CG000317 Rev E

USER GUIDE

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

with Feature Barcode technology for Cell Surface Protein

FOR USE WITH

Chromium Next GEM Single Cell 3' Kit v3.1, 16 rxns PN-1000268
Chromium Next GEM Single Cell 3' Kit v3.1, 4 rxns PN-1000269
3' Feature Barcode Kit, 16 rxns PN-1000262
Chromium Next GEM Chip G Single Cell Kit, 48 rxns PN-1000120
Chromium Next GEM Chip G Single Cell Kit, 16 rxns PN-1000127
Dual Index Kit TT Set A, 96 rxns PN-1000215
Dual Index Kit NT Set A, 96 rxns PN-1000242

Next GEM reagents are specific to Next GEM products and should not be used interchangeably with non-Next GEM reagents.

10x GENOMICS

Notices

Document Number

CG000317 • Rev E

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Document Revision Summary

Document Number	CG000317
Title	Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) with Feature Barcode technology for Cell Surface Protein User Guide
Revision	Rev E
Revision Date	April 2024

Specific Changes:

- Updated 10x Genomics Accessories table to add Magnetic Separator B (PN-2001212) on page 11
- Updated Thermal Cycler Recommendations on page 11
- Removed Qubit from Additional Kits, Reagents & Equipment table on page 12 and from all the QC steps on pages 42 & 70
- Updated the volume of 50% glycerol solution to be added to row labeled 3 on page 31

General Changes:

- Updated for general minor consistency of language and terms throughout.

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Chromium Next GEM Single Cell 3' Reagents Kits

Chromium Next GEM Single Cell 3' Kit v3.1, 16 rxns PN-1000268

Chromium Next GEM Single Cell 3' GEM Kit v3.1 16 rxns PN-1000123 (store at –20°C)

Chromium Next GEM Single Cell 3' GEM Kit v3.1

	#	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

10xGenomics.com 10x GENOMICS

Library Construction Kit 16 rxns PN-1000190 (store at –20°C)

Library Construction Kit

	#	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

10xGenomics.com 10x GENOMICS

Chromium Next GEM Single Cell 3' Gel Bead Kit v3.1, 16 rxns PN-1000122 (store at –80°C)

Chromium Next GEM Single Cell 3' v3.1 Gel Beads

	#	PN
Single Cell 3' v3.1 Gel Beads	2	2000164

10xGenomics.com 10x GENOMICS

Dynabeads™ MyOne™ SILANE PN-2000048 (store at 4°C)

	#	PN
Dynabeads MyOne SILANE	1	2000048

Chromium Next GEM Single Cell 3' Kit v3.1, 4 rxns PN-1000269

Chromium Next GEM Single Cell 3' GEM Kit v3.1 4 rxns PN-1000130 (store at –20°C)

Chromium Next GEM Single Cell 3' GEM Kit v3.1

	#	PN
RT Reagent B	1	2000165
RT Enzyme C	1	2000102
Template Switch Oligo	1	3000228
Reducing Agent B	1	2000087
Cleanup Buffer	1	2000088
cDNA Primers	1	2000089
Amp Mix	1	2000103

10xGenomics.com 10x GENOMICS

Library Construction Kit 4 rxns PN-1000196 (store at –20°C)

Library Construction Kit

	#	PN
Fragmentation Enzyme	1	2000104
Fragmentation Buffer	1	2000091
Ligation Buffer	1	2000092
DNA Ligase	1	220131
Adaptor Oligos	1	2000094

10xGenomics.com 10x GENOMICS

Chromium Next GEM Single Cell 3' Gel Bead Kit v3.1, 4 rxns PN-1000129 (store at –80°C)

Chromium Next GEM Single Cell 3' v3.1 Gel Beads

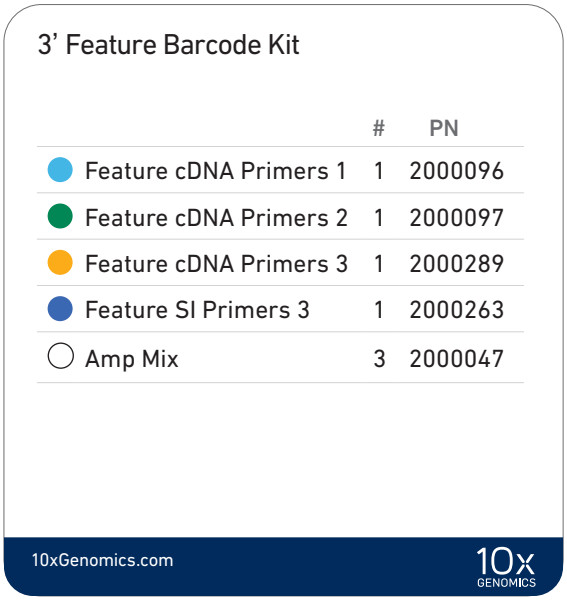
	#	PN
Single Cell 3' v3.1 Gel Beads (4 rxns)	1	2000164

10xGenomics.com 10x GENOMICS

Dynabeads™ MyOne™ SILANE PN-2000048 (store at 4°C)

	#	PN
Dynabeads MyOne SILANE	1	2000048

3’ Feature Barcode Kit, 16 rxns PN-1000262 (store at –20°C)



Dual Index Kit TT Set A, 96 rxns PN-1000215 (store at –20°C)

Dual Index Kit TT Set A

	#	PN
Dual Index Plate TT Set A	1	3000431

Dual Index Kit NT Set A, 96 rxns PN-1000242 (store at –20°C)

Dual Index Kit NT Set A

	#	PN
Dual Index Plate NT Set A	1	3000483

Chromium Next GEM Chip G Single Cell Kit, 48 rxns PN-1000120 (store at ambient temperature)

Chromium
Partitioning Oil

	#	PN
Partitioning Oil	6	2000190

Chromium
Recovery Agent

	#	PN
Recovery Agent	6	220016

Chromium
Next GEM
Chip G & Gaskets

	#	PN
Chromium Next GEM Chip G	6	2000177
Chip Gasket, 6-pack	1	370017

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Chromium Next GEM Chip G Single Cell Kit, 16 rxns PN-1000127 (store at ambient temperature)

Chromium
Partitioning Oil

	#	PN
Partitioning Oil	2	2000190

Chromium
Recovery Agent

	#	PN
Recovery Agent	2	220016

Chromium
Next GEM
Chip G & Gaskets

	#	PN
Chromium Next GEM Chip G	2	2000177
Chip Gasket, 2-pack	1	3000072

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10x Genomics Accessories

Product	PN (Kit)	PN (Item)
10x Vortex Adapter	120251	330002
Chromium Next GEM Secondary Holder	1000195	3000332
10x Magnetic Separator*	120250	230003
10x Magnetic Separator B*	1000709 (Chromium X/iX Accessory Kit)/1000707 (GEM-X Transition Kit)	2001212

**10x Magnetic Separator (PN-230003) and 10x Magnetic Separator B (PN-2001212) can be used interchangeably.*

Recommended Thermal Cyclers

The table below lists the thermal cyclers that have been validated by 10x Genomics. Thermal cyclers used must support uniform heating of 100 µl emulsion volumes.

Supplier	Description	Part Number
Analytik Jena	Biometra TAdvanced 96 SG/S*	846-x-070-241/846-x-070-251 *(x = 2 for 230 V; 4 for 115 V; 5 for 100 V, 50-60 Hz)*
Eppendorf	Mastercycler X50s/X50a**	6311000010/6313000018
ThermoFisher	VeritiPro***	A48141
Bio-Rad	PTC Tempo Deepwell	12015392
Bio-Rad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (discontinued)	1851197
Eppendorf	Mastercycler Pro (discontinued)	North America 950030010 International 6321 000.019
Thermo Fisher Scientific	Veriti 96-Well Thermal Cycler (discontinued)	4375786

For select instruments, ramp rates should be adjusted for all steps as described below:

**Analytik Jena Biometra TAdvanced 96 SG/S: 2°C/sec for both heating and cooling*

***Eppendorf Mastercycler X50s/X50a: 3°C/sec heating and 2°C/sec cooling*

****ThermoFisher VeritiPro requires FW 1.2.0, 96 well tray/retainer (PN 4381850), and "Cover Ramping" enabled*

Additional Kits, Reagents & Equipment

The items in the table below have been validated by 10x Genomics and are highly recommended for the Single Cell 3' protocols. Substituting materials may adversely affect system performance. This list may not include some standard laboratory equipment.

Supplier	Description	Part Number (US)
Plastics		
Eppendorf	PCR Tubes 0.2 ml 8-tube strips DNA LoBind Tubes, 1.5 ml DNA LoBind Tubes, 2.0 ml	951010022 022431021 022431048
USA Scientific	TempAssure PCR 8-tube strip	1402-4700
Thermo Fisher Scientific	MicroAmp 8-Tube Strip, 0.2 ml MicroAmp 8 -Cap Strip, clear	N8010580 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 VWR Mini Centrifuge (alternatively, use any equivalent mini centrifuge)	10153-838 41428-958 76269-066
Eppendorf	Eppendorf ThermoMixer C Eppendorf SmartBlock 1.5 ml, Thermoblock for 24 reaction vessel (alternatively, use a temperature-controlled Heat Block)	5382000023 5360000038
Quantification & Quality Control		
Agilent	2100 Bioanalyzer Instrument & Laptop Bundle High Sensitivity DNA Kit 4200 TapeStation High Sensitivity D1000 ScreenTape/Reagents High Sensitivity D5000 ScreenTape/Reagents	G2939BA & G2953CA 5067-4626 G2991AA 5067-5584/5067-5585 5067-5592/5067-5593
Advanced Analytical	Fragment Analyzer Automated CE System - 12 cap Fragment Analyzer Automated CE System - 48/96 cap High Sensitivity NGS Fragment Analysis Kit	FSv2-CE2F FSv2-CE10F 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

Choose either Eppendorf, USA Scientific or Thermo Fisher Scientific PCR 8-tube strips.

Choose Bioanalyzer, TapeStation, LabChip, or Fragment Analyzer based on availability & preference.

Recommended Pipette Tips

10x Genomics recommends using only validated emulsion-safe pipette tips for all Single Cell protocols. Rainin pipette tips have been extensively validated by 10x Genomics and are highly recommended for all single cell assays. If Rainin tips are unavailable, any of the listed alternate pipette tips validated by 10x Genomics may be used.

Supplier	Description	Part Number (US)
Recommended Pipettes & Pipette tips		
Rainin	**Pipettes**	
	Pipet-Lite Multi Pipette L8-50XLS+	17013804
	Pipet-Lite Multi Pipette L8-200XLS+	17013805
	Pipet-Lite Multi Pipette L8-10XLS+	17013802
	Pipet-Lite Multi Pipette L8-20XLS+	17013803
	Pipet-Lite LTS Pipette L-2XLS+	17014393
	Pipet-Lite LTS Pipette L-10XLS+	17014388
	Pipet-Lite LTS Pipette L-20XLS+	17014392
	Pipet-Lite LTS Pipette L-100XLS+	17014384
	Pipet-Lite LTS Pipette L-200XLS+	17014391
	Pipet-Lite LTS Pipette L-1000XLS+	17014382
	Pipette Tips	
	Tips LTS 200UL Filter RT-L200FLR	30389240
	Tips LTS 1ML Filter RT-L1000FLR	30389213
	Tips LTS 20UL Filter RT-L10FLR	30389226
Alternate Recommendations *(If Rainin pipette tips are unavailable, any of the listed pipette tips may be used)*		
Eppendorf	**Pipettes**	
	Eppendorf Research plus, 8-channel, epT.I.P.S. Box, 0.5 – 10 µL	3125000010
	Eppendorf Research plus, 8-channel, epT.I.P.S. Box, 10 – 100 µL	3125000036
	Eppendorf Research plus, 8-channel, epT.I.P.S. Box, 100 – 300 µL	3125000052
	Eppendorf Research plus, 1-channel, epT.I.P.S.® Box, 0.1 – 2.5 µL	3123000012
	Eppendorf Research plus, 1-channel, epT.I.P.S.® Box, 0.5 – 10 µL	3123000020
	Eppendorf Research plus, 1-channel, epT.I.P.S.® Box, 2 – 20 µL	3123000039
	Eppendorf Research plus, 1-channel, epT.I.P.S.® Box, 2 – 200 µL	3123000055
	Eppendorf Research plus, 1-channel, epT.I.P.S.® Box, 100 – 1000 µL	3123000063
	Pipette Tips (compatible with Eppendorf pipettes only)	
	ep Dualfilter T.I.P.S., 2-20 µL	0030078535
	ep Dualfilter T.I.P.S., 2-200 µL	0030078551
	ep Dualfilter T.I.P.S., 2-1,000 µL	0030078578
Labcon*	ZAP SLIK 20 µL Low Retention Aerosol Filter Pipet Tips for Rainin LTS	4-1143-965-008
	ZAP SLIK 200 µL Low Retention Aerosol Filter Pipet Tips for Rainin LTS	4-1144-965-008
	ZAP SLIK 1000 µL Low Retention Aerosol Filter Pipet Tips for Rainin LTS	4-1145-965-008
Biotix*	xTIP4 Racked Pipette Tips, Rainin LTS Pipette Compatible, 0.1-20uL	63300931
	xTIP4 Racked Pipette Tips, Rainin LTS Pipette Compatible, 200uL	63300001
	xTIP4 Racked Pipette Tips, Rainin LTS Pipette Compatible, 1000uL	63300003

**Compatible with Rainin pipettes*

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			
	1.1	Prepare Reaction Mix	20 min	
	1.2	Load Chromium Next GEM Chip	10 min	
4h	1.3	Run the Chromium Controller or X/iX	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	45 min	
	2.2	cDNA Amplification	40 min	STOP 4°C ≤72 h or –20°C ≤1 week
	2.3	cDNA Cleanup – SPRIselect		
6h		2.3A Pellet Cleanup	30 min	STOP 4°C ≤72 h or –20°C ≤4 weeks
		2.3B Transferred Supernatant Cleanup	20 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			
	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	30 min	
	3.5	Sample Index PCR	40 min	STOP 4°C ≤72 h
8 h Plus	3.6	Post Sample Index PCR Double Sided Size Selection- SPRIselect	30 min	STOP 4°C ≤72 h or -20°C long term
	3.7	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	

Stepwise Objectives

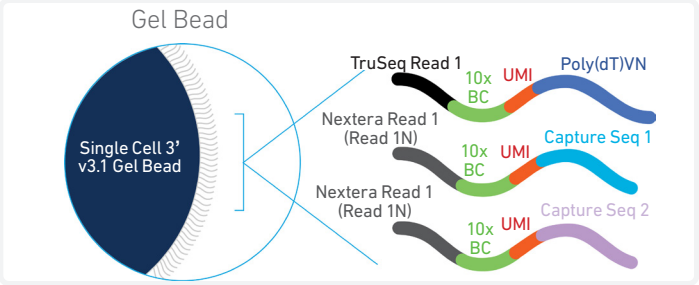
The Chromium Single Cell Gene Expression Solution with Feature Barcode technology upgrades short read sequencers to deliver a scalable microfluidic platform for assessing cell surface protein expression combined with 3' digital gene expression of the same single cell by profiling 500-10,000 individual cells per sample. A pool of ~3,500,000 10x Barcodes are sampled separately to index each cell's transcriptome and cell surface protein. It is done by partitioning thousands of cells into nanoliter-scale Gel Beads-in-emulsion (GEMs), where all generated DNA molecules share a common 10x Barcode. Libraries are generated and sequenced from the DNA molecules and 10x Barcodes are used to associate individual reads back to the individual partitions.

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

Single Cell 3' v3.1 Gel Beads

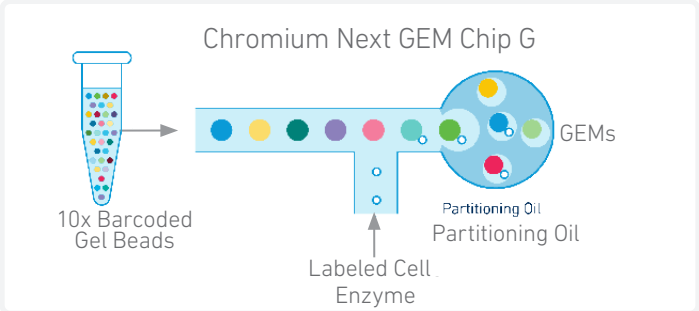
In addition to the poly(dT) primer that enables the production of barcoded, full-length cDNA from poly-adenylated mRNA, the Single Cell 3' v3.1 Gel Beads also include two additional primer sequences (Capture Sequence 1 and Capture Sequence 2), that enable capture and priming of Feature Barcode technology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



Step 1 GEM Generation & Barcoding

GEMs are generated by combining barcoded Single Cell 3' v3.1 Gel Beads, a Master Mix with cell surface protein labeled cells, and Partitioning Oil onto Chromium Chip G. 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.



Step 1 GEM Generation & Barcoding

Immediately following GEM generation, the Gel Bead is dissolved releasing the three types of primers 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 Capture Sequence 2 is not shown in the illustrated example).

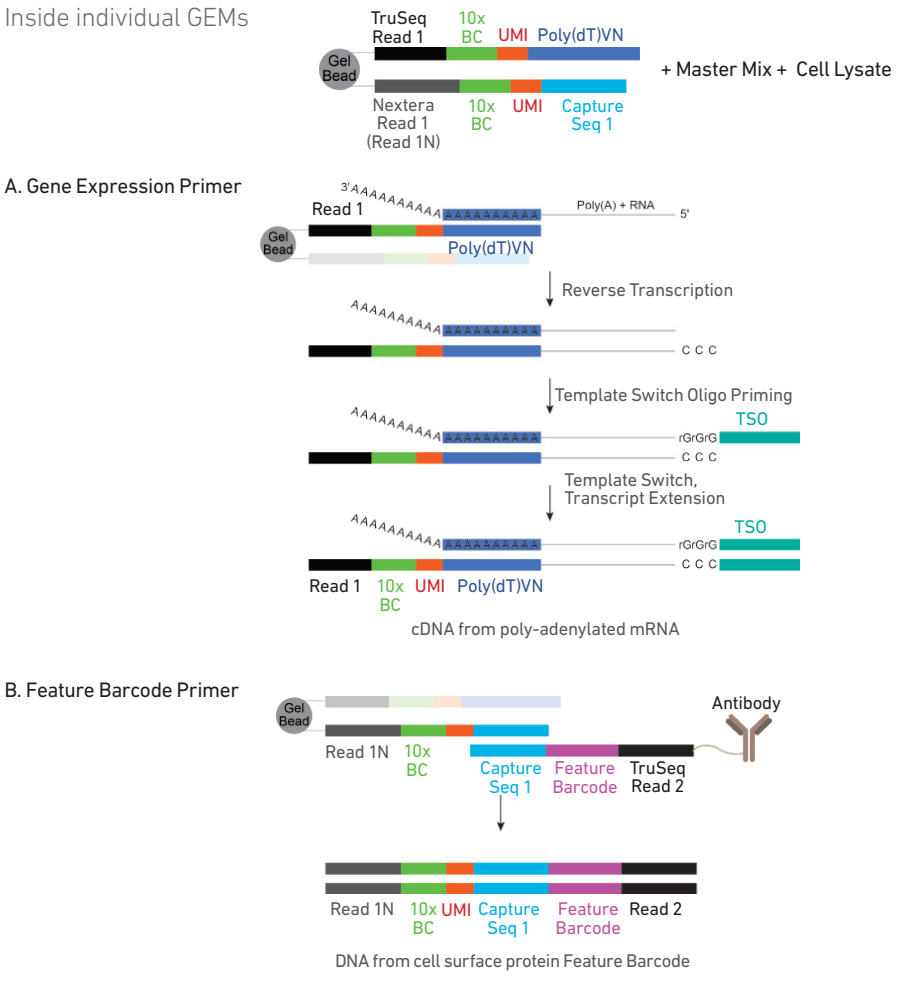
A. Primers containing:

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

B. Primers containing:

- an Illumina Nextera Read 1 (Read 1N; read 1 sequencing primer)
- 16 nt 10x Barcode
- 12 nt unique molecular identifier (UMI)
- Capture Sequence 1 or 2

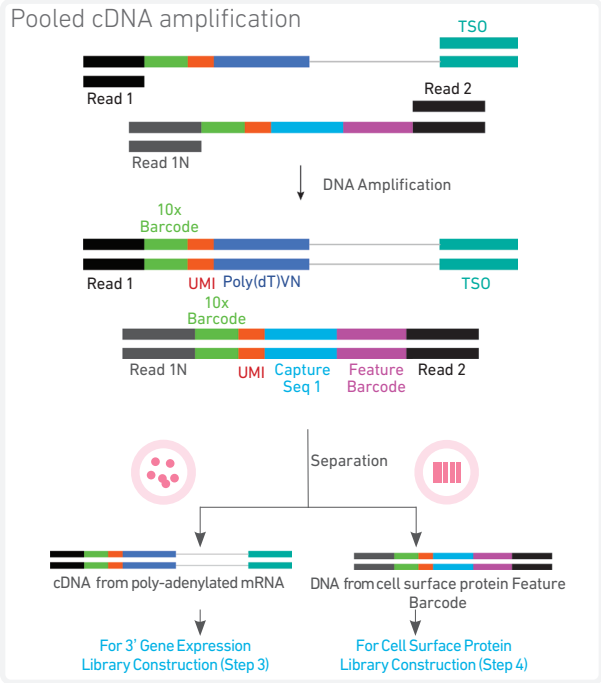
Both are mixed with cell lysate and Master Mix containing RT reagents. Incubation of the GEMs produces barcoded, full-length cDNA from poly-adenylated mRNA and barcoded DNA from the cell surface protein Feature Barcode.



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

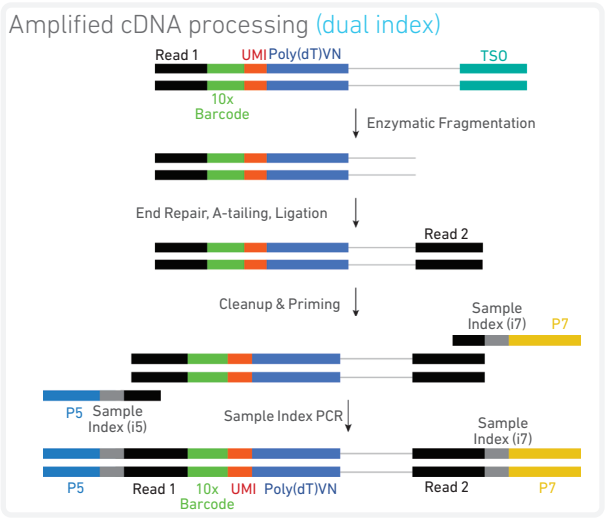
Step 2
Post GEM-RT Cleanup & cDNA Amplification

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



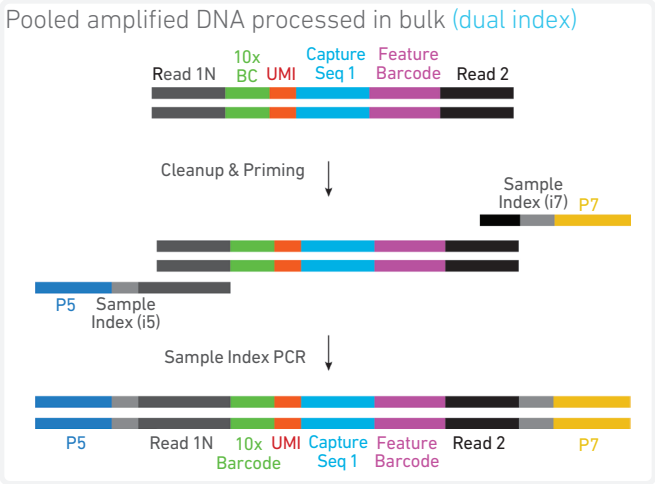
Step 3
3' Gene Expression Library Construction

Enzymatic fragmentation and size selection are used to optimize the cDNA amplicon size. P5, P7, 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.



Step 4 Cell Surface Protein Library Construction

Amplified DNA from cell surface protein Feature Barcodes is used for library construction. P5, P7, 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 bridge amplification.

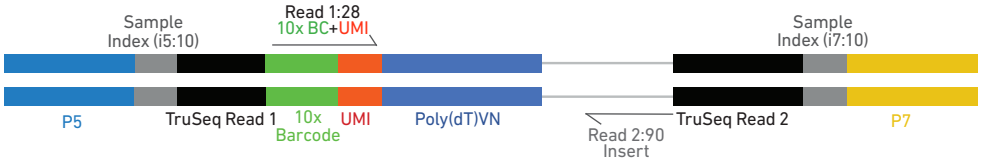


Step 5 Sequencing

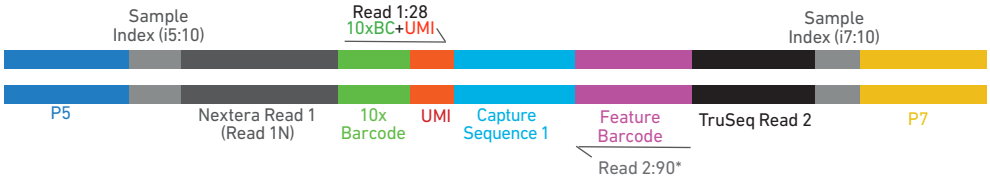
The Single Cell 3' libraries comprise standard Illumina paired-end constructs which begin and end with P5 and P7. The 16 bp 10x Barcode and 12 bp UMI are encoded in Read 1, while Read 2 is used to sequence the cDNA fragment in 3' Gene Expression libraries and the Feature Barcode in the Cell Surface Protein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

Illumina sequencer compatibility, sample indices, library loading and pooling, recommended read depths and run parameters are summarized in step 5.

Chromium Single Cell 3' Gene Expression Dual Index Library



Chromium Single Cell 3' Cell Surface Protein Dual Index Library



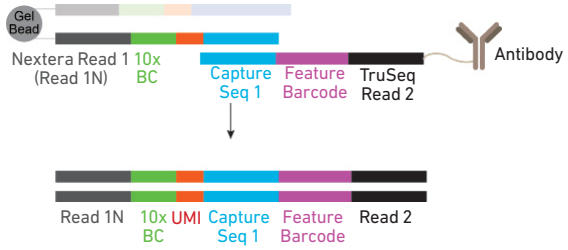
*Minimum required Read 2 length for Cell Surface Protein libraries is 25 bp

See Appendix for Oligonucleotide Sequences

Cell Surface Protein Labeling Guidelines

Overview

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



DNA from cell surface protein Feature Barcode

Demonstrated Protocols for cell surface protein labeling

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

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

Tips & Best Practices

Icons

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

Next GEM 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.

Cell Concentration

- Recommended starting point is to load ~1,600 cells per reaction, resulting in recovery of ~1000 cells, and a multiplet rate of ~0.8%. The optimal input cell concentration is 700-1,200 cells/µl.
- The presence of dead cells in the suspension may also reduce the recovery rate. Consult the 10x Genomics Single Cell Protocols Cell Preparation Guide and the Guidelines for Optimal Sample Preparation flowchart (Documents CG00053 and CG000126 respectively) for more information on preparing cells.

Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~0.4%	~825	~500
~0.8%	~1,650	~1,000
~1.6%	~3,300	~2,000
~2.4%	~4,950	~3,000
~3.2%	~6,600	~4,000
~4.0%	~8,250	~5,000
~4.8%	~9,900	~6,000
~5.6%	~11,550	~7,000
~6.4%	~13,200	~8,000
~7.2%	~14,850	~9,000
~8.0%	~16,500	~10,000

General Reagent Handling

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

50% Glycerol Solution

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

OR

- Prepare 50% glycerol solution:
  i. Mix an equal volume of water and 99% Glycerol, Molecular Biology Grade.
  ii. Filter through a 0.2 µm filter.
  iii. Store at –20°C in 1-ml LoBind tubes. 50% glycerol solution should be equilibrated to room temperature before use.

Pipette Calibration

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

Chromium Next GEM Chip Handling

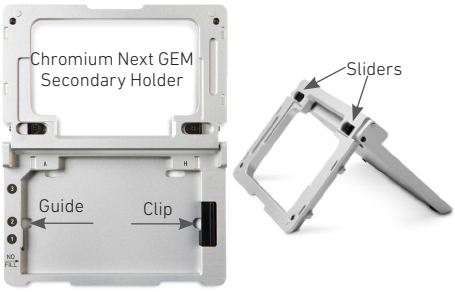
Next GEM

- 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 multiple chips are to be used, load, run, and collect the content from one chip before loading the next.
- Fill all unused input wells in rows labeled 1, 2, and 3 on a chip with an appropriate volume of 50% glycerol solution before loading the used wells. DO NOT add glycerol to the wells in the bottom NO FILL row.
- Avoid contacting the bottom surface of the chip with gloved hands and other surfaces. Frictional charging can lead to inadequate priming of the channels, potentially leading to either clogs or wetting failures.
- Minimize the distance that a loaded chip is moved to reach the Chromium Controller or X/iX.
- Keep the chip horizontal to prevent wetting the gasket with oil, which depletes the input volume and may adversely affect the quality of the resulting emulsion.

Chromium Next GEM Secondary Holders

Next GEM

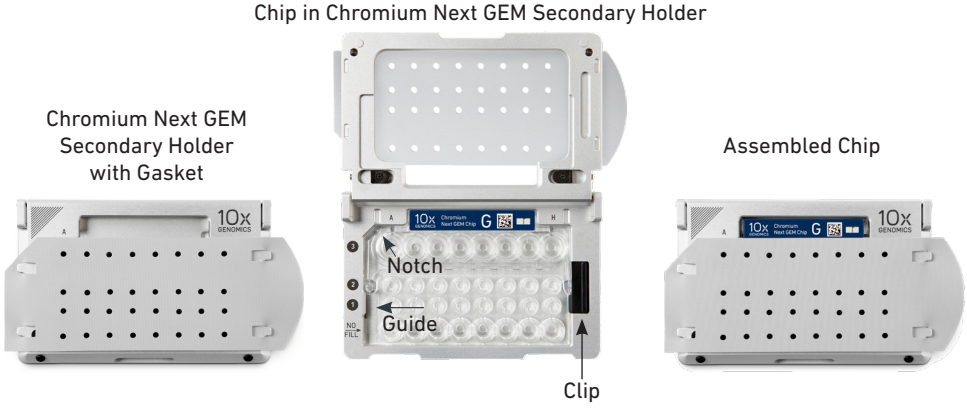
- Chromium Next GEM Secondary Holders encase Chromium Next GEM Chips.
- The holder lid flips over to become a stand, holding the chip at 45 degrees for optimal recovery well content removal.
- Squeeze the black sliders on the back side of the holder together to unlock the lid and return the holder to a flat position.



Chromium Next GEM Chip & Holder Assembly with Gasket

Next GEM

- Close the holder lid. Attach the gasket by holding the tongue (curved end, to the right) and hook the gasket on the left-hand tabs of the holder. Gently pull the gasket toward the right and hook it on the two right-hand tabs.
- DO NOT touch the smooth side of the gasket.
- Open the chip holder.
- Align notch on the chip (upper left corner) and the open holder with the gasket attached.
- Slide the chip to the left until the chip is inserted under the guide on the holder. Depress the right hand side of the chip until the spring-loaded clip engages.
- Keep the assembled unit with the attached gasket until ready for dispensing reagents into the wells.



Chromium Next GEM Chip Loading

Next GEM

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

Gel Bead Handling

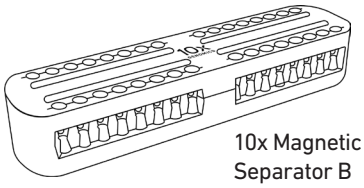
Next GEM

- Use one tube of Gel Beads per sample. DO NOT puncture the foil seals of tubes not used at the time.
- Equilibrate the Gel Beads strip to room temperature 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.

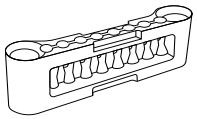
- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**.
- Centrifuge the Gel Bead strip for ~**5 sec** after removing from the holder. Confirm there are no bubbles at the bottom of the tubes and the liquid levels look even. Place the Gel Bead strip back in the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

10x Magnetic Separator

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



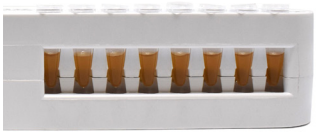
10x Magnetic Separator B



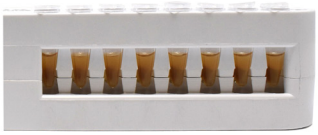
10x Magnetic Separator

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 need for the solution to clear may vary based on specific step, reagents, volume of reagents used etc.
- Images below are representative - actual color of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.



1. Magnetic beads mixed with reagent



2. Separation in progress

3. Separation continuing

4. Separation complete; solution clear

SPRIselect Cleanup & Size Selection

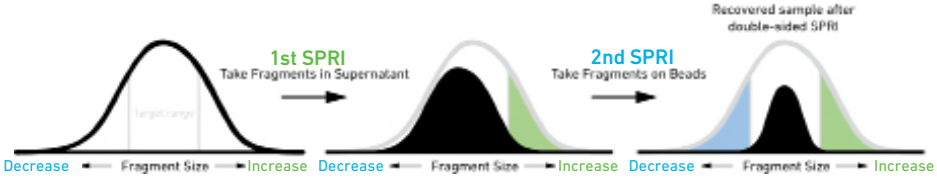
- After aspirating the desired volume of SPRIselect reagent, examine the pipette tips before dispensing to ensure the correct volume is transferred.
- Pipette mix thoroughly as insufficient mixing of sample and SPRIselect reagent will lead to inconsistent results.
- Use fresh preparations of 80% Ethanol.

Tutorial — SPRIselect Reagent:DNA Sample Ratios

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

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

Schematic of Double Sided Size Selection



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

Tutorial — Double Sided Size Selection

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

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

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

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

Enzymatic Fragmentation

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

Sample Indices in Sample Index PCR

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

Index Hopping Mitigation

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

- Remove adaptors 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 adaptors before sequencing.
- Hopped indices can be computationally removed from the data generated from single cell dual index libraries.

Step 1

GEM Generation & Barcoding

1.1 Prepare Single Cell Master Mix
1.2 Load Chromium Next GEM Chip G
1.3 Run the Chromium Controller or X/iX
1.4 Transfer GEMs
1.5 GEM-RT Incubation

1.0 GEM Generation & Barcoding

GET STARTED!				
Action	**Item**	**10x PN**	**Preparation & Handling**	**Storage**
Equilibrate to Room Temperature	**Single Cell 3' v3.1 Gel Beads**	2000164	Equilibrate to room temperature 30 min before loading the chip.	–80°C
	● **RT Reagent B**	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. Thaw at temperature for ≥ 30 minutes in subsequent uses.	–20°C
	○ **Reducing Agent B**	2000087	Vortex, verify no precipitate, centrifuge briefly.	–20°C
Place on Ice	● **RT Enzyme C**	2000085/ 2000102	Centrifuge briefly before adding to the mix.	–20°C
	Labeled Cell Suspension			
Obtain	● **Partitioning Oil**	2000190	-	Ambient
	Chromium Next GEM Chip G	2000177	-	Ambient
	10x Gasket	370017/ 3000072	See Tips & Best Practices.	Ambient
	Chromium Next GEM Secondary Holder	3000332	See Tips & Best Practices.	Ambient
	10x Vortex Adapter	330002	See Tips & Best Practices.	Ambient
	50% glycerol solution If using <8 reactions	-	See Tips & Best Practices.	-

Consult Demonstrated Protocol Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols with Feature Barcoding technology (CG000149)

Firmware Version 4.0 or higher is required in the Chromium Controller or the Chromium Single Cell Controller used for this Single Cell 3' v3.1 protocol.

1.1 Prepare Master Mix

Next GEM

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

Master Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
● **RT Reagent B**	2000165	18.8	82.7	165.4
● **Template Switch Oligo**	3000228	2.4	10.6	21.1
○ **Reducing Agent B**	2000087	2.0	8.8	17.6
● **RT Enzyme C**	2000085/ 2000102	8.7	38.3	76.6
Total	-	**31.9**	**140.4**	**280.7**

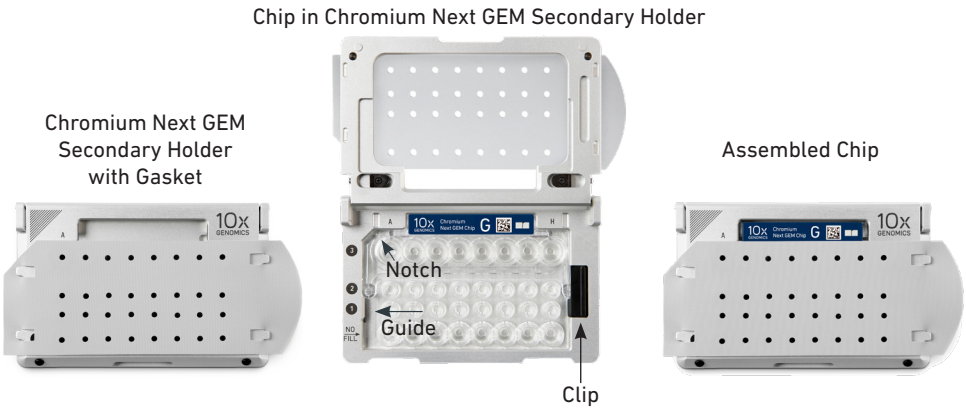
b. Add **31.9 µl** Master Mix into each tube of a PCR 8-tube strip on ice.

Assemble Chromium Next GEM Chip

TIPS

See Tips & Best Practices for chip handling instructions.

- Close the holder lid. Attach the gasket by holding the tongue (curved end, to the right) and hook the gasket on the left-hand tabs of the holder. Gently pull the gasket toward the right and hook it on the two right-hand tabs.
- DO NOT touch the smooth side of the gasket.
- Open the chip holder.
- Remove the chip from the sealed bag. Use the chip within ≤ 24 h.
- Align notch on the chip (upper left corner) and the open holder with the gasket attached.
- Slide the chip to the left until the chip is inserted under the guide on the holder. Depress the right hand side of the chip until the spring-loaded clip engages.
- Keep the assembled unit with the attached gasket open until ready for and while dispensing reagents into the wells. DO NOT touch the smooth side of the gasket. After loading reagents, close the chip holder. DO NOT press down on the top of the gasket.



For GEM generation, load the indicated reagents only in the specified rows, starting from row labeled 1, followed by rows labeled 2 and 3. DO NOT load reagents in the bottom row labeled NO FILL. See step 1.2 for details.

Cell Suspension Volume Calculator Table

(for step 1.2 of Chromium Next GEM Single Cell 3' v3.1 protocol)

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 1.2b.

Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery										
	500	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000
100	8.3 35.0	16.5 26.7	33.0 10.2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
200	4.1 39.1	8.3 35.0	16.5 26.7	24.8 18.5	33.0 10.2	41.3 2.0	n/a	n/a	n/a	n/a	n/a
300	2.8 40.5	5.5 37.7	11.0 32.2	16.5 26.7	22.0 21.2	27.5 15.7	33.0 10.2	38.5 4.7	n/a	n/a	n/a
400	2.1 41.1	4.1 39.1	8.3 35.0	12.4 30.8	16.5 26.7	20.6 22.6	24.8 18.5	28.9 14.3	33.0 10.2	37.1 6.1	41.3 2.0
500	1.7 41.6	3.3 39.9	6.6 36.6	9.9 33.3	13.2 30.0	16.5 26.7	19.8 23.4	23.1 20.1	26.4 16.8	29.7 13.5	33.0 10.2
600	1.4 41.8	2.8 40.5	5.5 37.7	8.3 35.0	11.0 32.2	13.8 29.5	16.5 26.7	19.3 24.0	22.0 21.2	24.8 18.5	27.5 15.7
700	1.2 42.0	2.4 40.8	4.7 38.5	7.1 36.1	9.4 33.8	11.8 31.4	14.1 29.1	16.5 26.7	18.9 24.3	21.2 22.0	23.6 19.6
800	1.0 42.2	2.1 41.1	4.1 39.1	6.2 37.0	8.3 35.0	10.3 32.9	12.4 30.8	14.4 28.8	16.5 26.7	18.6 24.6	20.6 22.6
900	0.9 42.3	1.8 41.4	3.7 39.5	5.5 37.7	7.3 35.9	9.2 34.0	11.0 32.2	12.8 30.4	14.7 28.5	16.5 26.7	18.3 24.9
1000	0.8 42.4	1.7 41.6	3.3 39.9	5.0 38.3	6.6 36.6	8.3 35.0	9.9 33.3	11.6 31.7	13.2 30.0	14.9 28.4	16.5 26.7
1100	0.8 42.5	1.5 41.7	3.0 40.2	4.5 38.7	6.0 37.2	7.5 35.7	9.0 34.2	10.5 32.7	12.0 31.2	13.5 29.7	15.0 28.2
1200	0.7 42.5	1.4 41.8	2.8 40.5	4.1 39.1	5.5 37.7	6.9 36.3	8.3 35.0	9.6 33.6	11.0 32.2	12.4 30.8	13.8 29.5
1300	0.6 42.6	1.3 41.9	2.5 40.7	3.8 39.4	5.1 38.1	6.3 36.9	7.6 35.6	8.9 34.3	10.2 33.0	11.4 31.8	12.7 30.5
1400	0.6 42.6	1.2 42.0	2.4 40.8	3.5 39.7	4.7 38.5	5.9 37.3	7.1 36.1	8.3 35.0	9.4 33.8	10.6 32.6	11.8 31.4
1500	0.6 42.7	1.1 42.1	2.2 41.0	3.3 39.9	4.4 38.8	5.5 37.7	6.6 36.6	7.7 35.5	8.8 34.4	9.9 33.3	11.0 32.2
1600	0.5 42.7	1.0 42.2	2.1 41.1	3.1 40.1	4.1 39.1	5.2 38.0	6.2 37.0	7.2 36.0	8.3 35.0	9.3 33.9	10.3 32.9
1700	0.5 42.7	1.0 42.2	1.9 41.3	2.9 40.3	3.9 39.3	4.9 38.3	5.8 37.4	6.8 36.4	7.8 35.4	8.7 34.5	9.7 33.5
1800	0.5 42.7	0.9 42.3	1.8 41.4	2.8 40.5	3.7 39.5	4.6 38.6	5.5 37.7	6.4 36.8	7.3 35.9	8.3 35.0	9.2 34.0
1900	0.4 42.8	0.9 42.3	1.7 41.5	2.6 40.6	3.5 39.7	4.3 38.9	5.2 38.0	6.1 37.1	6.9 36.3	7.8 35.4	8.7 34.5
2000	0.4 42.8	0.8 42.4	1.7 41.6	2.5 40.7	3.3 39.9	4.1 39.1	5.0 38.3	5.8 37.4	6.6 36.6	7.4 35.8	8.3 35.0

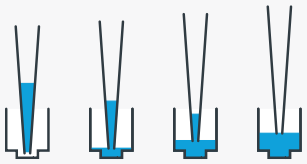
Grey boxes: Volumes that would exceed the allowable water volume in each reaction
Yellow boxes: Indicate a low transfer volume that may result in higher cell load variability
Blue boxes: Optimal range of cell stock concentration to maximize the likelihood of achieving the desired cell recovery target

1.2 Load Chromium Next GEM Chip G

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 pipette centered to each well and the tips slightly submerged.

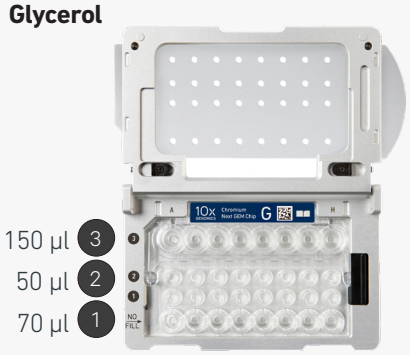


a. Add 50% glycerol solution to each unused well (if processing <8 samples/chip)

- **70 µl** in each unused well in row labeled **1**
- **50 µl** in each unused well in row labeled **2**
- **150 µl** in each unused well in row labeled **3**

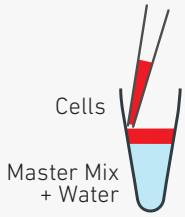
DO NOT add 50% glycerol solution to the bottom row of NO FILL wells. DO NOT use any substitute for 50% glycerol solution.

Glycerol



b. 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. Add corresponding volume of single cell suspension to Master Mix. Total of ***75 µl*** in each tube.
- Gently pipette mix the cell suspension before adding to the Master Mix.



c. Load Row Labeled 1

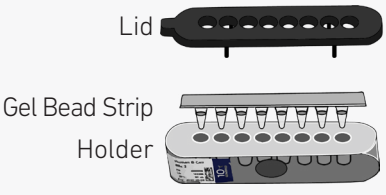
- Gently pipette mix the Master Mix + Cell Suspension
- Using the same pipette tip, dispense **70 µl** Master Mix + Cell Suspension into the bottom center of each well in **row labeled 1** without introducing bubbles.

Master Mix + Sample

d. Prepare Gel Beads

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

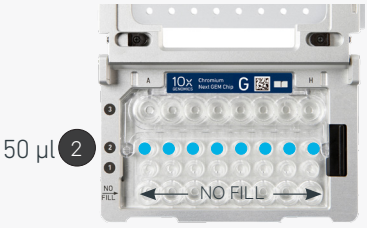
Prep Gel Beads



e. Load Row Labeled 2

- Puncture the foil seal of the Gel Bead tubes.
- Slowly aspirate **50 µl** Gel Beads.
- Dispense into the wells in **row labeled 2** without introducing bubbles.
- Wait **30 sec.**

Gel Beads

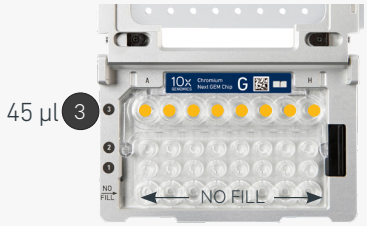


f. Load Row Labeled 3

- Dispense **45 µl** Partitioning Oil into the wells in **row labeled 3** from a reagent reservoir.

⚠ Failure to add Partitioning Oil to the top row labeled 3 will prevent GEM generation and can damage the Chromium Controller or X/iX.

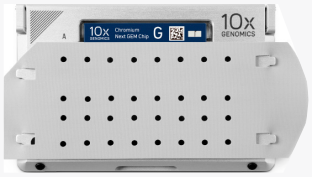
Partitioning Oil



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 the Chromium Controller or X/iX immediately after loading the Partitioning Oil



Keep horizontal to avoid wetting the gasket. DO NOT press down on the gasket.

1.3 Run the Chromium Controller or X/iX

If using Chromium Controller:

a. Press the eject button on the Controller to eject the tray.

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.

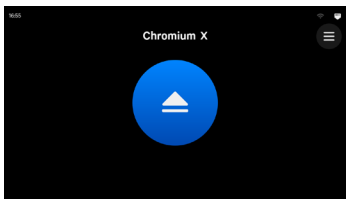
⚠ **d.** At completion of the run **(~18 min)**, the Controller will chime. **Immediately** proceed to the next step.

⚠ Firmware Version 4.0 or higher is required in the Chromium Controller or the Chromium Single Cell Controller used for this protocol.

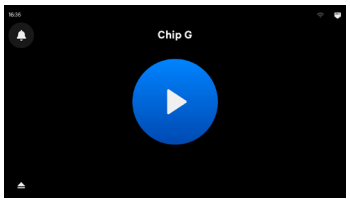
If using Chromium X/iX:

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

a. Press the eject button on Chromium X/iX 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.

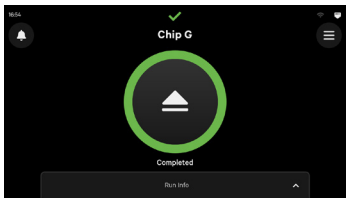


b. Place the assembled chip with the gasket in the tray, ensuring that the chip stays horizontal. Press the button to retract the tray.



c. Press the play button.

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



1.4 Transfer GEMs

Next GEM

a. Place a tube strip on ice.

b. Press the eject button of the Controller or X/iX and remove the chip.

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

Expose Wells at 45 Degrees

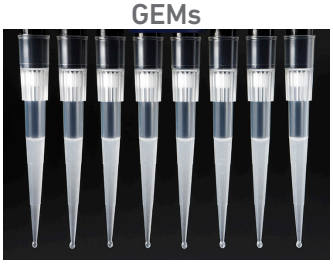
d. Visually compare the remaining volume in rows labeled 1-2. Abnormally high volume in one well relative to other wells may indicate a clog.

Transfer GEMs

e. Slowly aspirate **100 µl** GEMs from the lowest points of the recovery wells in the top row labeled 3 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.

GEMs



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

h. If multiple chips are run back-to-back, cap/cover the GEM-containing tube strip 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**
1	53°C	00:45:00
2	85°C	00:05:00
3	4°C	Hold

STOP

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 Post GEM-RT Cleanup & cDNA Amplification

GET STARTED!

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 2 Verify name & PN	2000097	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 and quantification	-	Manufacturer's recommendations.	-
	Agilent TapeStation ScreenTape and Reagents If used for QC and quantification	-	Manufacturer's recommendations.	-
Place on ice	**Amp Mix** Retrieve from Single Cell 3' GEM Kit	2000047/ 2000103	Vortex, centrifuge briefly.	–20°C
Thaw at 65°C	**Cleanup Buffer**	2000088	Thaw for 10 min at 65°C at max speed on a thermomixer. Verify no visible crystals. Cool to room temperature.	–20°C
Obtain	**Recovery Agent**	220016	-	Ambient
	Qiagen Buffer EB	-	Manufacturer's recommendations.	-
	Bio-Rad 10% Tween 20	-	Manufacturer's recommendations.	-
	10x Magnetic Separator/ 10x Magnetic Separator B	230003/ 2001212	-	Ambient
	Prepare 80% Ethanol Prepare 15 ml for 8 reactions.	-	-	-

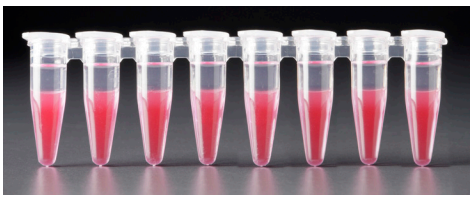
2.1 Post GEM-RT Cleanup – Dynabeads

Next GEM

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).

Biphasic Mixture

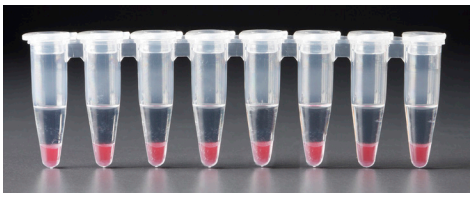


If biphasic separation is incomplete:
Firmly secure the cap on the tube strip, ensuring that no liquid is trapped between the cap and the tube rim. Mix by inverting the capped tube strip 5x, centrifuge briefly, and proceed to step b. DO NOT invert without firmly securing the caps.

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.

Remove Recovery Agent



c. Prepare Dynabeads Cleanup Mix.

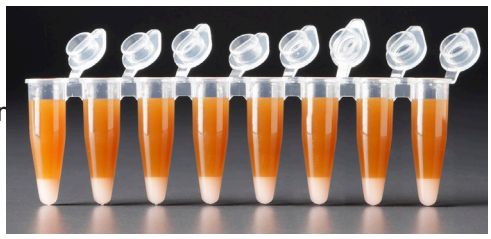
Dynabeads Cleanup Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Cleanup Buffer	2000088	182	801	1602
Dynabeads MyOne SILANE Vortex thoroughly (≥**30 sec**) **immediately** before adding to the mix. Aspirate the full liquid volume with a pipette tip to verify that the beads have not settled in the bottom of the tube. If clumps are present, pipette mix to resuspend completely. DO NOT centrifuge before use.	2000048	8	35	70
Reducing Agent B	2000087	5	22	44
Nuclease-free Water		5	22	44
Total	-	**200**	**880**	**1760**

Resuspend clump

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

e. Incubate **10 min** at **room temperature** (keep caps open). Pipette mix again at **~5 min** after start of incubation to resuspend settled beads.

Add Dynabeads Cleanup Mix



f. Prepare Elution Solution I. Vortex and centrifuge briefly.

	Elution Solution I *Add reagents in the order listed*	PN	1X (µl)	10X (µl)
	Buffer EB	-	98	980
	10% Tween 20	-	1	10
◯	**Reducing Agent B**	2000087	1	10
	Total	-	**100**	**1000**

TIPS

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

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

h. Remove the supernatant (aqueous phase and Recovery Agent).

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. Remove from the magnet. Immediately add **35.5 µl** Elution Solution I (prepared in step 2.1f).

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	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
○ **Amp Mix** Retrieve from Single Cell 3' GEM Kit	2000047	50	220	440
● **Feature cDNA Primers 2** Verify name & PN Use indicated primer only	2000097	15	66	132
Total	-	**65**	**286**	**572**

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**
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

The optimal number of cycles is a trade-off between generating sufficient final mass for library construction and minimizing PCR amplification artifacts. The number of cDNA cycles should also be reduced if large numbers of cells are sampled.

Recommended starting point for cycle number optimization.

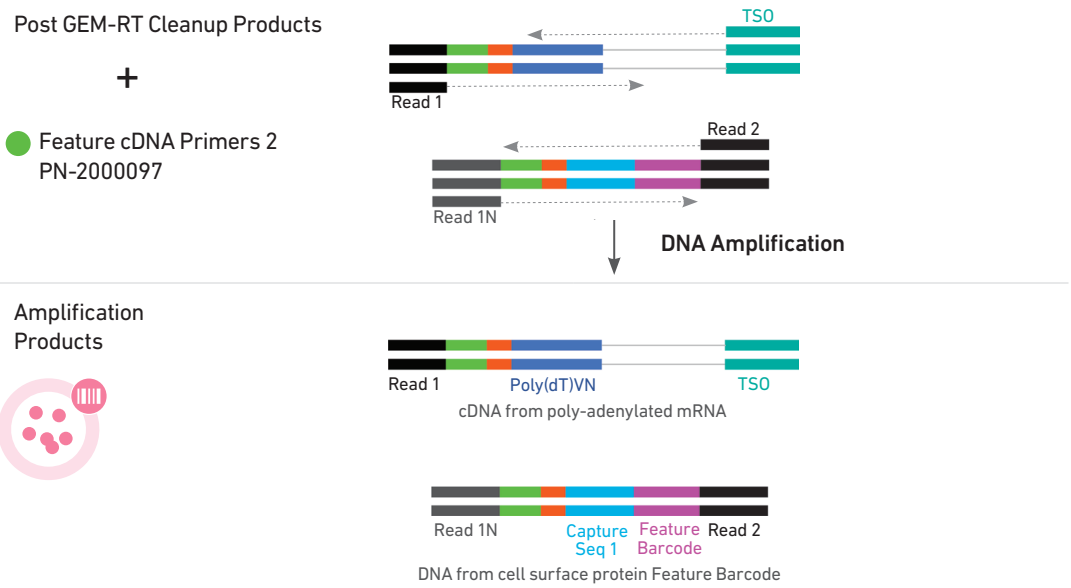
Targeted Cell Recovery	Total Cycles
<500	13
500–6,000	12
>6,000	11

STOP

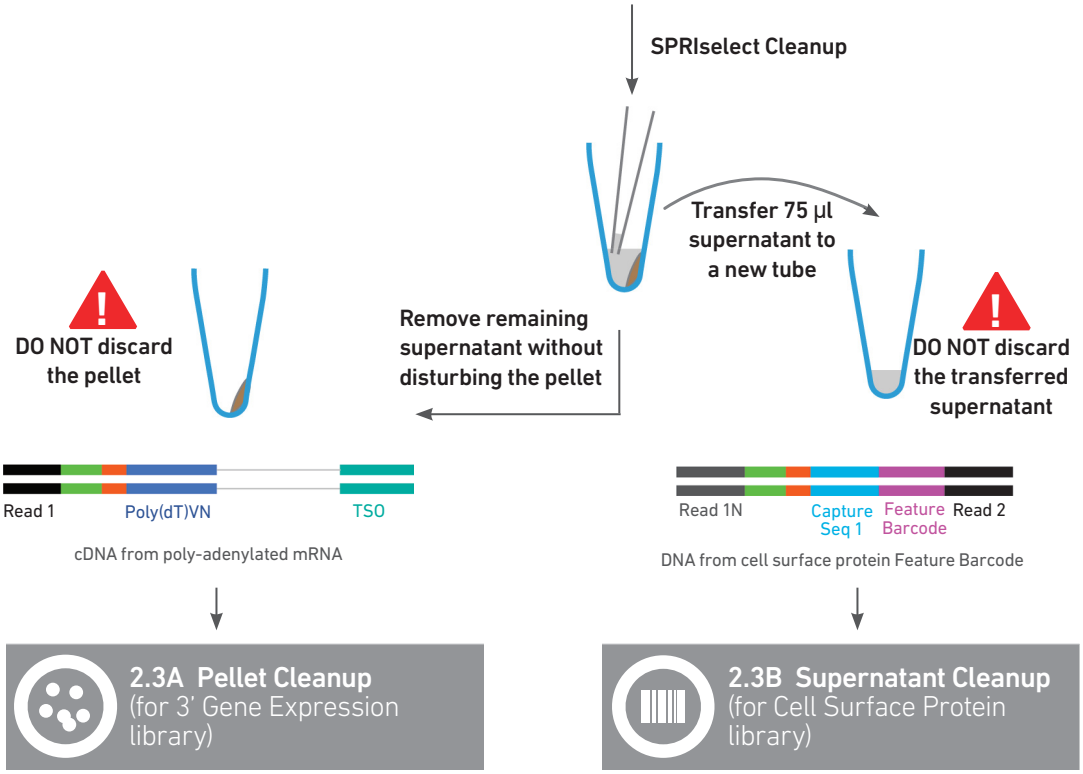
e. Store at **4°C** for up to **72 h** or **–20°C** for **≤1 week**, or proceed to the next step.

Step Overview (steps 2.2 & 2.3)

Amplification Products Generated in Step 2.2 – cDNA Amplification



Step 2.3 – cDNA Cleanup – SPRIselect Overview



2.3 cDNA Cleanup – SPRIselect

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

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

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

d. Transfer and save **75 µl** supernatant in a new tube strip without disturbing the pellet. Maintain at **room temperature**. DO NOT discard the transferred supernatant (cleanup for Cell Surface Protein library construction).

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

2.3A Pellet Cleanup (for 3' Gene Expression library)

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

ii. Remove the ethanol.

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

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

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

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

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

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

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

x. STOP 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 library)

i. Vortex to resuspend the SPRIselect reagent. Add **70 µl** SPRIselect reagent **(2.1X)** to **75 µl** of the transferred supernatant and pipette mix 15x (pipette set to 150 µl).

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

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

iv. Remove supernatant.

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

vi. Remove the ethanol.

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

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

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

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

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

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

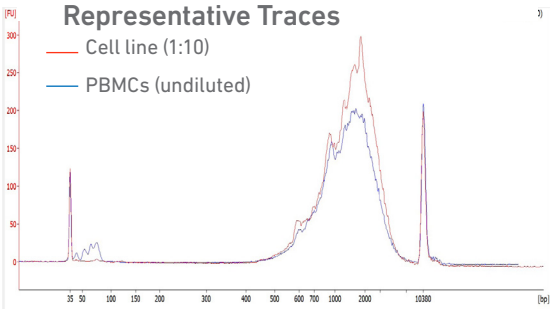
xiii. Transfer **40 µl** sample to a new tube strip.

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

2.4 Post cDNA Amplification QC & Quantification

a. Run **1 µl** of sample from **Pellet Cleanup** (step 2.3A-x), diluted 1:10 on an Agilent Bioanalyzer High Sensitivity chip. DO NOT run sample from 2.3B Transferred Supernatant Cleanup step.

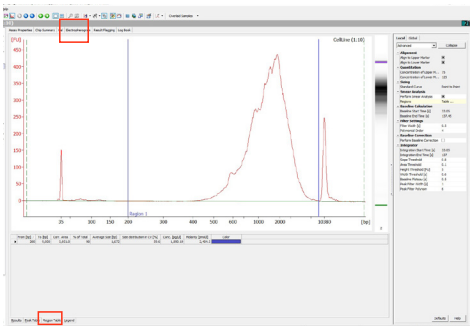
For input cells with low RNA content (<1pg total RNA/cell), 1 µl undiluted product may be run.
Lower molecular weight product (35 – 150 bp) may be present. This is normal and does not affect sequencing or application performance.



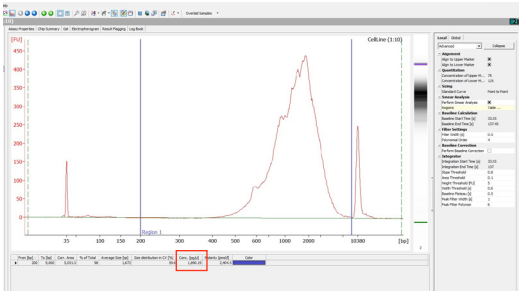
EXAMPLE CALCULATION

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 the Agilent 2100 Expert Software by the elution volume (40 µl) of the Post cDNA Amplification Reaction Clean Up sample (taking any dilution factors into account) and then divide by 1000 to obtain the total cDNA yield in ng.

Example Calculation of cDNA Total Yield

Concentration: 1890.19 pg/µl
Elution Volume: 40
Dilution Factor: 10

Total cDNA Yield

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

$$= \frac{1890.19\ \text{(pg/µl)} \times 40 \times 10}{1000\ \text{(pg/ng)}} = 756.08\ \text{ng}$$

Carry forward **ONLY 25%** of total cDNA yield into 3' Gene Expression Library Construction (step 3)

= 0.25 x Total cDNA yield

= 0.25 x 756.08= 189.02 ng

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

Alternate Quantification Methods See Appendix for representative traces

- Agilent TapeStation
- LabChip

Agilent Bioanalyzer, Agilent TapeStation, LabChip are the recommended methods for accurate quantification.

Step 3

3’ Gene Expression Library Construction

3.0 3' Gene Expression Library Construction

GET STARTED!				
Action	**Item**	**10x PN**	**Preparation & Handling**	**Storage**
Equilibrate to Room Temperature	**Fragmentation Buffer**	2000091	Vortex, verify no precipitate, centrifuge briefly.	–20°C
	Adaptor Oligos	2000094	Vortex, centrifuge briefly.	–20°C
	Ligation Buffer	2000092	Vortex, verify no precipitate, centrifuge briefly.	–20°C
	DUAL INDEX **Dual Index Plate TT Set A** Verify name & PN Use indicated plate only	3000431	-	–20°C
	Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendations.	-
	Agilent TapeStation Screen Tape and Reagents If used for QC	-	Manufacturer's recommendations.	-
	Agilent Bioanalyzer High Sensitivity kit If used for QC	-	Manufacturer's recommendations.	-
	DNA High Sensitivity Reagent Kit If LabChip used for QC	-	Manufacturer's recommendations.	-
Place on Ice	**Fragmentation Enzyme**	2000090/ 2000104	Centrifuge briefly.	–20°C
	DNA Ligase	220110/ 220131	Centrifuge briefly.	–20°C
	Amp Mix	2000047/ 2000103	Centrifuge briefly.	–20°C
	KAPA Library Quantification Kit for Illumina Platforms	-	Manufacturer's recommendations.	-
Obtain	**Qiagen Buffer EB**	-	-	Ambient
	10x Magnetic Separator/ 10x Magnetic Separator B	230003/ 2001212	-	Ambient
	Prepare 80% Ethanol Prepare 20 ml for 8 reactions	-	Prepare fresh.	Ambient

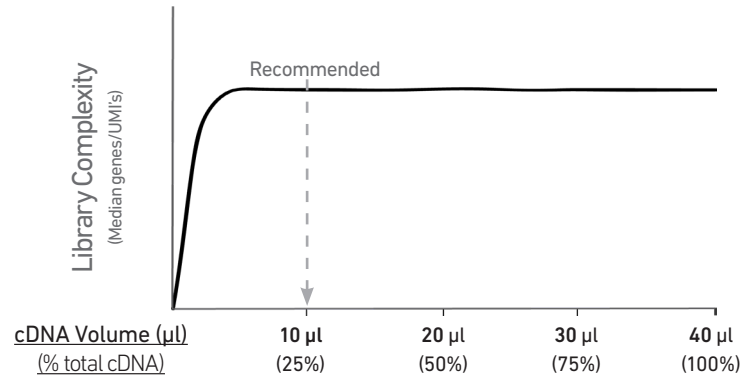
Ensure that Fragmentation Enzyme and Fragmentation Buffer from the same kit are used together. Lots are matched for optimal performance.

Step Overview (Step 3.1d)

Correlation between input & library complexity

A Single Cell 3' Gene Expression library is generated using a fixed proportion (10 µl, 25%) of the total cDNA (40 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 (30 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**).

Correlation: cDNA input & Library Complexity



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

Example: Library Construction Input Mass & SI PCR Cycles

Cell Type	Targeted Cell Recovery	Total cDNA Yield (ng)	cDNA Input into Fragmentation		SI PCR Cycle Number
			Volume (µl)	Mass (ng)	
High RNA Content	Low	250 ng	10 µl	62.5 ng	13
	High	1900 ng	10 µl	475 ng	10
Low RNA Content	Low	1 ng	10 µl	0.25 ng	16
	High	200 ng	10 µl	50 ng	12

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**
Pre-cool block Pre-cool block prior to preparing the Fragmentation Mix	4°C	Hold
Fragmentation	32°C	00:05:00
End Repair & A-tailing	65°C	00:30:00
Hold	4°C	Hold

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

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

Fragmentation Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Fragmentation Buffer	2000091	5	22	44
Fragmentation Enzyme	2000090/ 2000104	10	44	88
Total	-	**15**	**66**	**132**

d. Transfer **ONLY 10 µl** purified cDNA sample from Pellet Cleanup (step 2.3A-x) to a tube strip.
Note that only **10 µl** (25%) cDNA sample transfer is sufficient for generating 3' Gene Expression library.
The remaining **30 µl** (75%) 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 **25 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 Size Selection – 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 transferred supernatant. 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. DO NOT over dry to ensure maximum elution efficiency.

m. Remove from the magnet. Add **50.5 µl** Buffer EB to each sample. Pipette mix 15x (pipette set to 45 µl).

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.

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)	4X + 10% (µl)	8X + 10% (µl)
Ligation Buffer	2000092	20	88	176
DNA Ligase	220110/ 220131	10	44	88
Adaptor Oligos	2000094	20	88	176
Total	-	**50**	**220**	**440**

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

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

Lid Temperature	Reaction Volume	Run Time
30°C	100 µl	15 min
Step	**Temperature**	**Time**
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 **30.5 µl** Buffer EB. Pipette mix 15x.

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

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

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

3.5 Sample Index PCR

DUAL INDEX

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

b. Add **50 µl** Amp Mix (PN-2000047/2000103) 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**
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 cDNA QC & Quantification (step 2.4)

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

STOP

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

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

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

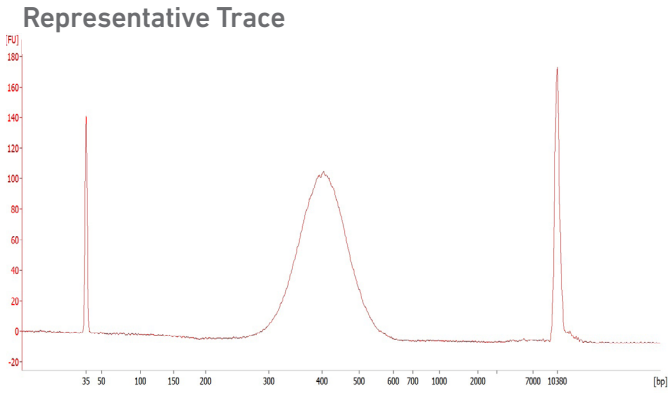
o. Place on the magnet•**Low** until the solution clears.

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 s**ample at **1:10 dilution** on an Agilent Bioanalyzer High Sensitivity chip.



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

If additional peaks below 200 bp are observed, repeat step 3.6 Post Sample Index PCR Double Sided Size Selection - SPRIselect. Add nuclease-free water to bring the library volume to **100 µl** before performing step 3.6a. Note that ~40% of material may be lost when repeating step 3.6.

Alternatively, libraries that will be sequenced together can first be pooled and then used as input into step 3.6.

See Troubleshooting for further details.

Alternate QC Method:

- Agilent TapeStation
- LabChip

See Appendix for representative traces

See Appendix for Post Library Construction Quantification

Step 4

Cell Surface Protein Library Construction

4.1 Sample Index PCR

4.2 Post Sample Index PCR Size Selection – SPRIselect

4.3 Post Library Construction QC

4.0 Cell Surface Protein Library Construction

GET STARTED!					
Action		**Item**	**10x PN**	**Preparation & Handling**	**Storage**
Equilibrate to Room Temperature	DUAL INDEX	**Dual Index Plate NT Set A** Verify name & PN Use indicated plate only	3000483	-	–20°C
		Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendations.	-
		Agilent TapeStation Screen Tape and Reagents If used for QC	-	Manufacturer's recommendations.	-
		Agilent Bioanalyzer High Sensitivity kit If used for QC	-	Manufacturer's recommendations.	-
		DNA High Sensitivity Reagent Kit If LabChip used for QC	-	Manufacturer's recommendations.	-
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**	-	-	Ambient
		10x Magnetic Separator/ 10x Magnetic Separator B	230003/ 2001212	See Tips & Best Practices.	Ambient
		Prepare 80% Ethanol Prepare 20 ml for 8 reactions	-	Prepare fresh.	Ambient

4.1 Sample Index PCR

DUAL INDEX

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)	4X + 10% (µl)	8X + 10% (µl)
◯ **Amp Mix** Retrieve from 3' Feature Barcode Kit	2000047	50	220	440
Buffer EB	-	25	110	220
Total	-	**75**	**330**	**660**

c. Transfer **ONLY 5 µl** DNA sample from the Transferred Supernatant Cleanup (step 2.3B-xiv) to a new tube strip.

Note that only **5 µl** DNA sample is sufficient for generating Cell Surface Protein library. The remaining **35 µl** DNA 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 Surface Protein libraries.

d. Add **75 µl** Sample Index PCR Mix to each sample.

e. Add **20 µl** of an individual Dual Index to each sample and record their assignment. 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**
1	98°C	00:00:45
2	98°C	00:00:20
3	54°C	00:00:30
4	72°C	00:00:20
5	Go to step 2, repeat 9X for a total of 10 cycles*	
6	72°C	00:01:00
7	4°C	Hold

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

4.2 Post Sample Index PCR Size Selection – SPRIselect

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

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

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

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.

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

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

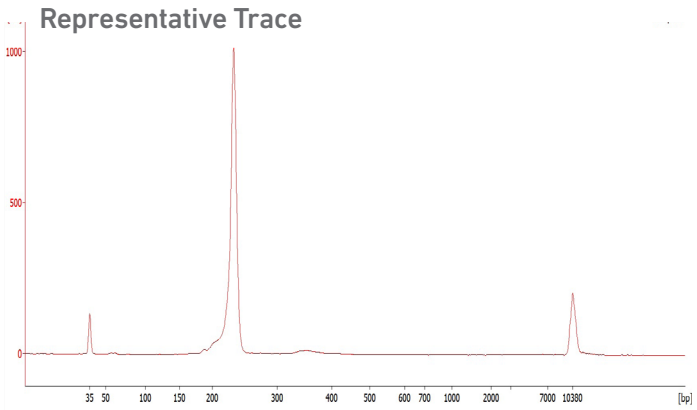
l. Place on the magnet•**Low** until the solution clears.

m. Transfer **40 µl** 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 s**ample at **1:10 dilution** on an Agilent Bioanalyzer High Sensitivity chip.



Select the region between 150-300 bp to 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 representative traces

See Appendix for Post Library Construction Quantification

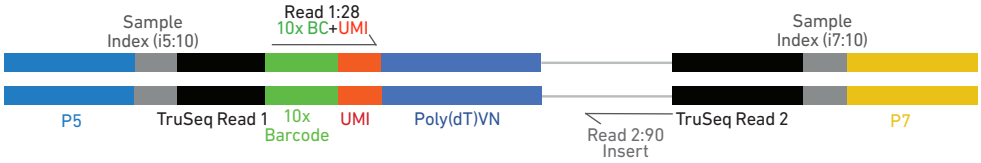
Sequencing

5

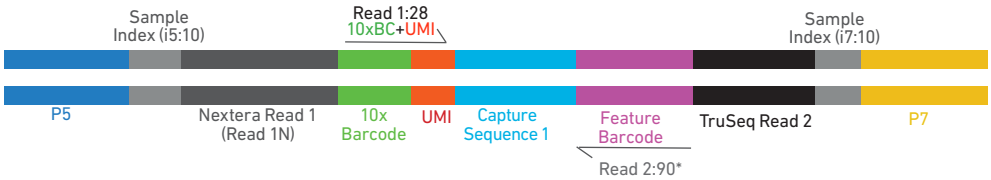
Sequencing Libraries

Chromium Single Cell 3' Gene Expression and Cell Surface Protein Dual Index libraries comprise standard Illumina paired-end constructs which begin with P5 and end with P7. These libraries include 16 bp 10x Barcodes at the start of TruSeq Read 1 and Nextera Read 1 (Read 1N) respectively 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 TruSeq Read 2 are used for paired-end sequencing of Single Cell 3' Cell Surface Protein libraries. Sequencing these libraries produce 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



*Minimum required Read 2 length for Cell Surface Protein libraries is 25 bp

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 6000

Sample Indices

Each sample index in the Dual Index Kit TT Set A (PN-1000215) 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 sequencing 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 with "cellranger mkfastq". 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 Depth & Run Parameters

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

Cell Surface Protein Library Sequencing Depth & Run Parameters†

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

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

Library Loading

Once quantified and normalized, the 3' Gene Expression and Cell Surface Protein libraries should be denatured and diluted as recommended for Illumina sequencing platforms. Refer to Illumina documentation for denaturing and diluting libraries. Refer to the 10x Genomics Support website, for more information.

Library quantification should be done with the KAPA DNA Quantification Kit using the average insert size determined by Agilent Bioanalyzer, Perkin Elmer LabChip, or Agilent TapeStation QC. Alternate methods to KAPA qPCR for final library quantification may result in under quantification, and consequently overloading.

Instrument	3' Gene Expression libraries only or 3' Gene Expression + Cell Surface Protein libraries		Cell Surface Protein libraries only	
	Loading Concentration (pM)	PhiX (%)	Loading Concentration (pM)	PhiX (%)
MiSeq	11	1	11	1
NextSeq 500/550	1.8	1	1.8	1
NextSeq 1000/2000	650	1	650	1
HiSeq 2500 (RR)	11	1	11	1
HiSeq 4000	240	1	240	1
*NovaSeq 6000 standard workflow	300	1	300	1
*NovaSeq 6000 Xp workflow	150	1	150	1

**The NovaSeq 6000 standard workflow permits loading one library pool across all lanes of the flow cell; whereas the Xp workflow enables sequencing various library pools in each lane of the NovaSeq 6000 flow cell. The recommended loading concentrations for the standard workflow are higher than the Xp workflow. For more information on both workflows, refer to the NovaSeq 6000 Denature and Dilute Guide*

Library Pooling

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

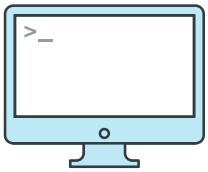
Library Pooling Example:

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

Data Analysis and Visualization

Sequencing data may be analyzed using Cell Ranger 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

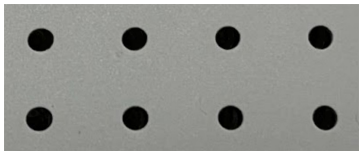
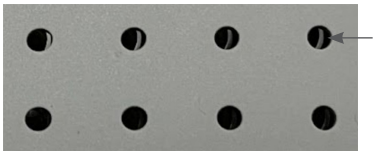
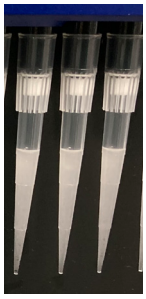
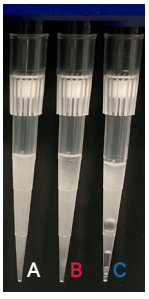
Loupe Browser is an interactive data visualization tool that requires no prior programming knowledge.

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

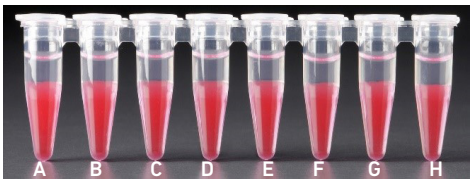
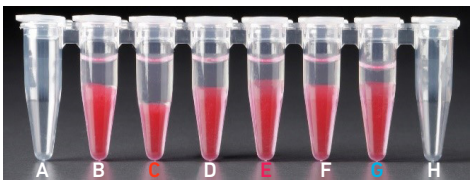
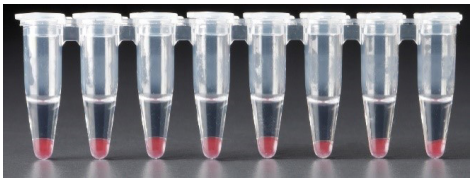
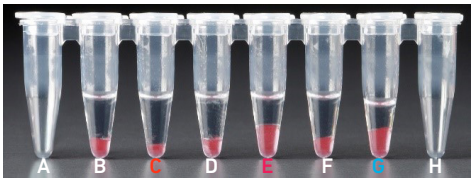
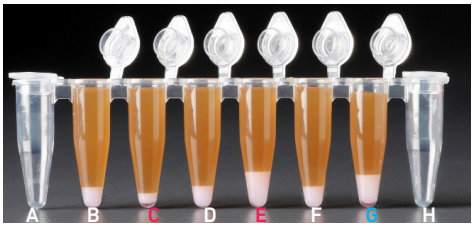
Troubleshooting

6

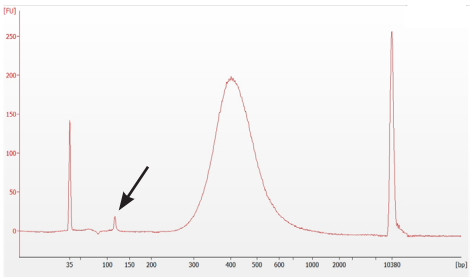
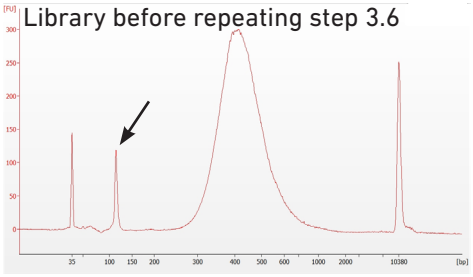
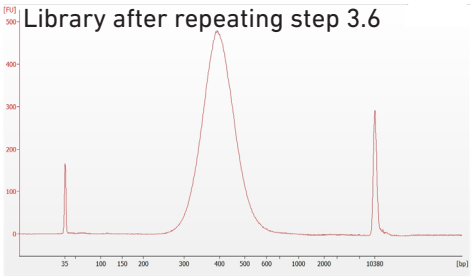
GEM Generation & Barcoding

STEP	NORMAL	IMPACTED
1.2 Load Chromium Next GEM Chip	 Gasket holes are aligned with the sample and gel bead wells.	 Gasket holes are misaligned with the gel bead wells. Open and close the chip holder slowly once.
1.4 d After Chip G is removed from the Controller or X/iX and the wells are exposed	 All 8 recovery wells are similar in volume and opacity.	 Recovery well G indicates a reagent clog. Recovery well C and E indicate a wetting failure. Recovery wells B, D, and F are normal. Wells A and H contain 50% Glycerol Solution.
1.4 f Transfer GEMs from Chip G Row Labeled 3	 All liquid levels are similar in volume and opacity without air trapped in the pipette tips.	 Pipette tip A shows normal GEM generation, pipette tip B indicates a wetting failure, and pipette tip C shows a clog and wetting failure.

Consult the Best Practices to Minimize Chromium Next GEM Chip Clogs and Wetting Failures (Technical Note CG000479) for more information.

STEP	NORMAL	IMPACTED
2.1 a After transfer of the GEMs + Recovery Agent	 All liquid levels are similar in the aqueous sample volume (clear) and Recovery Agent/Partitioning Oil (pink).	 Tube G indicates a reagent clog has occurred. There is a decreased volume of aqueous layer (clear). Tube C and E indicate a wetting failure has occurred. There is an abnormal volume of Recovery Agent/Partitioning Oil (pink).
2.1 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. Replacement reagents and chips may be provided for properly documented clogs or wetting failures if they are associated with runs of unexpired reagents and chips, and are reported within 30 days of the expiration date. Consult the Best Practices to Minimize Chromium Next GEM Chip Clogs and Wetting Failures (Technical Note CG000479) for more information.

STEP	ACCEPTABLE	PROMINENT ADAPTER DIMER PEAKS
3.7 Post Library Consctruction QC	 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.

Chromium Controller Errors

If the Chromium Controller or the Chromium Single Cell Controller fails to start, an error tone will sound and one of the following error messages will be displayed:

a. **Chip not read – Try again:** Eject the tray, remove and/or reposition the Chromium Next GEM Secondary Holder assembly and try again. If the error message is still received after trying this more than twice, contact support@10xgenomics.com for further assistance.

b. **Check gasket:** Eject the tray by pressing the eject button to check that the 10x Gasket is correctly installed on the Chromium Next GEM Chip. If the error message persists, contact support@10xgenomics.com for further assistance.

c. **Error Detected: Row _ Pressure:**

   i. If this message is received within a few seconds of starting a run, eject the tray by pressing the eject button and check for dirt or deposits on the 10x Gasket. If dirt is observed, replace with a new 10x Gasket, open and close the lid to ensure the gasket is properly aligned, and try again. If the error message is still received after trying this more than twice, contact support@10xgenomics.com for further assistance.

   ii. If this message is received after a few minutes into the run, the Chromium Next GEM Chip must be discarded. **Do not try running this Chromium Next GEM Chip again as this may damage the Chromium Controller.**

d. **Invalid Chip CRC Value:** This indicates that a Chromium Next GEM Chip has been used with an older firmware version. The chip must be discarded. Contact support@10xgenomics.com for further assistance.

e. **Chip Holder Not Present:** Open the controller drawer and check if chip holder is present. Insert chip properly into chip holder and retry.

f. **Unauthorized Chip:** This indicates that an incompatible non-Next GEM chip has been used with an instrument that only can run Next GEM assays. Use only Chromium Controller (PN-120223;120246) or Chromium Single Cell Controller (PN-120263;120212) to run that chip or chip must be discarded. Contact support@10xgenomics.com for further assistance.

g. **Endpoint Reached Early:** If this message is received, contact support@10xgenomics.com for further assistance.

Chromium X Series Errors

The Chromium X touchscreen will guide the user through recoverable errors. If the error continues, or if the instrument has seen critical or intermediate errors, email support@10xgenomics.com with the displayed error code. Support will request a troubleshooting package. Upload pertinent logs to 10x Genomics by navigating to the Logs menu option on screen.

There are two types of errors:

Critical Errors — When the instrument has seen a critical error, the run will immediately abort. Do not proceed with any further runs. Contact support@10xgenomics.com with the error code.

a. System Error

b. Pressure Error

c. Chip Error

d. Run Error

e. Temperature Error

f. Software Error

User Recoverable Errors — Follow error handling instructions through the touchscreen and continue the run.

a. Gasket Error

b. Tray Error

c. Chip Error

d. Unsupported Chip Error

e. Update Error

Consult the Chromium X Series (X/iX) User Guide (CG000396) for additional information and follow the Chromium X touchscreen prompts for execution.

Appendix

Post Library Construction Quantification

Agilent TapeStation Traces

LabChip Traces

Oligonucleotide Sequences

7

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.

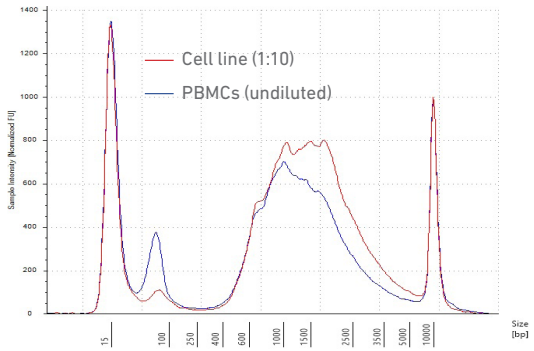
Agilent TapeStation Traces

Agilent TapeStation Traces

Agilent TapeStation High Sensitivity D5000 ScreenTape was used. Protocol steps correspond to the Chromium Next GEM Single Cell 3' (Dual Index) v3.1 User Guide with Feature Barcode technology for Cell Surface Protein (CG000317)

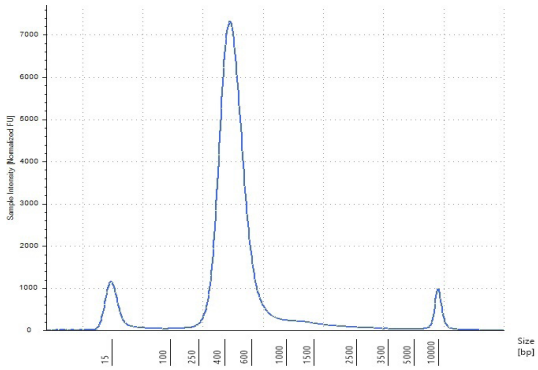
Protocol Step 2.4 – cDNA QC & Quantification

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



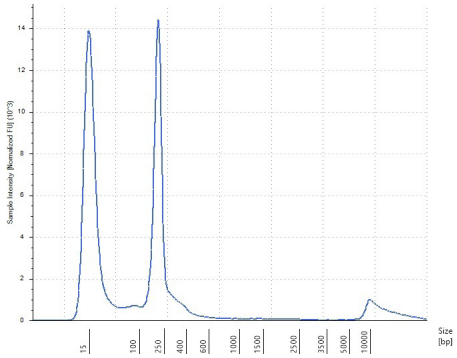
Protocol Step 3.7 – Post Library Construction QC

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.

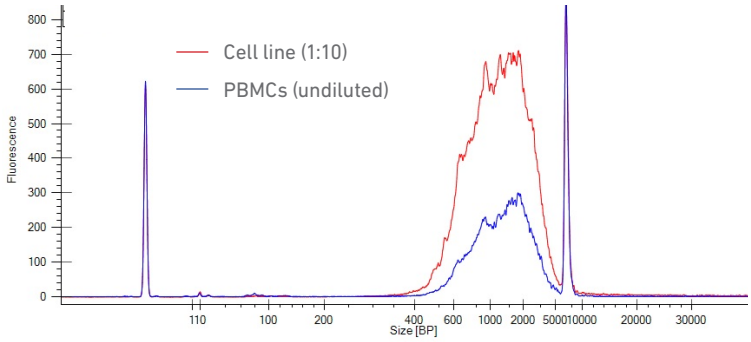
LabChip Traces

LabChip Traces

DNA High Sensitivity Reagent Kit was used.
Protocol steps correspond to the Chromium Next GEM Single Cell 3' v3.1 (Dual Index) User Guide with Feature Barcode technology for Cell Surface Protein (CG000317)

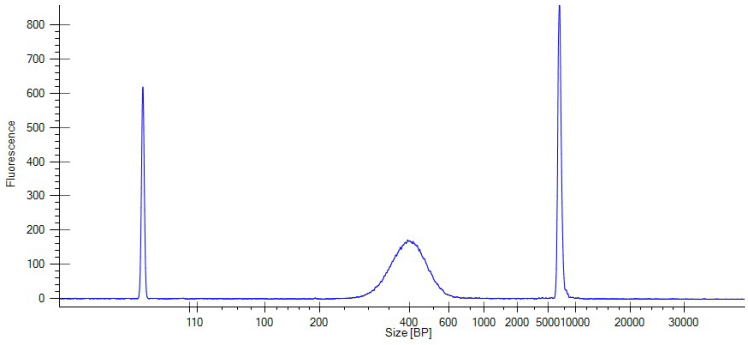
Protocol Step 2.4 – cDNA QC & Quantification

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



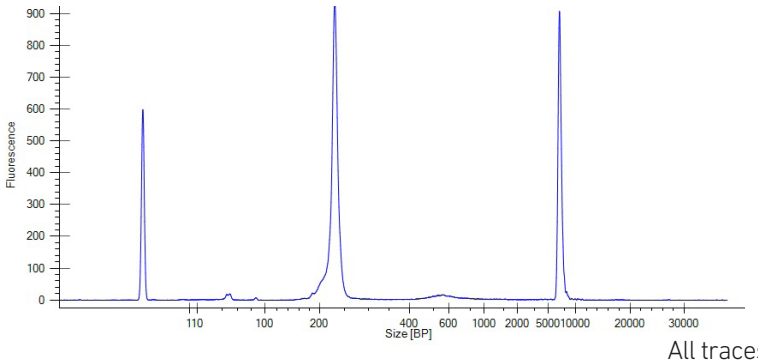
Protocol Step 3.7 – Post Library Construction QC

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).



All traces are representative.

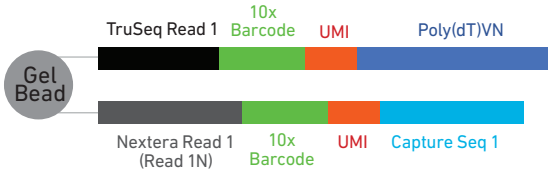
Oligonucleotide Sequences

Protocol steps correspond to the Chromium Next GEM Single Cell 3' (Dual Index) v3.1 User Guide with Feature Barcode technology for Cell Surface Protein (CG000317)

Protocol Step 1.5 – GEM-RT Incubation

Gel Bead Primers

5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'



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

Template Switch Oligo PN-3000228

5'-AAGCAGTGGTATCAACGCAGAGTACATrGrGrG-3'

Cell Surface Protein Feature Barcode

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

GEM-RT Products

cDNA from poly-adenylated mRNA

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

DNA from cell surface protein Feature Barcode

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

Protocol Step 2.2 – cDNA Amplification

Feature cDNA Primers 2 PN-2000097

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

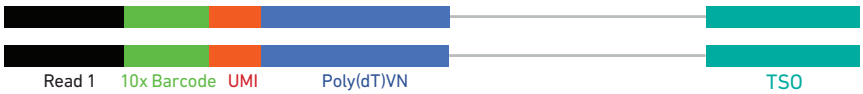
5'-GCAGCGTCAGATGTGTATAAGAGACAG-3'

Reverse primer: Partial Read 2

5'-GTGACTGGAGTTCAGACGT-3'

Amplification Products

Amplified cDNA from poly-adenylated mRNA



5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-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'

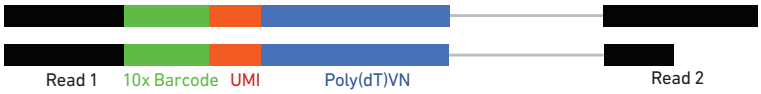
Protocol Step 3.3 – Adaptor Ligation (for 3' Gene Expression Library Construction)

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'

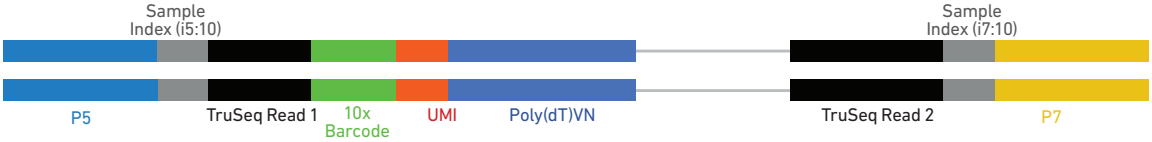
Protocol Step 3.5 – Sample Index PCR (for 3' Gene Expression Library Construction)

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'

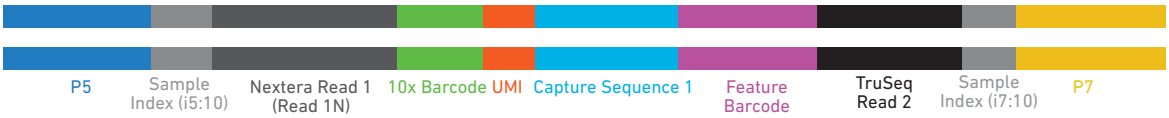
Protocol Step 4.1 – Sample Index PCR (for Cell Surface Protein Library Construction)

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-N12AACGATCCTGGCCGGAATTTCG-N9-N15-N10-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'