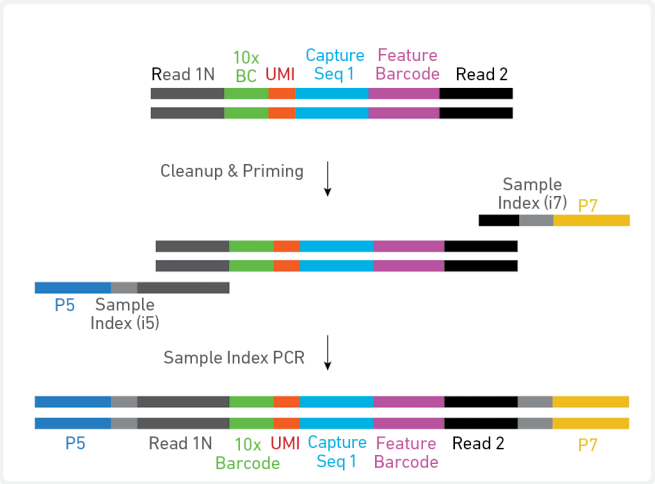
Amplified cDNA Processing (dual index)



Step 4: Cell Surface Protein Library Construction

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

Amplified DNA Processing (dual index)



Step 5: Cell Multiplexing Library Construction

Amplified DNA from Cell Multiplexing Feature Barcode oligonucleotides is used for library construction. P5 and P7 adapters, i7 and i5 sample indexes, and Nextera Read 2 (read 2N primer sequence) are added via PCR. The final libraries contain the P5 and P7 sequences necessary for amplification on the Illumina flow cell.

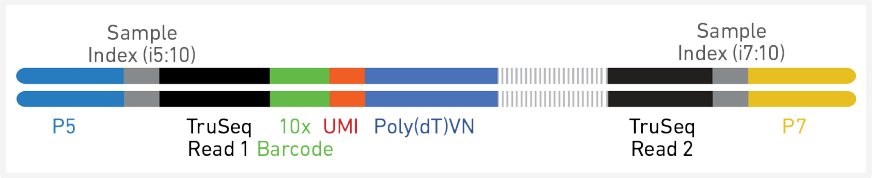
Pooled Amplified DNA Processing in Bulk (dual index)

Sequencing

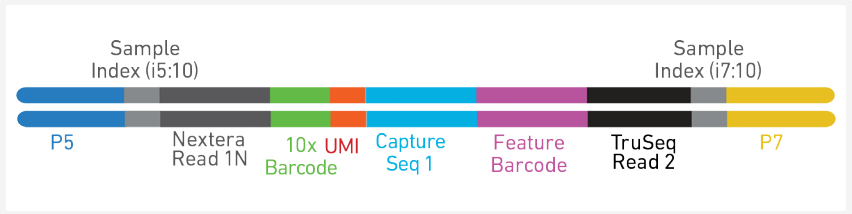
The Single Cell 3' library comprises standard Illumina paired-end constructs which begin and end with P5 and P7 adapters. The 16 bp 10x Barcode and 12 bp UMI are encoded in Read 1, while Read 2 is used to sequence the cDNA fragment in 3' Gene Expression libraries and the Feature Barcode in Cell Surface Protein libraries. Read 2N is used to sequence the DNA from Cell Multiplexing Oligo Feature Barcode in the Cell Multiplexing libraries. i7 and i5 index sequences are incorporated as the sample index reads. Standard Illumina sequencing primer sites TruSeq Read 1 and TruSeq Read 2 in the 3' Gene Expression libraries, Nextera Read 1 and TruSeq Read 2 in Cell Surface Protein libraries, and Nextera Read 1 and Nextera Read 2 in the Cell Multiplexing libraries are used in paired-end sequencing.

Illumina sequencer compatibility, sample indices, library loading and pooling for sequencing are summarized in the sequencing chapter.

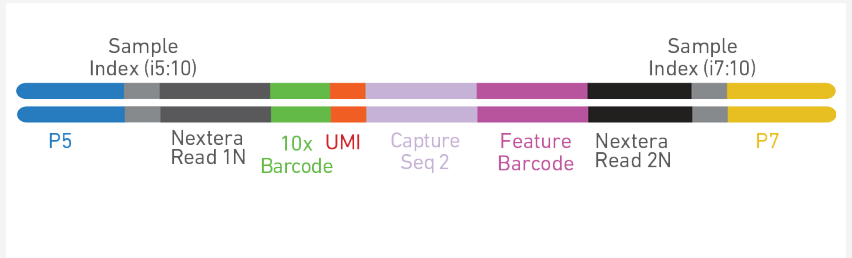
Chromium Single Cell 3' Gene Expression Dual Index Library



Chromium Single Cell 3' Cell Surface Protein Dual Index Library



Chromium Single Cell 3' Cell Multiplexing Dual Index Library



See Appendix for Oligonucleotide Sequences on page 111

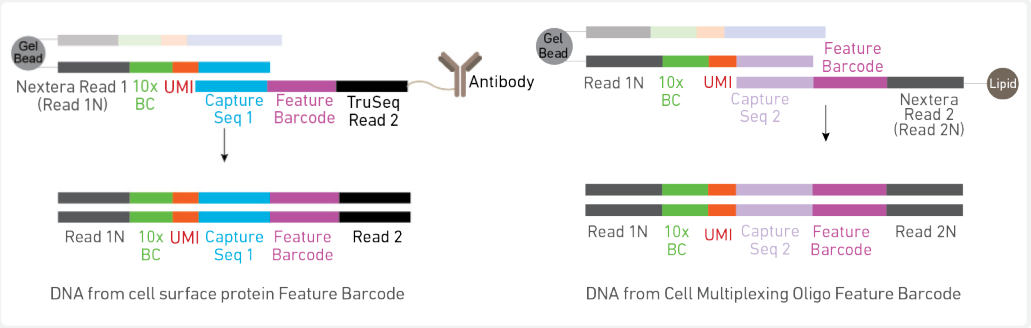
Cell Surface Protein & Cell Multiplexing Labeling Guidelines

Cell Surface Protein Overview

Cell surface proteins can be labeled using a Feature Barcode oligonucleotide conjugated to a specific protein binding molecule, such as an antibody. The Feature Barcode conjugated molecule bound to the cell surface protein can be directly captured by the Gel Bead inside a GEM during GEM generation and amplified (see Stepwise Objectives on page 19 for assay scheme specifics). The amplified DNA generated from the Feature Barcode can be used for Cell Surface Protein Library Construction.

Cell Multiplexing Overview

The 10x Genomics 3' CellPlex Kit provides a species agnostic sample multiplexing solution through the use of a set of 12 Feature Barcode oligonucleotides each conjugated to a lipid. Individual cells or nuclei samples can be labeled with a Cell Multiplexing Oligo (CMO) and then pooled together prior to loading onto a 10x Genomics chip. The Feature Barcode molecules can be directly captured by the oligos present on the Gel Beads inside a GEM during GEM-RT and subsequently amplified (see Stepwise Objectives on page 19 for assay scheme specifics). The amplified DNA generated from the Feature Barcode molecules can be used for Cell Multiplexing Library Construction. Upon sequencing and processing the data through Cell Ranger, pooled samples can be bioinformatically demultiplexed and analyzed as individual samples, with identified cell multiplets excluded.



Demonstrated Protocols for labeling

- For cell multiplexing oligo and cell surface protein labeling guidance, consult Demonstrated Protocol Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (Document CG000391). For antibody oligonucleotide conjugation guidance, consult Demonstrated Protocol Cell Surface Protein Labeling for

Single Cell RNA Sequencing Protocols with Feature Barcode technology (Document CG000149).

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

- Failure to label cell or nuclei with a Feature Barcode oligonucleotide conjugated to a lipid molecule prior to using the cells for GEM Generation & Barcoding will preclude generation of Cell Multiplexing library.

Tips & Best Practices

Icons

TIPS			HT	DUAL INDEX
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	Dual index specific protocol step updates

Emulsion-safe Plastics

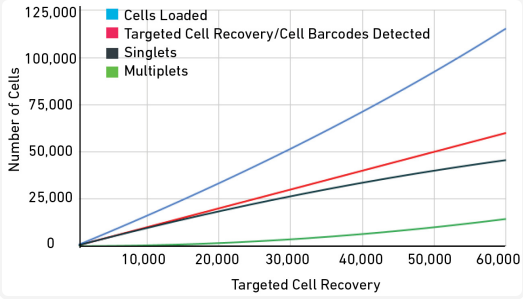
Use validated emulsion-safe plastic consumables when handling GEMs as some plastics can destabilize GEMs.

HT Cell Concentration

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

Optimal Input Cell Concentration	Cell Recovery Target
700-1,200 cells/ µl	2,000-20,000 cells
1,300-1,600 cells/ µl	20,000-60,000 cells

- 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.



Target Cell Recovery	# of Cells Loaded	Multiplet Rate (%)	Singlets	Multiplets
~2,000	~3,130	~0.8%	1,980	20
~4,000	~6,320	~1.6%	3,940	60
~6,000	~9,550	~2.4%	5,860	140
~8,000	~12,800	~3.2%	7,740	260
~10,000	~16,100	~4.0%	9,600	400
~12,000	~19,500	~4.8%	11,420	580
~14,000	~22,900	~5.6%	13,220	780
~16,000	~26,300	~6.4%	15,000	1,000
~18,000	~29,800	~7.2%	16,700	1,300
~20,000	~33,300	~8.0%	18,400	1,600
~24,000	~40,500	~9.6%	21,700	2,300
~28,000	~ 47,800	~11.2%	24,900	3,100
~32,000	~55,400	~ 12.8%	27,900	4,100
~36,000	~ 63,200	~14.4%	30,800	5,200
~40,000	~71,200	~ 16.0%	33,600	6,400
~44,000	~79,500	~ 17.6%	36,300	7,700
~48,000	~ 88,100	~ 19.2%	38,800	9,200
~52,000	~ 96,900	~ 20.8%	41,200	10,800
~56,000	~106,000	~ 22.4%	43,500	12,500
~60,000	~115,000	~ 24.0%	45,600	14,400

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 M on page 51 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.

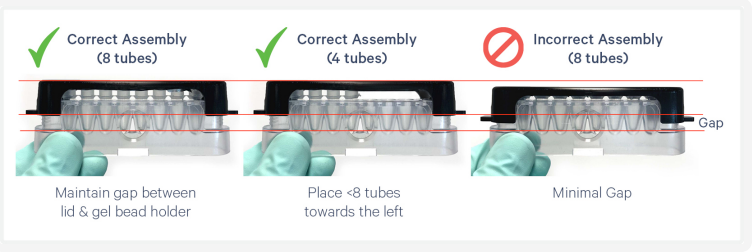
Chromium Next GEM Chip Loading

- 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 M on page 51 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**.

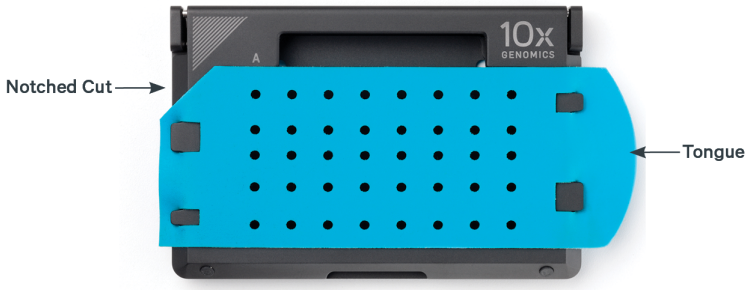
- Centrifuge the Gel Bead strip for **~5 sec** after removing from the holder. Confirm there are no bubbles at the bottom of tubes and the liquid levels look even. Place Gel Bead strip back in the holder and secure the holder lid.
- Ensure that the gel bead strip is positioned with one tube in the left-most 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

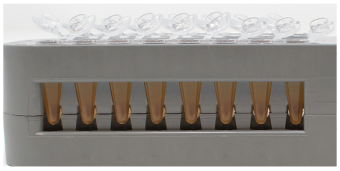
Low

Magnetic Bead Cleanup Steps

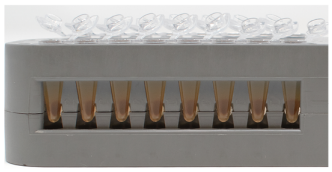
- During magnetic bead based cleanup steps that specify waiting “until the solution clears”, visually confirm clearing of solution before proceeding to the next step. See adjacent panel for an example.
- The time needed for the solution to clear may vary based on specific step, reagents, volume of reagents 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

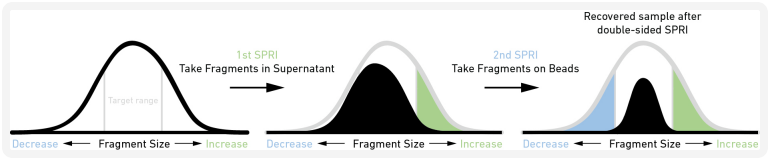
- After aspirating the desired volume of SPRIselect reagent, examine the pipette tips before dispensing to ensure the correct volume is transferred.
- Pipette mix thoroughly as insufficient mixing of sample and SPRIselect reagent will lead to inconsistent results. Use fresh preparations of 80% Ethanol.

Tutorial — SPRIselect Reagent:DNA Sample Ratios

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

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

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**).

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

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

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

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.

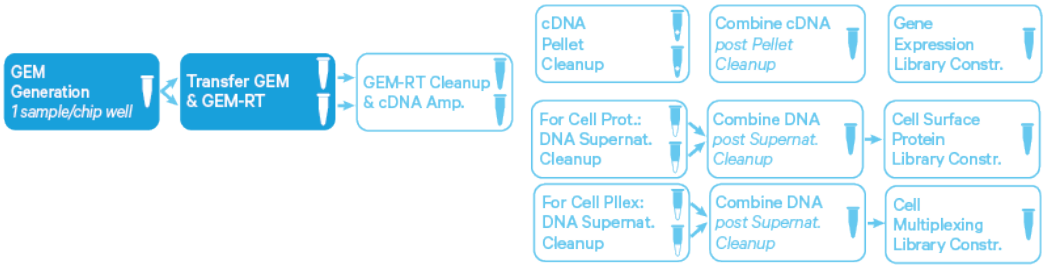
Step 1:

GEM Generation and Barcoding

1

1.0 Get Started

Overview



1 sample/chip well may be a pool of up to 12 labeled samples for Cell Multiplexing

Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐	**Single Cell 3' HT v3.1 Gel Beads**	2000443	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
☐ ●	**Template Switch Oligo**	3000228	Centrifuge briefly, resuspend in 80 µl Low TE Buffer. Vortex 15 sec at maximum speed, centrifuge briefly, leave at room temperature for ≥ 30 min. After resuspension, store at −80°C.	−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**	—	Consult Demonstrated Protocol Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (CG000149) Consult Demonstrated	—

Action	Item	10x PN	Preparation & Handling	Storage
			Protocol Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (CG000391)	
Obtain				
☐	**Partitioning Oil**	220088	—	Ambient
☐	**Chromium Next GEM Chip M**	2000417	See Tips & Best Practices.	Ambient
☐	**10x Gasket, HT**	3000614/ 3000656	See Tips & Best Practices.	Ambient
☐	**10x Vortex Adapter**	330002	See Tips & Best Practices.	Ambient
☐	**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
●	**Template Switch Oligo**	3000228	10.3	41.4	82.7
○	**Reducing Agent B**	2000087	8.5	34.3	68.6
●	**RT Enzyme C**	2000085/ 2000436	38.5	154.0	308.0
	Total	–	**139.8**	**559.7**	**1119.3**

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

a. Add **63.6 µl** Master Mix into each tube of a PCR 8-tube strip on ice.

Assemble Chromium Next GEM Chip M

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.

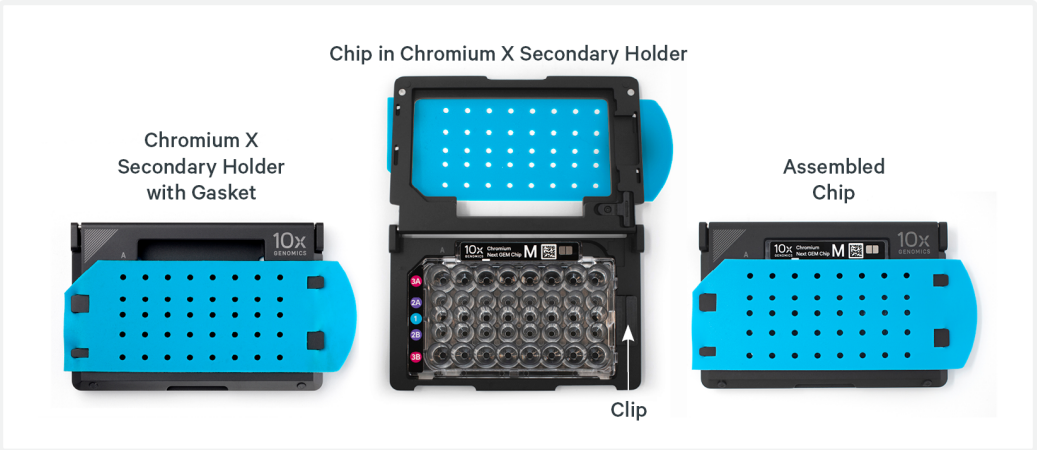
f. Slide the chip to the left until the guide on the holder is inserted into the chip. Depress the right hand side of the chip until the spring-loaded clip engages.

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.

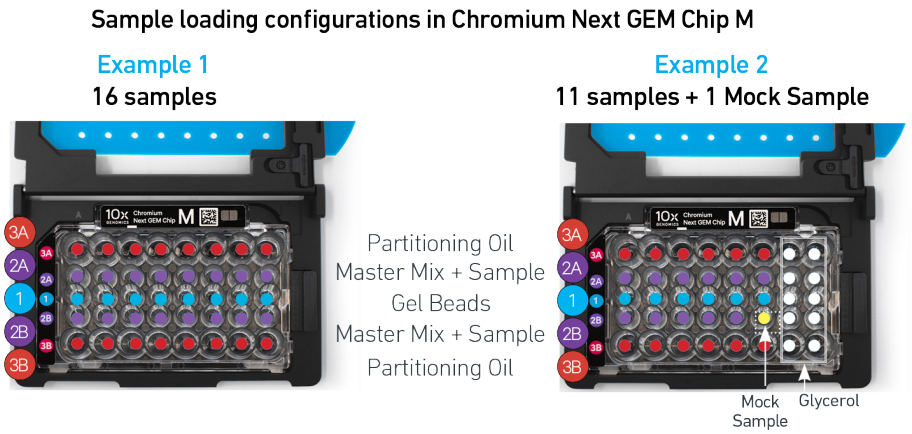


Sample Loading Guidelines

Read these guidelines before loading Chromium Next GEM Chip M

- 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 1.2c.

Cell Stock Conc. (Cells/µl)	Targeted Cell Recovery									
	2000	4000	6000	8000	10000	12000	14000	16000	18000	20000
100	31.3 55.1	63.2 23.2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
200	15.6 70.8	31.6 54.8	47.8 38.6	64.1 22.3	80.7 5.7	n/a	n/a	n/a	n/a	n/a
300	10.4 76.0	21.1 65.3	31.8 54.6	42.7 43.7	53.8 32.6	64.9 21.5	76.2 10.2	n/a	n/a	n/a
400	7.8 78.6	15.8 70.6	23.9 62.5	32.1 54.3	40.3 46.1	48.7 37.7	57.2 29.2	65.8 20.6	74.4 12.0	83.2 3.2
500	6.3 80.1	12.6 73.8	19.1 67.3	25.6 60.8	32.3 54.1	39.0 47.4	45.7 40.7	52.6 33.8	59.6 26.8	66.6 19.8
600	5.2 81.2	10.5 75.9	15.9 70.5	21.4 65.0	26.9 59.5	32.5 53.9	38.1 48.3	43.8 42.6	49.6 36.8	55.5 30.9
700	4.5 81.9	9.0 77.4	13.6 72.8	18.3 68.1	23.0 63.4	27.8 58.6	32.7 53.7	37.6 48.8	42.5 43.9	47.6 38.8
800	3.9 82.5	7.9 78.5	11.9 74.5	16.0 70.4	20.2 66.2	24.4 62.0	28.6 57.8	32.9 53.5	37.2 49.2	41.6 44.8
900	3.5 82.9	7.0 79.4	10.6 75.8	14.2 72.2	17.9 68.5	21.6 64.8	25.4 61.0	29.2 57.2	33.1 53.3	37.0 49.4
1000	3.1 83.3	6.3 80.1	9.6 76.8	12.8 73.6	16.1 70.3	19.5 66.9	22.9 63.5	26.3 60.1	29.8 56.6	33.3 53.1
1100	2.8 83.6	5.7 80.7	8.7 77.7	11.7 74.7	14.7 71.7	17.7 68.7	20.8 65.6	23.9 62.5	27.1 59.3	30.3 56.1
1200	2.6 83.8	5.3 81.1	8.0 78.4	10.7 75.7	13.4 73.0	16.2 70.2	19.1 67.3	21.9 64.5	24.8 61.6	27.7 58.7
1300	2.4 84.0	4.9 81.5	7.3 79.1	9.9 76.5	12.4 74.0	15.0 71.4	17.6 68.8	20.2 66.2	22.9 63.5	25.6 60.8
1400	2.2 84.2	4.5 81.9	6.8 79.6	9.2 77.2	11.5 74.9	13.9 72.5	16.3 70.1	18.8 67.6	21.3 65.1	23.8 62.6
1500	2.1 84.3	4.2 82.2	6.4 80.0	8.5 77.9	10.8 75.6	13.0 73.4	15.2 71.2	17.5 68.9	19.9 66.5	22.2 64.2
1600	2.0 84.4	4.0 82.4	6.0 80.4	8.0 78.4	10.1 76.3	12.2 74.2	14.3 72.1	16.4 70.0	18.6 67.8	20.8 65.6
1700	1.8 84.6	3.7 82.7	5.6 80.8	7.5 78.9	9.5 76.9	11.5 74.9	13.5 72.9	15.5 70.9	17.5 68.9	19.6 66.8
1800	1.7 84.7	3.5 82.9	5.3 81.1	7.1 79.3	9.0 77.4	10.8 75.6	12.7 73.7	14.6 71.8	16.5 69.9	18.5 67.9
1900	1.6 84.8	3.3 83.1	5.0 81.4	6.7 79.7	8.5 77.9	10.3 76.1	12.0 74.4	13.8 72.6	15.7 70.7	17.5 68.9
2000	1.6 84.8	3.2 83.2	4.8 81.6	6.4 80.0	8.1 78.3	9.7 76.7	11.4 75.0	13.2 73.2	14.9 71.5	16.6 69.8

Grey boxes: Exceeds allowable volume	**Blue boxes:** Optimal cell stock conc. for cell recovery target of 2,000-20,000	**Purple boxes:** Optimal cell stock conc. for cell recovery target of 24,000-60,000	**Yellow boxes:** Low transfer volume that may result in higher cell load variability

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 1.2c.

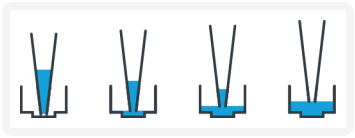
Cell Stock Conc. (Cells/µl)	Targeted Cell Recovery										
	20000	24000	28000	32000	36000	40000	44000	48000	52000	56000	60000
400	83.2 3.1	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
500	66.6 19.8	81.0 5.4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
600	55.5 30.9	67.5 18.9	79.7 6.7	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
700	47.6 38.8	57.8 28.6	68.4 18.0	79.2 7.2	n/a	n/a	n/a	n/a	n/a	n/a	n/a
800	41.6 44.8	50.6 35.8	59.8 26.6	69.3 17.1	79.0 7.4	n/a	n/a	n/a	n/a	n/a	n/a
900	37.0 49.4	45.0 41.4	53.2 33.2	61.6 24.8	70.2 16.2	79.2 7.2	n/a	n/a	n/a	n/a	n/a
1000	33.3 53.1	40.5 45.9	47.8 38.6	55.4 31.0	63.2 23.2	71.2 15.2	79.5 6.9	n/a	n/a	n/a	n/a
1100	30.3 56.1	36.8 49.6	43.5 42.9	50.4 36.0	57.5 28.9	64.8 21.6	72.3 14.1	80.0 6.4	n/a	n/a	n/a
1200	27.7 58.7	33.7 52.7	39.9 46.5	46.2 40.2	52.7 33.7	59.4 27.0	66.3 20.1	73.4 13.0	80.7 5.7	n/a	n/a
1300	25.6 60.8	31.1 55.3	36.8 49.6	42.6 43.8	48.6 37.8	54.8 31.6	61.2 25.2	67.7 18.7	74.5 11.9	81.5 4.9	n/a
1400	23.8 62.6	28.9 57.5	34.2 52.2	39.6 46.8	45.2 41.2	50.9 35.5	56.8 29.6	62.9 23.5	69.2 17.2	75.7 10.7	82.4 4.0
1500	22.2 64.2	27.0 59.4	31.9 54.5	37.0 49.4	42.1 44.3	47.5 38.9	53.0 33.4	58.7 27.7	64.6 21.8	70.7 15.7	76.9 9.5
1600	20.8 65.6	25.3 61.1	29.9 56.5	34.6 51.8	39.5 46.9	44.5 41.9	49.7 36.7	55.0 31.4	60.5 25.9	66.2 20.2	72.1 14.3
1700	19.6 66.8	23.8 62.6	28.1 58.3	32.6 53.8	37.2 49.2	41.9 44.5	46.8 39.6	51.8 34.6	57.0 29.4	62.3 24.1	67.9 18.5
1800	18.5 67.9	22.5 63.9	26.6 59.8	30.8 55.6	35.1 51.3	39.6 46.8	44.2 42.2	48.9 37.5	53.8 32.6	58.9 27.5	64.1 22.3
1900	17.5 68.9	21.3 65.1	25.2 61.2	29.2 57.2	33.3 53.1	37.5 48.9	41.9 44.5	46.3 40.1	51.0 35.4	55.8 30.6	60.7 25.7
2000	16.6 69.8	20.2 66.2	23.9 62.5	27.7 58.7	31.6 54.8	35.6 50.8	39.8 46.6	44.0 42.4	48.4 38.0	53.0 33.4	57.7 28.7

Grey boxes: Exceeds allowable volume	**Blue boxes:** Optimal cell stock conc. for cell recovery target of 2,000-20,000	**Purple boxes:** Optimal cell stock conc. for cell recovery target of 24,000-60,000	**Yellow boxes:** Low transfer volume that may result in higher cell load variability

1.2 HT Load Chromium Next GEM Chip M

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

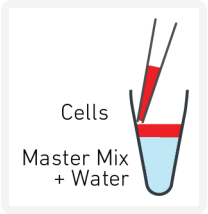
DO NOT use any substitute for 50% glycerol solution.
For odd number of samples, if a sample is loaded in a well in row 2A, load a mock-sample (Master Mix + 86.4 µl water) and NOT glycerol in the corresponding well in row 2B.

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

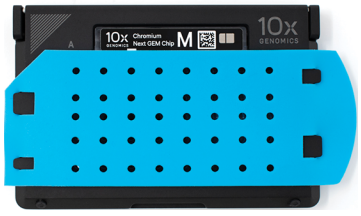
- Dispense **140 µl** Partitioning Oil into the wells in **rows labeled 3A & 3B** from a reagent reservoir.

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 48 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.

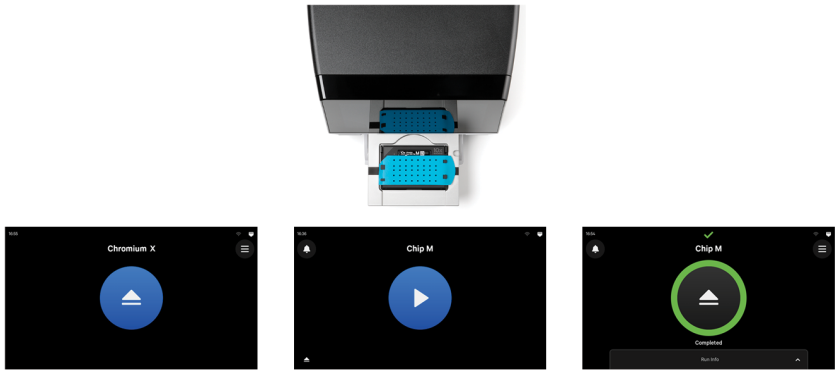
a. Press the eject button on the Chromium X to eject the tray.
If the eject button is not touched within **1 min**, tray will close automatically. System requires a few seconds before the tray can be ejected again.

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.

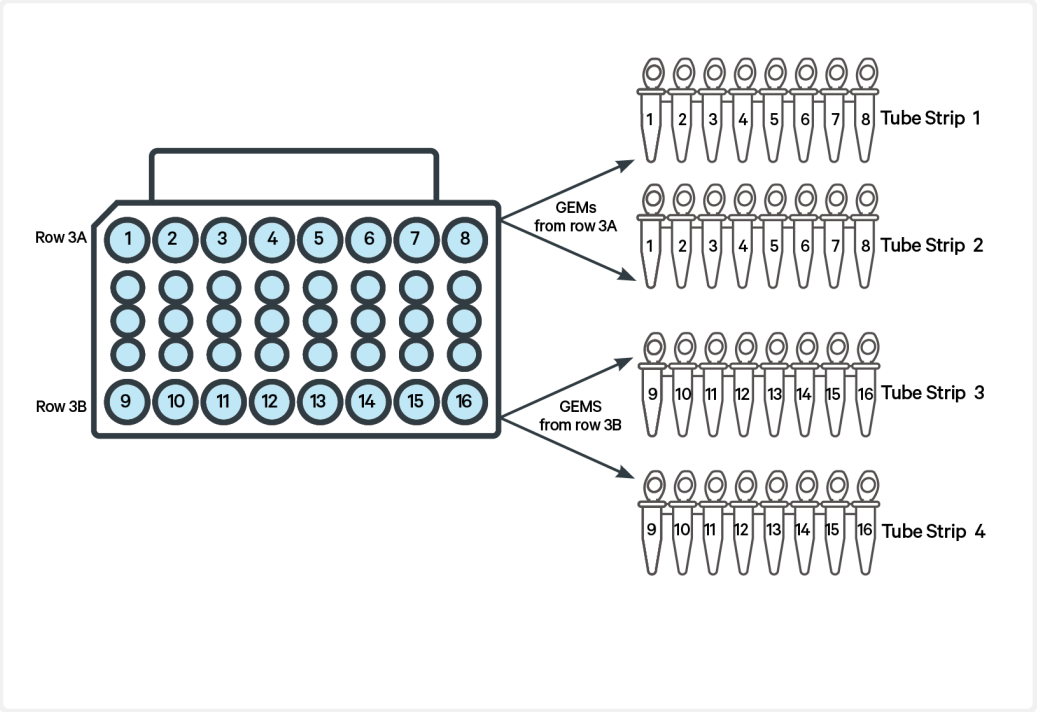
d. At completion of the run **(~18 min)**, Chromium X will chime. **Immediately** proceed to the next step.

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

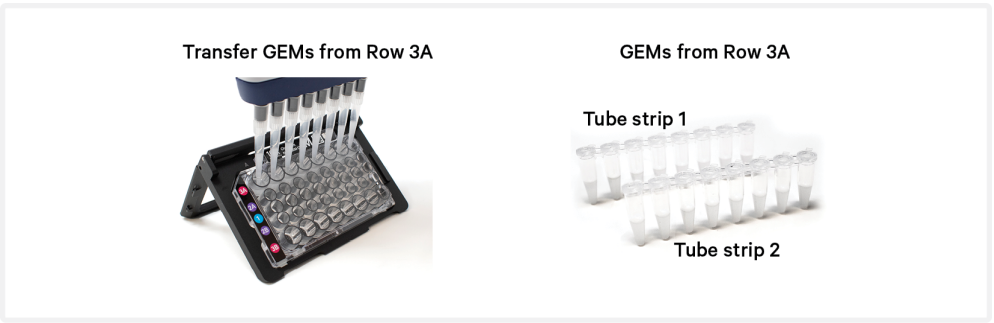
a. Label four tube strips and place on ice.

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

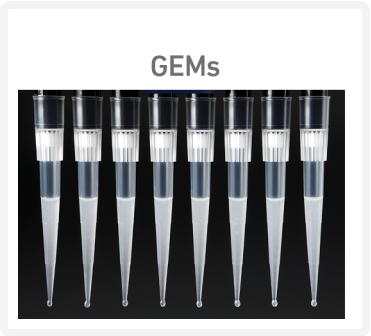
c. Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees.

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.



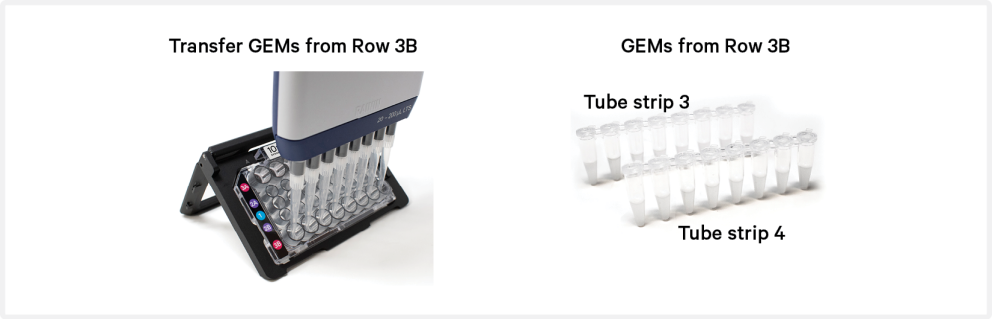
f. Withdraw pipette tips from the wells. GEMs should appear opaque and uniform across all channels. Excess Partitioning Oil (clear) in the pipette tips indicates a potential clog.



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

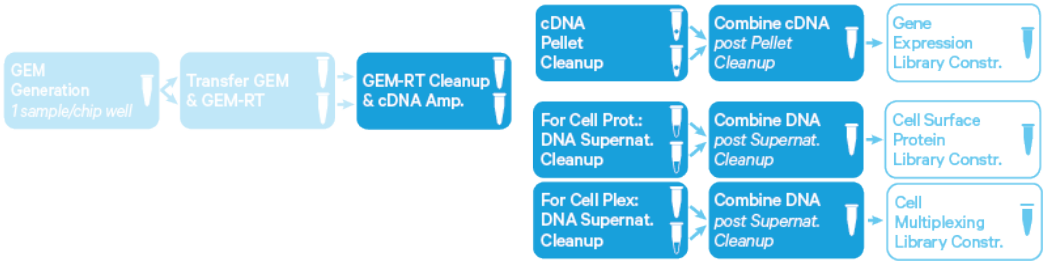
b. Store at **4°C** for up to **72 h** or at **−20°C** for up to **a week**, or proceed to the next step.

Step 2:

Post GEM–RT Cleanup & cDNA Amplification

2.0 Get Started

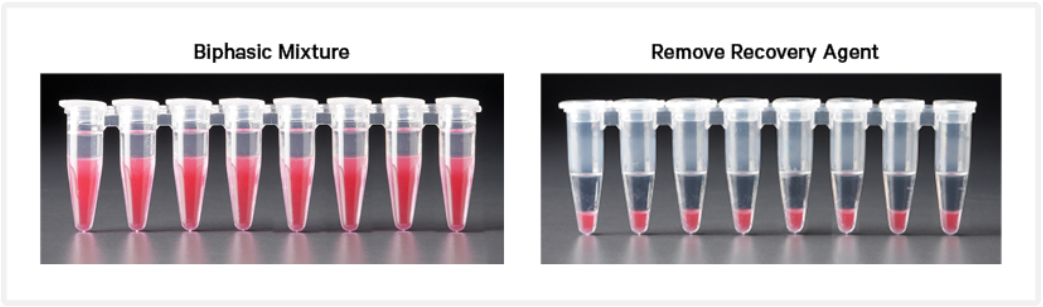
Overview



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 3**	2000289	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 3' 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.

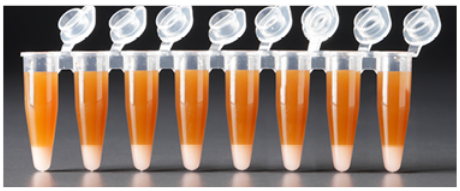
Before using Dynabeads MyOne SILANE to prepare the Dynabeads Cleanup Mix:

- 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 3' HT GEM kit*	2000047/ 2000440	110	880	1760
●	**Feature cDNA Primers 3**	2000289	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	00:03:00
2	98°C	00:00:15
3	63°C	00:00:20
4	72°C	00:01:00
5	Go to Step 2, see table below for total # of cycles	
6	72°C	00:01:00
7	4°C	Hold

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
<12,000	12
>12,000	11

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

Step Overview (steps 2.2 & 2.3)

For each original sample loaded on the chip, there will be two tubes of pellet and four tubes of supernatant at the end of step 2.3e. Note that the illustration only shows one set of tubes per sample.

2.3 cDNA Cleanup –SPRIselect

a. Vortex to resuspend the SPRIselect reagent. Add **60 µl** SPRIselect reagent **(0.6X)** to each sample and pipette mix 15x (pipette set to 150 µl).

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

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

d. Transfer and save **75 µl** supernatant each into two new tube strips (one for Cell Surface Protein and one for Cell Multiplexing) without disturbing the pellet. Maintain at room temperature. DO NOT discard the transferred supernatant (cleanup for Cell Surface Protein and Cell Multiplexing-step 2.3B).

For each original sample loaded on the chip, there will be two tubes of pellet and four tubes of supernatant at the end of this step. When processing multiple tube strips, maintain supernatant tubes on ice until ready to process. The tubes for each step should be processed sequentially. For longer storage, follow the guidelines provided in the relevant steps.

e. Remove the remaining supernatant from the pellet without disturbing the pellet. DO NOT discard the pellet (cleanup for 3' Gene Expression library construction). **Immediately** proceed to Pellet Cleanup (step 2.3A).

2.3A Pellet Cleanup (for 3' Gene Expression)

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.

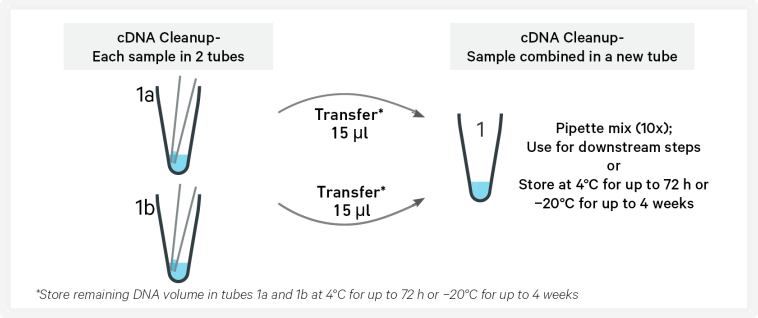
vi. Remove from the magnet. Add **41 µl** Buffer EB. Pipette mix 15x (pipette set to 35 µl).

vii. Incubate 2 min at room temperature.

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

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

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



x. Store at **4°C** for up to **72 h** or at **−20°C** for up to **4 weeks**, or proceed to step 2.4 followed by step 3 for 3' Gene Expression Library Construction.

2.3B Transferred Supernatant Cleanup (for Cell Surface Protein & Multiplexing)

i. Vortex to resuspend the SPRIselect reagent. Add **70 µl** SPRIselect reagent (2.0X) to **75 µl** of the transferred supernatant and pipette mix 15x (pipette

set to 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.

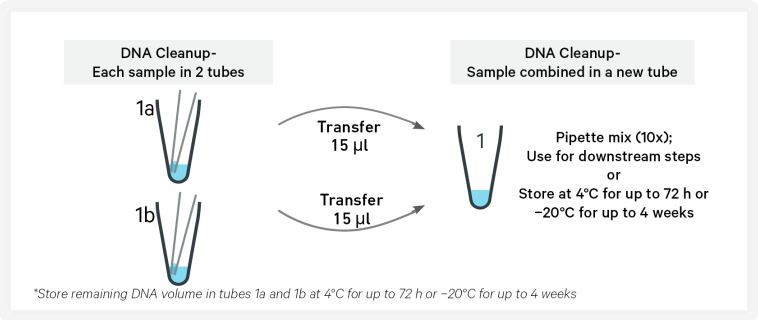
x. Remove from the magnet. Add **41 µl** Buffer EB. Pipette mix 15x (pipette set to 35 µl).

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

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

xiii. Transfer **40 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 and 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

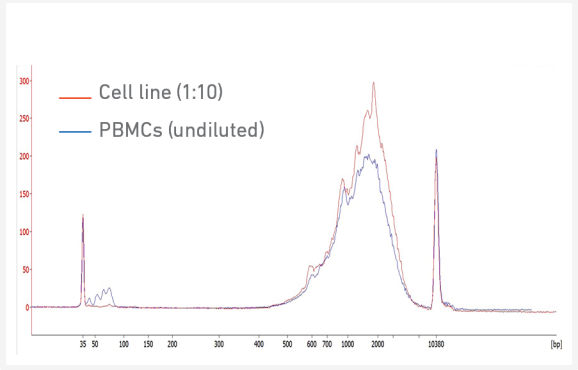
xiv. Store at **4°C** for up to **72 h** or at **−20°C** for up to **4 weeks**, or proceed directly to Cell Surface Protein and Cell Multiplexing Library Construction.

2.4 Post cDNA Amplification QC & Quantification

a. Run **1 µl** sample at **1:10 dilution** 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 Trace



See example calculation in the following page.

Alternate Quantification Methods

Agilent TapeStation

LabChip

Qubit Fluoromter and Qubit dsDNA HS Assay Kit

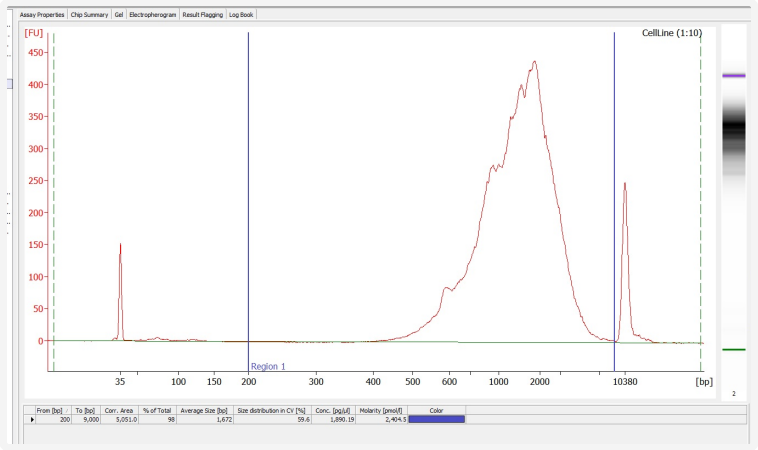
See Appendix for

- Agilent TapeStation Traces on page 107
- LabChip Traces on page 109

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 elution volume and dilution factor and divide by 1000 to obtain the total cDNA yield in ng. Carry forward ONLY 25% of total cDNA yield into 3' Gene Expression Library Construction.

Example Calculation of cDNA Total Yield

Concentration: **1890.19 pg/µl** Elution volume: 80 µl; Dilution Factor: 10

Total cDNA Yield

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

$$= \frac{\text{1890.19 x 80 x 10}}{\text{1000 (pg/ng)}}$$

=1512.15 ng

Carrying Forward ONLY 25% of total cDNA yield for 3' GEX Library

=Total cDNA x 0.25 =1512.15 ng x 0.25

=378.03 ng

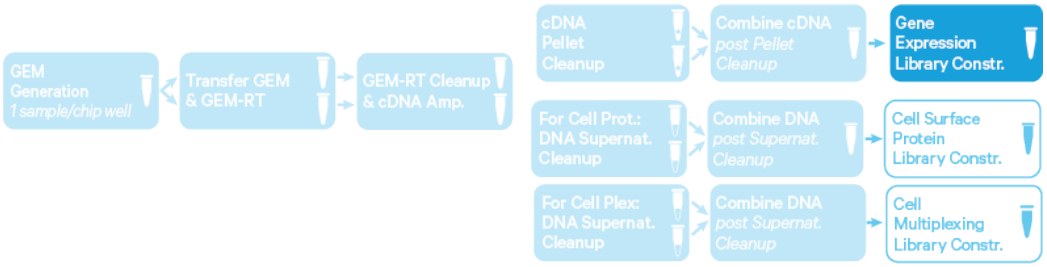
Refer to step 3.5 for appropriate number of Sample Index PCR cycles based on carry forward cDNA yield/input cDNA.

Step 3:

3' Gene Expression Library Construction

3.0 Get Started

Overview



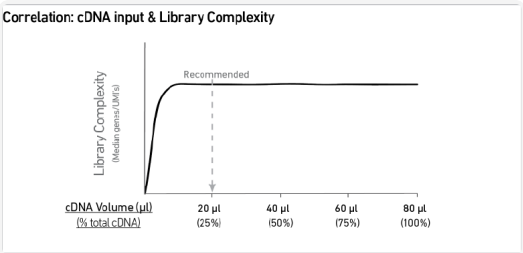
Action		Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature					
☐	●	**Fragmentation Buffer**	2000091	Thaw, vortex, verify no precipitate, centrifuge.	–20°C
☐	●	**Adaptor Oligos**	2000094	Thaw, vortex, centrifuge briefly.	–20°C
☐	●	**Ligation Buffer**	2000092	Thaw, vortex thoroughly (≥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*	2000090	Centrifuge briefly.	–20°C

Action	Item	10x PN	Preparation & Handling	Storage
	used together. Lots are matched for optimal performance			
☐	**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 3.1d)

Correlation between input & library complexity

A Single Cell 3' Gene Expression library is generated using a fixed proportion (20 µl, 25%) of the total cDNA (80 µl) obtained at step 2.3A-ix. The complexity of this library will be comparable to one generated using a higher proportion (>25%) of the cDNA. The remaining proportion (60 µl, 75%) 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 3.5d) should be optimized based on carrying forward a fixed proportion (20 µl, 25%) of the total cDNA yield calculated during Post cDNA Amplification QC & Quantification (step 2.4).

Example: Library Construction Input Mass & SI PCR Cycles

Cell	Targeted Cell Recovery	Total cDNA Yield (ng)	cDNA Input into Fragmentation		SI PCR Cycle Number
			Volume (µl)	Mass (ng)	
High RNA Content	Low	500 ng	20 µl	125 ng	12
	High	3800 ng	20 µl	950 ng	9
Low RNA Content	Low	2 ng	20 µl	0.5 ng	16
	High	400 ng	20 µl	100 ng	13

3.1 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)
Fragmentation Buffer	2000091	5	44	88
Fragmentation Enzyme	2000090	10	88	176
Total		**15**	**132**	**264**

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

- Note that only **20 µl** (25%) cDNA sample is sufficient for generating 3' 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 3' Gene Expression libraries.

e. Add **15 µl** Buffer EB to each sample.

f. Add **15** µl Fragmentation Mix to each sample.

g. Pipette mix 15x (pipette set to 35 µl) on ice. Centrifuge briefly.

h. Transfer into the pre-cooled thermal cycler (**4°C**) and press "SKIP" to initiate the protocol.

3.2 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.

3.3 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

3.4 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.

3.5 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 25% 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

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

3.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect

a. Vortex to resuspend the SPRIselect reagent. Add **60 µl** SPRIselect Reagent **(0.6X)** to each sample. Pipette mix 15x (pipette set to 150 µl).

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

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

d. Transfer **150 µl** supernatant to a new tube strip.

e. Vortex to resuspend the SPRIselect reagent. Add **20 µl** SPRIselect Reagent **(0.8X)** to each transferred supernatant. Pipette mix 15x (pipette set to 150 µl).

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

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

h. Remove **165 µl** supernatant. DO NOT discard any beads.

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

j. Remove the ethanol.

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

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

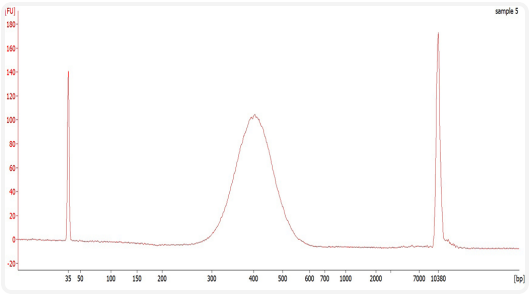
p. Transfer **35 µl** to a new tube strip.

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

3.7 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.

If peaks below 200 bp are more prominent, repeat step 3.6 Post Sample Index PCR Double Sided Size Selection - SPRIselect.

Alternate QC Method

Agilent TapeStation

LabChip

See Appendix for:

- Post Library Construction Quantification on page 106
- Agilent TapeStation Traces on page 107
- LabChip Traces on page 109

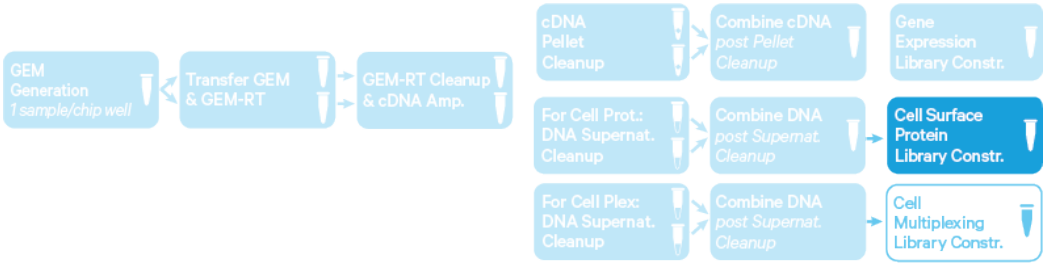
Step 4:

Cell Surface Protein Library Construction

4

4.0 Get Started

Overview



Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐ ⚠	**Dual Index Plate NT Set A** *Verify name & PN. Use indicated plate only*	3000483	—	−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 3' 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

4.1 Sample Index PCR

a. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x sample index name (PN-3000483 Dual Index Plate NT Set A well ID) used.

b. Prepare Sample Index PCR Mix.

Sample Index PCR Mix *Add reagents in the order listed*		**PN**	**1X (µl)**	**8X + 10% (µl)**	**16X + 10% (µl)**
◯	**Amp Mix** *Retrieve from 3' Feature Barcode Kit*	2000047	50	440	880
	Buffer EB	—	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 NT 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 9X for a total of 10 cycles*	
6	72°C	00:01:00
7	4°C	Hold

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

4.2 Post Sample Index PCR Size Selection – SPRIselect

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

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

c. Place 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 **41 µl** Buffer EB. Pipette mix 15x.

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

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

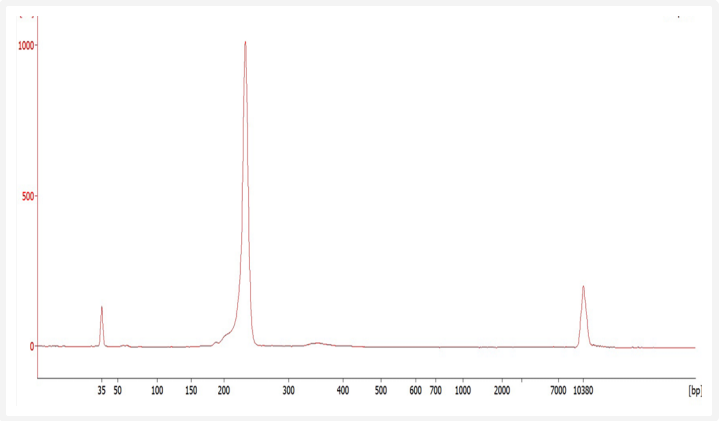
m. Transfer **40 µl** to a new tube strip.

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

4.3 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.

Minor peaks below 200 bp may be observed. Significant peaks below 200 bp may impact sequencer performance. Additional PCR cycles may enrich on-target library and minimize the impact on sequencer performance.

Alternate QC Method

- Agilent TapeStation
- LabChip

See Appendix for:

- Post Library Construction Quantification on page 106
- Agilent TapeStation Traces on page 107
- LabChip Traces on page 109

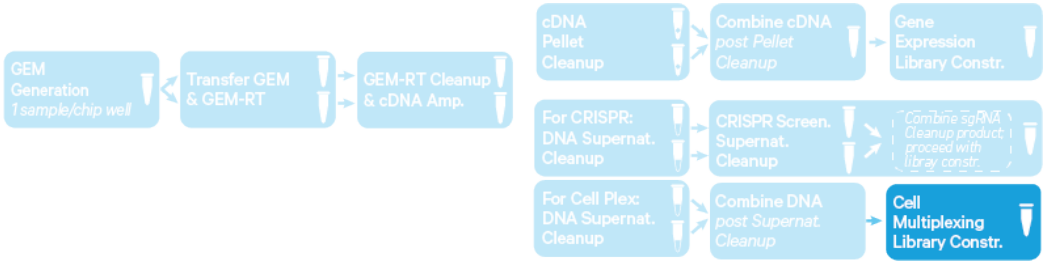
Step 5:

Cell Multiplexing Library Construction

5

5.0 Get Started

Overview



Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐ ⚠	**Dual Index Plate NN Set A** *Verify name & PN. Use indicated plate only*	3000482	—	−20°C
☐	**Beckman Coulter SPRIselect Reagent**	—	Manufacturer's recommendations.	—
☐	**Agilent Bioanalyzer High Sensitivity Kit If used for QC**	—	Manufacturer's recommendations.	—
☐	**Agilent TapeStation ScreenTape & Reagents** *If used for QC*	—	Manufacturer's recommendations.	—
☐	**DNA High Sensitivity Reagent Kit** *If LabChip used for QC*	—	Manufacturer's recommendations.	—
Place on Ice				
☐ ◯	**Amp Mix** *Retrieve from 3' 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

5.1 Sample Index PCR

a. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x sample index name (PN-3000482 Dual Index Plate NN Set A well ID) used.

b. Prepare Sample Index PCR Mix.

Sample Index PCR *Add reagents in the order listed*		**PN**	**1X (µl)**	**8X + 10% (µl)**	**16X + 10% (µl)**
○	**Amp Mix** *Retrieve from Single Cell 3'Feature Barcode kit*	2000047	50	440	880
	Buffer EB	—	20	176	352
Total			**70**	**616**	**1232**

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

- Note that only **10 µl** DNA sample is sufficient for generating Cell Multiplexing 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 Cell Multiplexing libraries.

d. Add **70 µl** Sample Index PCR Mix to each sample.

e. Add **20 µl** of an individual Dual Index NN 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	~10-15 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 5X for a total of 6 cycles	
6	72°C	00:01:00
7	4°C	Hold

5.2 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.

d. Remove the supernatant.

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

f. Remove the ethanol.

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

h. Remove the ethanol.

i. Centrifuge briefly. Place on the magnet•**Low**. Remove remaining ethanol. Air dry for **2 min**.

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

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

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

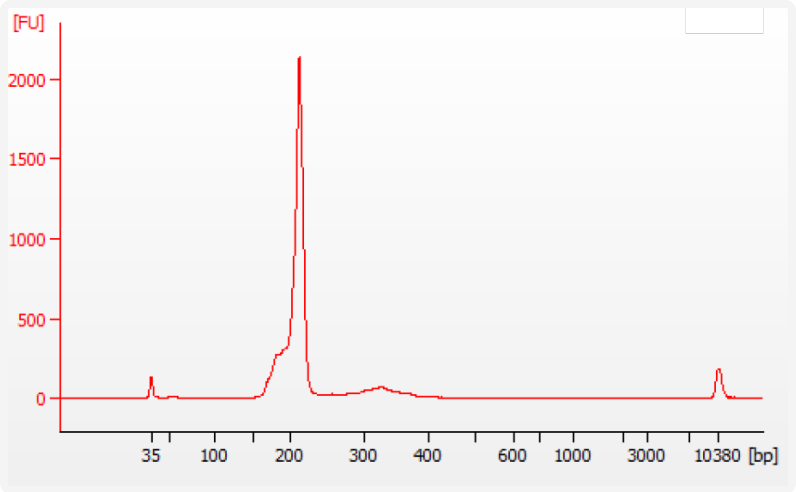
m. Transfer **40 µl** to a new tube strip.

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

5.3 Post Library Construction QC

Run **1 µl** sample at **1:20** 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 106
- Agilent TapeStation Traces on page 107
- LabChip Traces on page 109

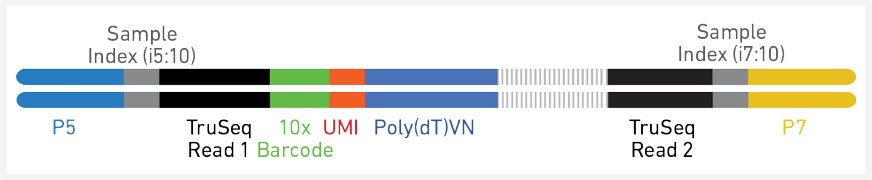
Step 6:

Sequencing

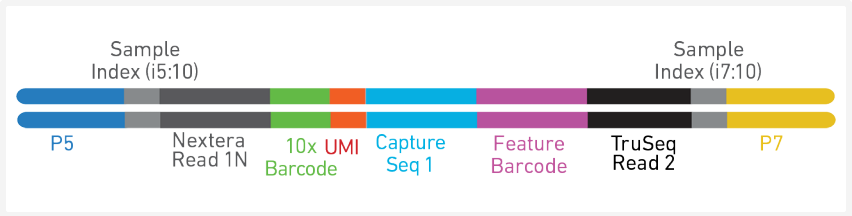
Sequencing Libraries

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

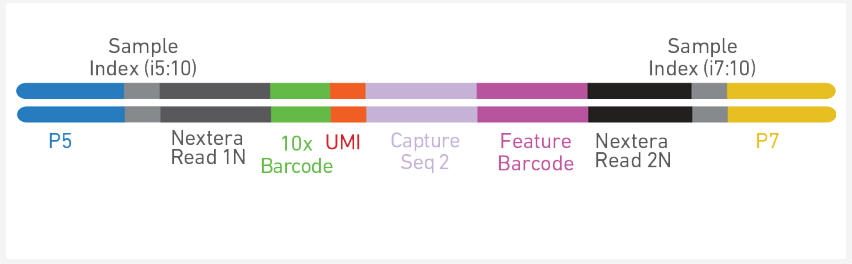
Chromium Single Cell 3' Gene Expression Dual Index Library



Chromium Single Cell 3' Cell Surface Protein Dual Index Library



Chromium Single Cell 3' Cell Multiplexing 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 NN Set A (PN-1000243)or Dual Index Kit NT Set A (PN-1000242) 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.

3' Gene Expression Library Sequencing Parameters

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

Cell Surface Protein & Cell Multiplexing Library Sequencing Parameters

Pooling Single Cell 3' Gene Expression, CRISPR Screening, & Cell Multiplexing dual index libraries is recommended for sequencing to maintain nucleotide diversity.

Parameter	Description
Sequencing Depth	Minimum 5,000 read pairs per cell
Sequencing Type	Paired-end, dual indexing
Sequencing Read	Recommended Number of Cycles
Read 1 i7 Index i5 Index Read 2	28 cycles 10 cycles 10 cycles 90 cycles *(Minimum required Read 2 length for Cell Surface Protein Screening libraries is 25 bp* *Minimum required Read 2 length for Cell Multiplexing libraries is 15 bp)*

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.

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

Library Loading

Instrument	Loading Concentration (pM)	PhiX (%)
MiSeq	11	1
NextSeq 500/550	1.8	1
NextSeq 1000/2000	650	1
HiSeq 2500 (RR)	11	1
HiSeq 4000	240	1
NovaSeq	150*/300	1

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

Library Pooling

The 3' Gene Expression libraries maybe pooled for sequencing, taking into account the differences in cell number and per-cell read depth requirements between each library. Samples utilizing the same sample index should not be pooled together or run on the same flow cell lane, as this would not enable correct sample demultiplexing.

Library Pooling Example

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

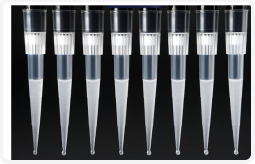
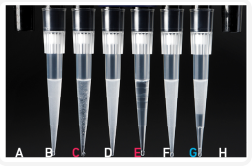
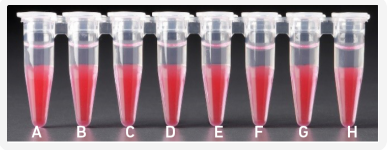
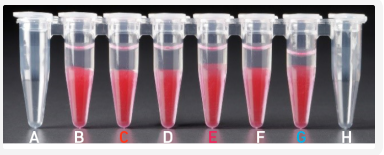
Loupe Browser

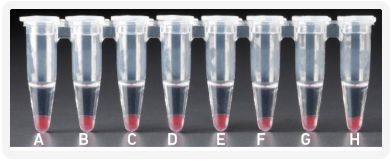
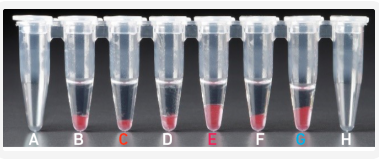
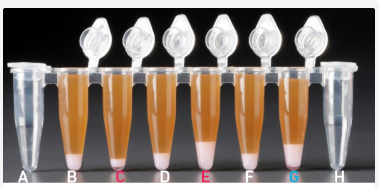
Loupe Browser is an interactive data visualization tool that requires no prior programming knowledge.

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

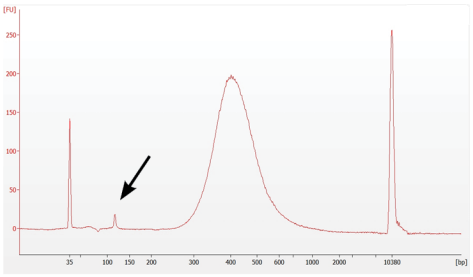
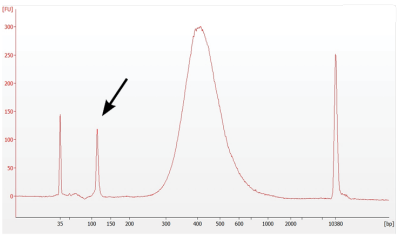
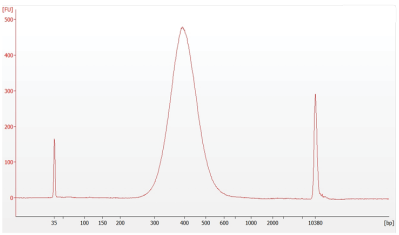
Troubleshooting

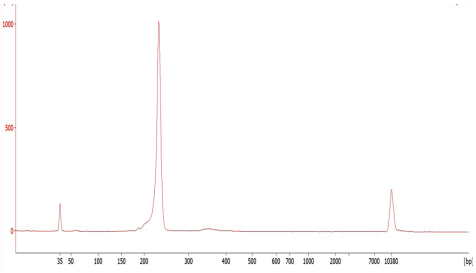
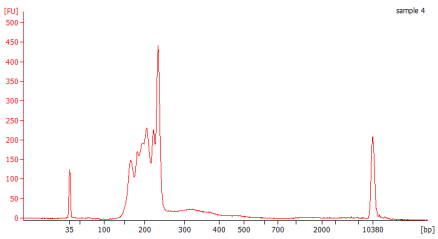
GEMs

Step	Normal	Reagent Clogs & Wetting Failures
1.4d After Chip M 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.

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

Step	Normal	Additional Peaks
4.3 Post Library Construction QC (Cell Surface Protein)	 Minor peaks below 200 bp may be observed.	 Peaks below 200 bp (as shown) in Cell Surface Protein trace may impact sequencer performance. Additional PCR cycles may enrich on-target library and minimize the impact on sequencer performance.

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

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)	

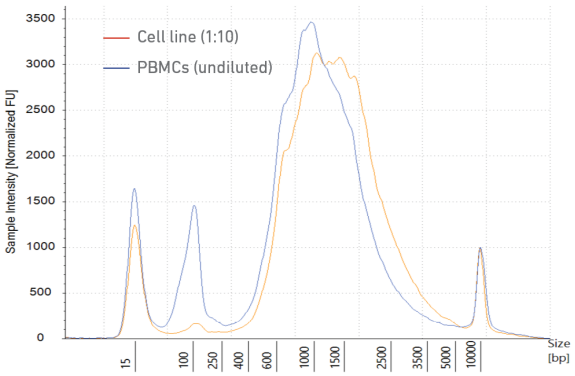
g. Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on insert size derived from the Bioanalyzer/TapeStation trace.

Agilent TapeStation Traces

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

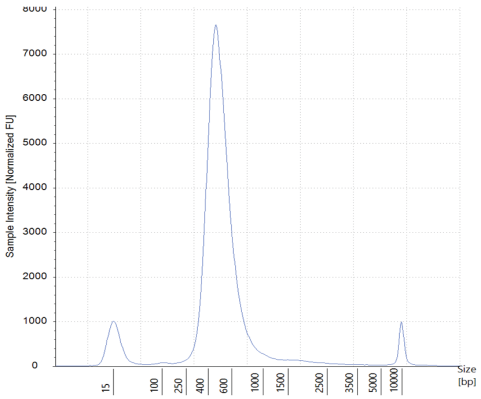
Protocol Step 2.4 – cDNA QC & Quantification

Run 2 µl sample mixed with 2 µl loading buffer. Ensure dilution factor is factored in when calculating cDNA yield.



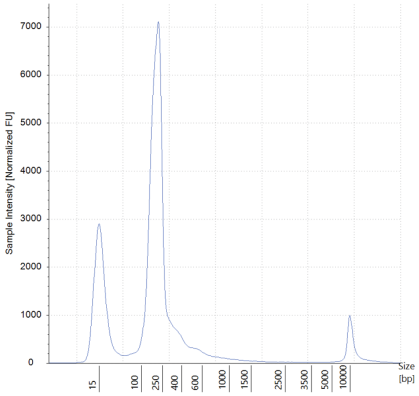
Protocol Step 3.7 – Post Library Construction QC

Run 2 µl diluted sample (1:10 dilution) mixed with 2 µl loading buffer.



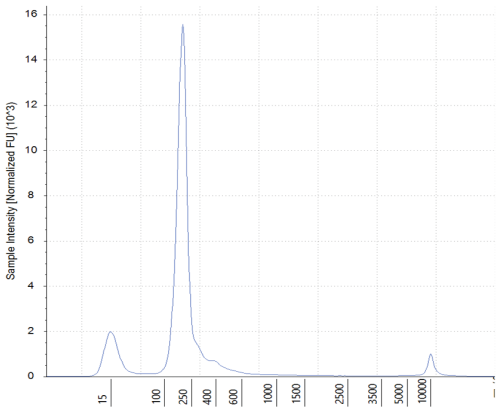
Protocol Step 4.3 – Post Library Construction QC (Cell Surface Protein Library)

Run 2 µl diluted sample (1:10 dilution) mixed with 2 µl loading buffer.



All traces are representative.

Protocol Step 5.3 – Post Library Construction QC (Cell Multiplexing library)



Run 2 µl diluted sample (1:20 dilution) mixed with 2 µl loading buffer.

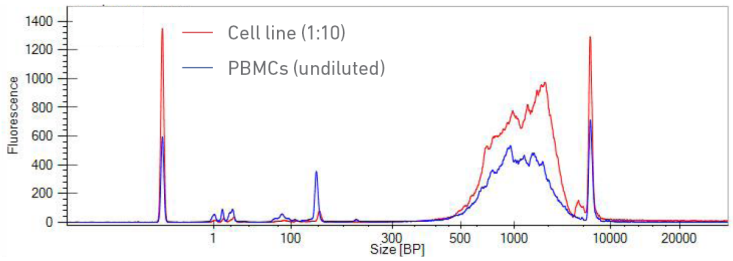
All traces are representative.

LabChip Traces

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

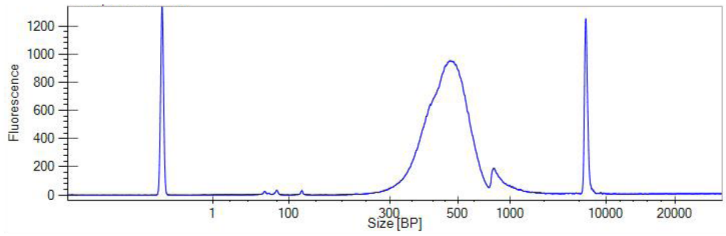
Protocol Step 2.4 – cDNA QC & Quantification

Run 10 µl sample. cDNA yield calculation is same as Agilent Bioanalyzer traces.



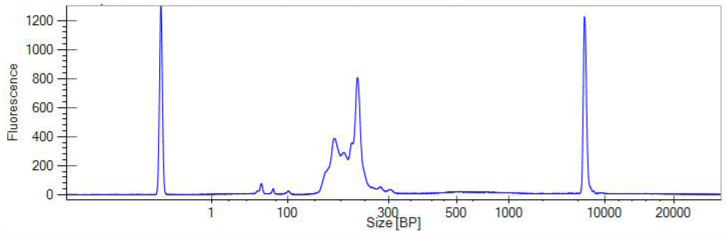
Protocol Step 3.7 – Post Library Construction QC

Run 10 µl diluted sample (1:10 dilution).



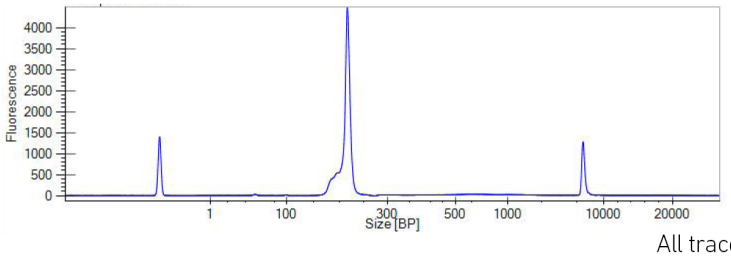
Protocol Step 4.3 – Post Library Construction QC (Cell Surface Protein Library)

Run 10 µl diluted sample (1:10 dilution).



Protocol Step 5.3 – Post Library Construction QC (Cell Multiplexing library)

Run 10 µl diluted sample (1:20 dilution).



All traces are representative.

Alternate QC Method:

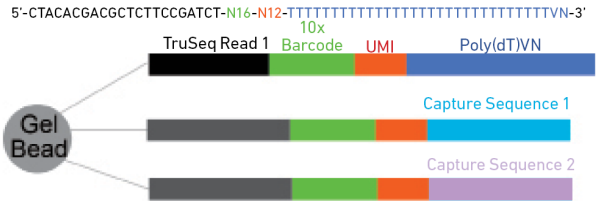
Qubit Fluorometer and Qubit dsDNA HS Assay Kit

Multiply the cDNA concentration reported via the Qubit Fluorometer by the elution volume (80 µl) to obtain the total cDNA yield in ng. To determine the equivalent range using the Agilent 2100 Expert Software, select the region encompassing 35-10,000 bp.

Oligonucleotide Sequences

Protocol Step 1.5 – GEM-RT Incubation

Gel Bead Primers



5'-GTCAGATGTGTATAAGAGACAG-N16-N12-TTGCTAGGACCGGCCTTAAAGC-3'

5'-GTCAGATGTGTATAAGAGACAG-N16-N12-CCTTAGCCGCTAATAGGTGAGC-3'

Template Switch Oligo PN-3000228

5'-AAGCAGTGGTATCAACGCAGAGTACATrGrGrG-3'

Cell Multiplexing Oligo Feature Barcode

3'-GGAATCGGCGATTATCCACTCG-N15-GACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-5'

Cell Surface Protein Feature Barcode

3'-AACGATCCTGGCCGGAATTTCG-N9-N15-N10-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-5'

GEM-RT Products

cDNA from poly-adenylated mRNA

5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-cDNA_Insert-CCCATGTACTCTGCGTTGATACCACTGCTT-3'

DNA from cell surface protein Feature Barcode

5'-GTCAGATGTGTATAAGAGACAG-N16-N12-TTGCTAGGACCGGCCTTAAAGC-N9-N15-N10-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'

DNA from Cell Multiplexing Oligo Feature Barcode

5'-GTCAGATGTGTATAAGAGACAG-N16-N12-CCTTAGCCGCTAATAGGTGAGC-N15-CTGTCTCTTATACACATCTCCGAGCCCACGAGAC-3'

Protocol Step 2.2 – cDNA Amplification

Feature cDNA Primers 3
PN-2000289

Amplifies cDNA

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

Reverse Primer: Partial TSO
5'-AAGCAGTGGTATCAACGCAGAG-3'

Amplifies DNA from cell surface protein Feature Barcode

Forward Primer: Partial Read 1N
5'-GCAGCGTCAGATGTGTATAAGAGACAG-3'

Reverse primer: Partial Read 2
5'-GTGACTGGAGTTCAGACGT-3'

Amplifies DNA from Cell Multiplexing Oligo Feature Barcode

Forward Primer: Partial Read 1N
5'-GCAGCGTCAGATGTGTATAAGAGACAG-3'

Reverse primer: Partial Read 2N
5'-GTCTCGTGGGCTCGGAGATGTGTATAA-3'

Amplification Products

Amplified cDNA from poly-adenylated mRNA

5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-cDNA_Insert-CCCATGTACTCTGCGTTGATACCACTGCTT-3'
3'-GATGTGCTGCGAGAAGGCTAGA-N16-N12-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAABN-cDNA_Insert-GGGTACATGAGACGCAACTATGGTGACGAA-5'

Amplified DNA from cell surface protein Feature Barcode

5'-GCAGCGTCAGATGTGTATAAGAGACAG-N16-N12-TTGCTAGGACCGGCCTTAAAGC-N9-N15-N10-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'
3'-CGTCGCAGTCTACACATATTCTCTGTC-N16-N12-AACGATCCTGGCCGGAATTTCG-N9-N15-N10-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-5'

Amplified DNA from Cell Multiplexing Oligo Feature Barcode

5'-GCAGCGTCAGATGTGTATAAGAGACAG-N16-N12-CCTTAGCCGCTAATAGGTGAGC-N15-CTGTCTCTTATACACATCTCCGAGCCCACGAGAC-3'
3'-CGTCGCAGTCTACACATATTCTCTGTC-N16-N12-GGAATCGGCGATTATCCACTCG-N15-GACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-5'

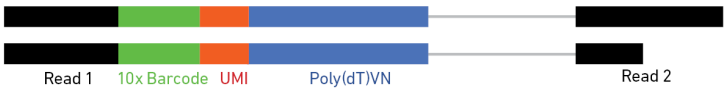
Protocol Step 3.3 – Adaptor Ligation (for 3' Gene Expression Library Construction)

Adaptor Oligos
PN -2000094

Partial Read 2

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

Ligation Product



5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-cDNA_Insert-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'
3'-GATGTGCTGCGAGAAGGCTAGA-N16-N12-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAABN-cDNA_Insert-TCTAGCCTTCTCG-5'

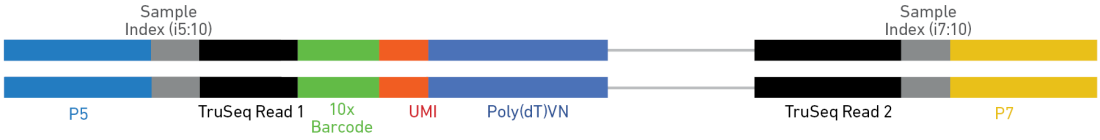
Protocol Step 3.5 – Sample Index PCR (for 3' Gene Expression Library Construction)

Dual Index Kit TT Set A
PN-1000215

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTC-3'

5'-CAAGCAGAAGACGGCATACGAGAT-N10-GTGACTGGAGTTCAGACGTGT-3'

Sample Index PCR Product



5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-cDNA_Insert-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'
3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAABN-cDNA_Insert-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-N10__-TAGAGCATACGGCAGAAGACGAAC-5'

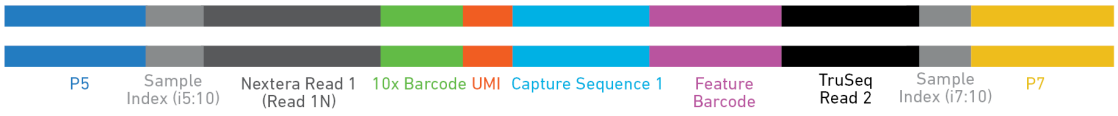
Protocol Step 4.1 – Sample Index PCR (for Cell Surface Protein Library Construction)

Dual Index Kit NT Set A
PN-1000242

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'

5'-CAAGCAGAAGACGGCATACGAGAT-N10-GTGACTGGAGTTCAGACGTGT-3'

Sample Index PCR Product



5'-AATGATACGGCGACCACCGAGATCTACAC-N10-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-N16-N12-TTGCTAGGACCGGCCTTAAAGC-N9-N15-N10-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'
3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-AGCAGCCGTCGCAGTCTACACATATTCTCTGTC-N16-N12AACGATCCTGGCCGGAATTTCC-N9-N15-N10-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'

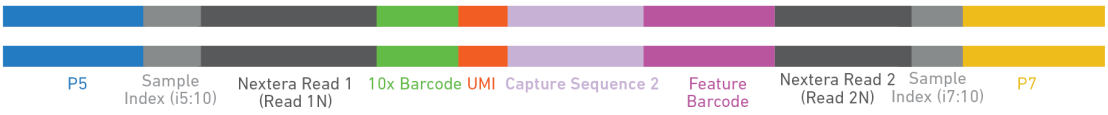
Protocol Step 5.1 – Sample Index PCR (for Cell Multiplexing Library Construction)

Dual Index Kit NN
Set A
PN-1000243

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'

5'-CAAGCAGAAGACGGCATACGAGAT-N10-GTCTCGTGGGCTCGG-3'

Sample Index PCR Product



5'-AATGATACGGCGACCACCGAGATCTACAC-N10-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-N16-N12-CCTTAGCCGCTAATAGGTGAGC-N15-CTGTCTCTTATACACATCTCCGAGCCCACGAGAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'
3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-AGCAGCCGTCGCAGTCTACACATATTCTCTGTC-N16-N12-GGAATCGGCGATTATCCACTCG-N15-GACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'