USER GUIDE

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

with Feature Barcode technology for Cell Multiplexing



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

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

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 NN Set A, 96 rxns PN-1000243



Notices

Document Number

CG000388 • Rev B

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Document Revision Summary

Document Number CG000388

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

Title Feature Barcode technology for

Cell Multiplexing User Guide

Revision Rev B

Revision Date August 2021

Specific Changes:

• Updated Step 1.3 - Run the Chromium Controller or X/iX

General Changes:

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

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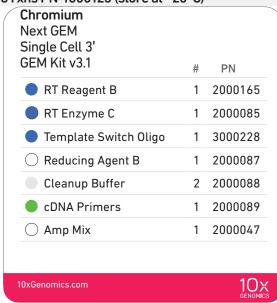
Introduction

Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index)
10x Genomics Accessories
Recommended Thermal Cyclers
Additional Kits, Reagents & Equipment
Protocol Steps & Timing
Stepwise Objectives
Cell Multiplexing Labeling Guidelines

Chromium NextGEM Single Cell 3' Reagent Kits

Chromium NextGEM 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)



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



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

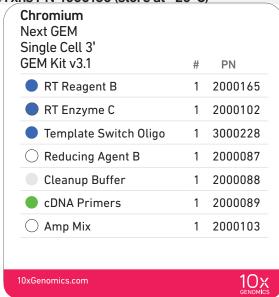


Dynabeads™ MyOne™ SILANE PN-2000048 (store at 4°C)

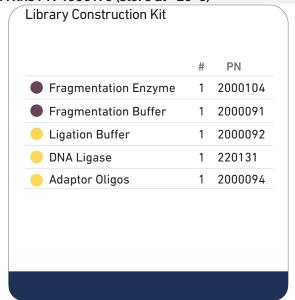


Chromium Next GEM Single Cell 3' Kit v3.1, 4 rxns PN-1000269

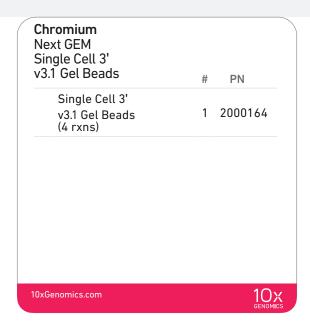
Chromium Next GEM Single Cell 3' GEM Kit v3.1 4 rxns PN-1000130 (store at -20°C)



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



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



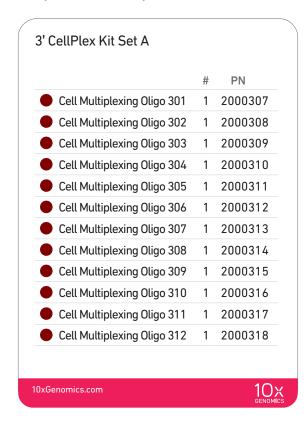
Dynabeads™ MyOne™ SILANE PN-2000048 (store at 4°C)



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



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



3' CellPlex Kit is used for labeling cells. Consult Demonstrated Protocol Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (Document CG000391).

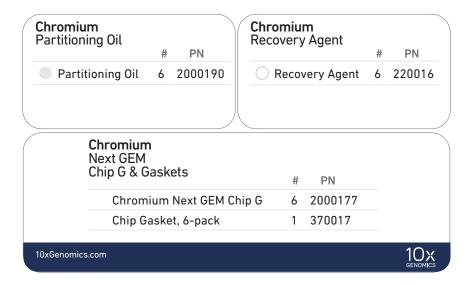
Dual Index Kit TT Set A, 96 rxns PN-1000215 (store at -20°C)



Dual Index Kit NN Set A, 96 rxns PN-1000243 (store at -20°C)



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



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



10x Genomics Accessories

Product	Part Number (Kit)	Part Number (Item)
10x Vortex Adapter	120251	330002
10x Magnetic Separator	120250	230003
Chromium Next GEM Secondary Holder	1000142	3000332

Recommended Thermal Cyclers

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

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

Additional Kits, Reagents & Equipment

The items in the table below have been validated by 10x Genomics and are highly recommended for 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	DNA LoBind Tubes, 1.5 ml				
USA Scientific	TempAssure PCR 8-tube strip	Thermo Fisher Scientific PCR	1402-4700			
Thermo Fisher Scientific	MicroAmp 8-Tube Strip, 0.2 ml MicroAmp 8 -Cap Strip, clear	8-tube strips.	N8010580 N8010535			
Rainin	Tips LTS 200UL Filter RT-L200FLR Tips LTS 1ML Filter RT-L1000FLR Tips LTS 20UL Filter RT-L10FLR		30389240 30389213 30389226			
Kits & Reagents						
Thermo Fisher Scientific	Nuclease-free Water Low TE Buffer (10 mM Tris-HCl pH 8.0, 0.1 m	nM EDTA)	AM9937 12090-015			
Millipore Sigma	Ethanol, Pure (200 Proof, anhydrous)		E7023-500ML			
Beckman Coulter	SPRIselect Reagent Kit	B23318				
Bio-Rad	10% Tween 20	10% Tween 20				
Ricca Chemical Company	Glycerin (glycerol), 50% (v/v) Aqueous Soluti	3290-32				
Qiagen	Qiagen Buffer EB	19086				
Equipment						
VWR	Vortex Mixer Divided Polystyrene Reservoirs VWR Mini Centrifuge (alternatively, use any equivalent mini centrif	- Tuge)	10153-838 41428-958 76269-066			
Eppendorf	Eppendorf ThermoMixer C Eppendorf SmartBlock 1.5 ml, Thermoblock (alternatively, use a temperature-controlled l		5382000023 5360000038			
Rainin	Pipet-Lite Multi Pipette L8-50XLS+ Pipet-Lite Multi Pipette L8-200XLS+ Pipet-Lite Multi Pipette L8-10XLS+ Pipet-Lite Multi Pipette L8-20XLS+ Pipet-Lite LTS Pipette L-2XLS+ Pipet-Lite LTS Pipette L-10XLS+ Pipet-Lite LTS Pipette L-20XLS+ Pipet-Lite LTS Pipette L-100XLS+ Pipet-Lite LTS Pipette L-100XLS+ Pipet-Lite LTS Pipette L-200XLS+ Pipet-Lite LTS Pipette L-1000XLS+		17013804 17013805 17013802 17013803 17014393 17014388 17014392 17014384 17014391 17014382			

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)
Quantification & Quality Control			
Agilent	2100 Bioanalyzer Laptop Bundle High Sensitivity DNA Kit 4200 TapeStation High Sensitivity D1000 ScreenTape/Reagents High Sensitivity D5000 ScreenTape/Reagents	Choose Bioanalyzer, TapeStation, LabChip, Fragment Analyzer or Qubit	G2943CA 5067-4626 G2991AA 5067-5584/5067-5585 5067-5592/5067-5593
Thermo Fisher Scientific	Qubit 4.0 Flourometer Qubit dsDNA HS Assay Kit	based on availability & preference.	Q33238 Q32854
Advanced Analytical	Fragment Analyzer Automated CE System - 12 c Fragment Analyzer Automated CE System - 48/ 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 Plat	forms	KK4824

Protocol Steps & Timing

Day	Steps Steps & Timin	Timing Stop & Store
	Cell Preparation & Labeling	
1 h	Dependent on Cell Type	~1-2 h
	Step 1 – GEM Generation & Barcoding	
	 1.1 Prepare Reaction Mix 1.2 Load Chromium Next GEM Chip G 1.3 Run the Chromium Controller or X/iX 1.4 Transfer GEMs 	20 min 10 min 18 min 3 min
4h	1.5 GEM-RT Incubation	55 min ⁵⁰⁰ 4°C ≤72 h or −20°C ≤1 week
	Step 2 – Post GEM-RT Cleanup & cDNA Amplification 2.1 Post GEM RT-Cleanup – Dynabead 2.2 cDNA Amplification	45 min 40 min
	 2.3 cDNA Cleanup – SPRIselect 2.3A Pellet Cleanup 2.3B Transferred Supernatant Cleanup cDNA QC & Quantification 	15 min $4^{\circ}C \le 72 \text{ h or } -20^{\circ}C \le 4 \text{ weeks}$ 20 min $4^{\circ}C \le 72 \text{ h or } -20^{\circ}C \le 4 \text{ weeks}$ 50 min
6h	Step 3 – 3' Gene Expression Library Construction	
	 3.1 Fragmentation, End Repair & A-tailing 3.2 Post Fragmentation, End Repair & A-tailing Double Sided Size Selection – SPRIselect 	45 min 30 min
8h plus	 3.3 Adaptor Ligation 3.4 Post Ligation Cleanup- SPRIselect 3.5 Sample Index PCR 3.6 Post Sample Index PCR Double Sided Size Selection-SPRIselect 3.6 Post Library Construction QC 	25 min 20 min 40 min 30 min 50 min 50 min 25 min 4°C ≤72 h 50 min
	Step 4 – Cell Multiplexing Library Construction	
	 4.1 Sample Index PCR 4.2 Post Sample Index PCR Size Selection- SPRIselect 4.3 Post Library Construction QC 	15 min 20 min 50P 4°C ≤72 h or -20°C long term 50 min

Stepwise Objectives



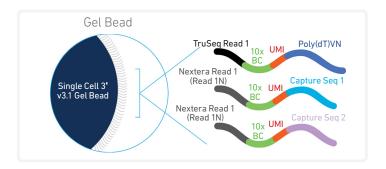
The Chromium Single Cell Gene Expression Solution with Feature Barcode technology for cell multiplexing upgrades short read sequencers to deliver a scalable microfluidic platform for assessing cell multiplexing combined with 3' digital gene expression of the same single cell by profiling 500-30,000 individual cells per sample. A pool of ~3,500,000 10x Barcodes are sampled separately to index each cell's transcriptome. 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 Multiplexing libraries from the same cells.

Single Cell 3' v3.1 Gel Beads

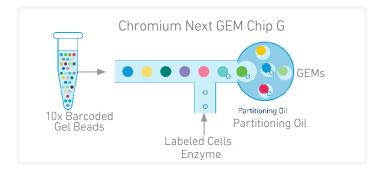
In addition to the poly(dT) primer that enables the production of barcoded, full-length cDNA from poly-adenylated mRNA, the Single Cell 3' v3.1 Gel Beads also include two additional primer sequences (Capture Sequence 1 and Capture Sequence 2), that enable capture and priming of Feature Barcode technology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



Step 1 GEM Generation & Barcoding

GEMs are generated by combining barcoded Single Cell 3' v3.1 Gel Beads, a Master Mix with Cell Multiplexing Oligo 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 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 1 is not shown in the illustrated example).

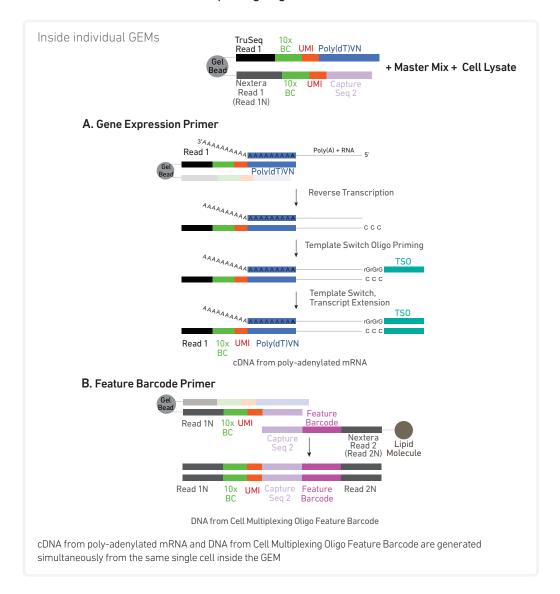
A. Primers containing:

- 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

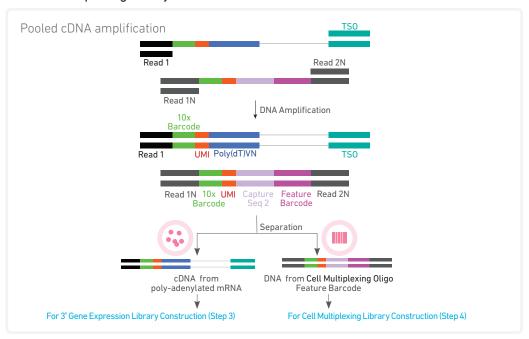
Both are mixed with the cell lysate and the Master Mix containing the RT reagents. Incubation of the GEMs produces barcoded, full-length cDNA from poly-adenylated mRNA and barcoded DNA from the Cell Multiplexing Oligo Feature Barcode.



Step 2 Post GEM-RT Cleanup & cDNA Amplification



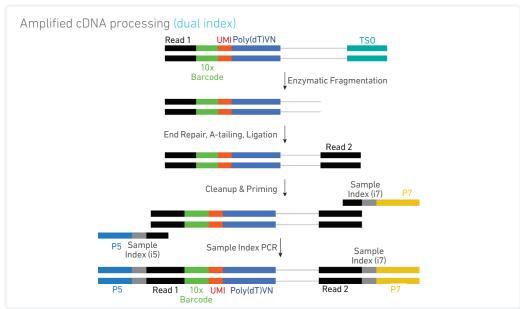
After incubation, GEMs are broken and pooled fractions are recovered. Silane magnetic beads are used to purify the cell barcoded products from the post GEM-RT reaction mixture, which includes leftover biochemical reagents and primers. The cell barcoded cDNA molecules are amplified via PCR to generate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



Step 3 3' Gene Expression Library Construction



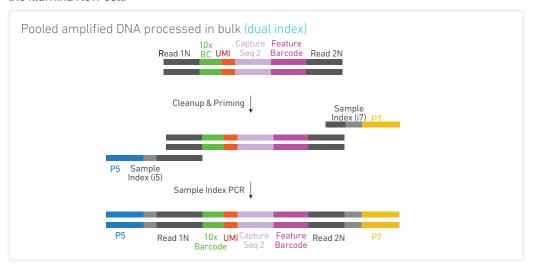
Enzymatic fragmentation and size selection are used to optimize the cDNA amplicon size. P5, P7, i7 and i5 sample indexes, and TruSeq Read 2 (read 2 primer sequence) are added via End Repair, A-tailing, Adaptor Ligation, and PCR. The final libraries contain the P5 and P7 primers used in Illumina amplification.



Step 4 Cell Multiplexing Library Construction



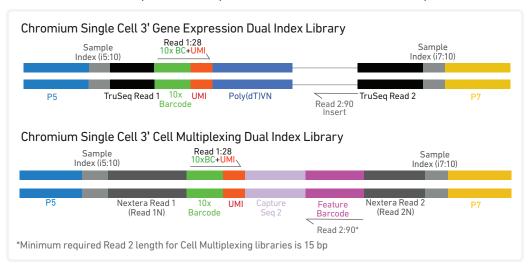
Amplified DNA from Cell Multiplexing Oligo Feature Barcodes is used for library construction. P5, P7, i7 and i5 sample indexes, and Nextera Read 2 (read 2N primer sequence) are added via PCR. The final libraries contain the P5 and P7 sequences necessary for amplification on the Illumina flow cell.



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. Read 2 is used to sequence the cDNA fragment in 3' Gene Expression libraries while Read 2N is used to sequence the DNA from Cell Multiplexing Oligo Feature Barcode in the Cell Multiplexing 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 Nextera Read 2 in the Cell Multiplexing 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.

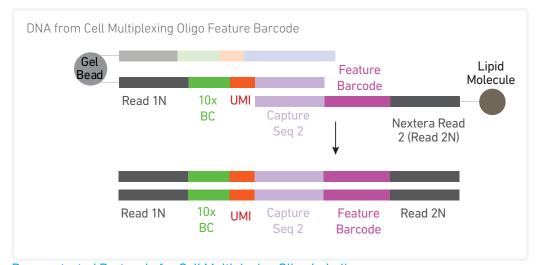


See Appendix for Oligonucleotide Sequences

Cell Multiplexing Oligo Labeling Guidelines

Overview

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



Demonstrated Protocols for Cell Multiplexing Oligo Labeling



For guidance on cell multiplexing oligo labeling protocol, consult Demonstrated Protocol Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (Document CG000391).



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

Tips & Best Practices



Icons











Emulsion-safe Plastics

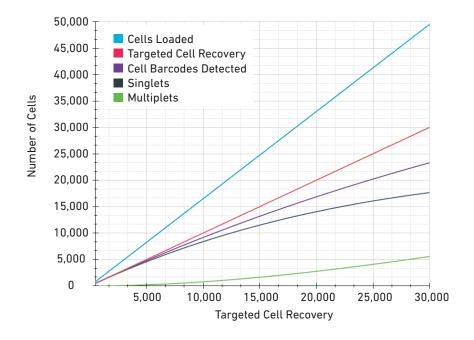
• Use validated emulsion-safe plastic consumables when handling GEMs as some plastics can destabilize GEMs.

Cell Concentration

• The optimal input cell concentration depends upon the desired cell recovery target.

Optimal Input Cell Concentration	Cell Recovery Target
700-1,200 cells/μl	500-10,000 cells
1,300-1,600 cells/µl	10,000-30,000 cells

- 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.
- Multiplets occur when more than one cell is partitioned into a single GEM. The
 multiplet rate increases linearly with increasing cell loads. When performing cell
 multiplexing, Cell Ranger can identify and filter multiplets if they contain cells
 labeled with different Cell Multiplexing Oligos (CMOs). For further details, consult the
 10x Genomics Cell Multiplexing Technical Note (CG000383).



Cell Concentration

Targeted Cell Recovery	# of Cells Loaded	Cell Barcodes Detected	Singlets	Multiplets	Multiplet Rate
500	825	~500	~500	~3	~0.4%
1,000	1,650	~1,000	~1,000	~10	~0.8%
2,000	3,300	~2,000	~2,000	~40	~1.6%
3,000	4,950	~3,000	~2,900	~80	~2.4%
4,000	6,600	~3,900	~3,800	~140	~3.2%
5,000	8,250	~4,800	~4,600	~210	~4.0%
6,000	9,900	~5,700	~5,400	~300	~4.8%
7,000	11,550	~6,600	~6,200	~400	~5.6%
8,000	13,200	~7,500	~7,000	~510	~6.4%
9,000	14,850	~8,400	~7,700	~640	~7.2%
10,000	16,500	~9,200	~8,400	~780	~8.0%
12,000	19,800	~10,900	~9,800	~1,100	~9.6%
14,000	23,100	~12,500	~11,000	~1,500	~11.2%
16,000	26,400	~14,000	~12,100	~1,900	~12.8%
18,000	29,700	~15,500	~13,100	~2,300	~14.4%
20,000	33,000	~16,900	~14,100	~2,800	~16.0%
22,000	36,300	~18,300	~15,000	~3,300	~17.6%
24,000	39,600	~19,600	~15,800	~3,900	~19.2%
26,000	42,900	~20,900	~16,500	~4,400	~20.8%
28,000	46,200	~22,200	~17,100	~5,000	~22.4%
30,000	49,500	~23,400	~17,700	~5,600	~24.0%

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.
- 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



- 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





- 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



- Align notch on the chip (upper left corner) and the holder.
- Insert the left-hand side of the chip under the guide. Depress the right-hand side of the chip until the spring-loaded clip engages.
- Close the lid before dispensing reagents into the wells.



Chromium Next GEM Chip Loading



- Place the assembled chip and holder flat on the bench with the lid closed.
- 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



- 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 Gasket Attachment

- After reagents are loaded, attach the gasket by holding the tongue (curved end, to the right) and hook it 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. DO NOT press down on the top of the gasket after attachment.
- Keep the assembly horizontal to avoid wetting the gasket with Partitioning Oil.



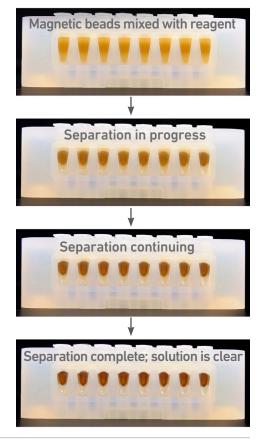
10x Magnetic Separator

- 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.



Magnetic Bead Cleanup Steps

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



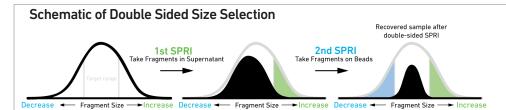
SPRIselect Cleanup & Size Selection

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



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

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

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

Enzymatic Fragmentation

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

Sample Indices in Sample Index PCR

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

Index Hopping Mitigation

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

- · Remove adapters during cleanup steps.
- Ensure no leftover primers and/or adapters are present when performing post-Library Construction QC.
- Store each library individually at 4°C for up to 72 h or at -20°C for long-term storage.
 DO NOT pool libraries during storage.
- Pool libraries prior to sequencing. An additional 0.8X SPRI may be performed for the pooled libraries to remove any free adapters before sequencing.
- Hopped indices can be computationally removed from the data generated from single cell dual index libraries.

Step 1

GEM Generation & 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 STARTI	ED!	ltem	10x PN	Preparation & Handling	Storage
Action		item	IUX PN	Preparation & nanuting	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.	-20°C
	\bigcirc	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
		Labalad Call Communi	•		
Λ		Labeled Cell Suspensions as the Cell Suspension of the Cell Suspensi	col Cell Multiple:	xing Oligo Labeling for Single Ce technology (CG000391)	ll RNA
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.	-



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



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.

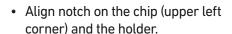
Assemble Chromium Next GEM Chip G



After removing the chip from the sealed bag, use the chip in ≤ 24 h.



See Tips & Best Practices for chip handling instructions.



- Insert the left-hand side of the chip under the guide. Depress the righthand side of the chip until the springloaded clip engages.
- Close the lid before dispensing reagents into the wells.
- The assembled chip is ready for loading the indicated reagents. Refer to step 1.2 for reagent volumes and loading order.



Chromium Next GEM Chip G

Notch



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 (Cell Recovery Target – 500-10,000) (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					Targete	ed Cell Re	ecovery				
Concentration (Cells/µl)	500	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000
100	8.3	16.5	33.0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
100	35.0	26.7	10.2	11/4	117 G	117 G	117 G	117 G	11/4	117 G	11/ 0
200	4.1	8.3	16.5	24.8	33.0	41.3	n/a	n/a	n/a	n/a	n/a
	39.1	35.0	26.7	18.5	10.2	2.0					
300	2.8	5.5	11.0	16.5	22.0	27.5	33.0	38.5	n/a	n/a	n/a
	40.5 2.1	37.7 4.1	32.2 8.3	26.7 12.4	21.2 16.5	15.7 20.6	10.2 24.8	28.9	33.0	37.1	41.3
400	41.1	39.1	35.0	30.8	26.7	22.6	18.5	14.3	10.2	6.1	2.0
	1.7	3.3	6.6	9.9	13.2	16.5	19.8	23.1	26.4	29.7	33.0
500	41.6	39.9	36.6	33.3	30.0	26.7	23.4	20.1	16.8	13.5	10.2
	1.4	2.8	5.5	8.3	11.0	13.8	16.5	19.3	22.0	24.8	27.5
600	41.8	40.5	37.7	35.0	32.2	29.5	26.7	24.0	21.2	18.5	15.7
700	1.2	2.4	4.7	7.1	9.4	11.8	14.1	16.5	18.9	21.2	23.6
700	42.0	40.8	38.5	36.1	33.8	31.4	29.1	26.7	24.3	22.0	19.6
800	1.0	2.1	4.1	6.2	8.3	10.3	12.4	14.4	16.5	18.6	20.6
000	42.2	41.1	39.1	37.0	35.0	32.9	30.8	28.8	26.7	24.6	22.6
900	0.9	1.8	3.7	5.5	7.3	9.2	11.0	12.8	14.7	16.5	18.3
700	42.3	41.4	39.5	37.7	35.9	34.0	32.2	30.4	28.5	26.7	24.9
1000	8.0	1.7	3.3	5.0	6.6	8.3	9.9	11.6	13.2	14.9	16.5
	42.4	41.6	39.9	38.3	36.6	35.0	33.3	31.7	30.0	28.4	26.7
1100	8.0	1.5	3.0	4.5	6.0	7.5	9.0	10.5	12.0	13.5	15.0
	42.5	41.7	40.2	38.7	37.2	35.7	34.2	32.7	31.2	29.7	28.2
1200	0.7	1.4	2.8	4.1	5.5	6.9	8.3	9.6	11.0	12.4	13.8
	42.5	41.8	40.5	39.1	37.7	36.3	35.0	33.6	32.2	30.8	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
	0.6	1.2	2.4	3.5	4.7	5.9	7.1	8.3	9.4	10.6	11.8
1400	42.6	42.0	40.8	39.7	38.5	37.3	36.1	35.0	33.8	32.6	31.4
	0.6	1.1	2.2	3.3	4.4	5.5	6.6	7.7	8.8	9.9	11.0
1500	42.7	42.1	41.0	39.9	38.8	37.7	36.6	35.5	34.4	33.3	32.2
	0.5	1.0	2.1	3.1	4.1	5.2	6.2	7.2	8.3	9.3	10.3
1600	42.7	42.2	41.1	40.1	39.1	38.0	37.0	36.0	35.0	33.9	32.9
1700	0.5	1.0	1.9	2.9	3.9	4.9	5.8	6.8	7.8	8.7	9.7
1700	42.7	42.2	41.3	40.3	39.3	38.3	37.4	36.4	35.4	34.5	33.5
1800	0.5	0.9	1.8	2.8	3.7	4.6	5.5	6.4	7.3	8.3	9.2
1000	42.7	42.3	41.4	40.5	39.5	38.6	37.7	36.8	35.9	35.0	34.0
1900	0.4	0.9	1.7	2.6	3.5	4.3	5.2	6.1	6.9	7.8	8.7
1700	42.8	42.3	41.5	40.6	39.7	38.9	38.0	37.1	36.3	35.4	34.5
2000	0.4	8.0	1.7	2.5	3.3	4.1	5.0	5.8	6.6	7.4	8.3
	42.8	42.4	41.6	40.7	39.9	39.1	38.3	37.4	36.6	35.8	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 (500-10,000 cells) Purple boxes: Optimal range of cell stock concentration to maximize the likelihood of achieving the desired cell recovery target (10,000-30,000 cells)



Cell Suspension Volume Calculator Table (Cell Recovery Target – 10,000-30,000) (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	Targeted Cell Recovery										
(Cells/µl)	10000	12000	14000	16000	18000	20000	22000	24000	26000	28000	30000
100	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
200	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
300	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
400	41.3 2.0	n/a									
500	33.0 10.2	39.6 3.6	n/a								
600	27.5 15.7	33.0 10.2	38.5 4.7	n/a							
700	23.6 19.6	28.3 14.9	33.0 10.2	37.7 5.5	42.4 0.8	n/a	n/a	n/a	n/a	n/a	n/a
800	20.6 22.6	24.8 18.5	28.9 14.3	33.0 10.2	37.1 6.1	41.3 2.0	n/a	n/a	n/a	n/a	n/a
900	18.3 24.9	22.0 21.2	25.7 17.5	29.3 13.9	33.0 10.2	36.7 6.5	40.3 2.9	n/a	n/a	n/a	n/a
1000	16.5 26.7	19.8 23.4	23.1 20.1	26.4 16.8	29.7 13.5	33.0 10.2	36.3 6.9	39.6 3.6	n/a	n/a	n/a
1100	15.0 28.2	18.0 25.2	21.0 22.2	24.0 19.2	27.0 16.2	30.0 13.2	33.0 10.2	36.0 7.2	39.0 4.2	42.0 1.2	n/a
1200	13.8 29.5	16.5 26.7	19.3 24.0	22.0 21.2	24.8 18.5	27.5 15.7	30.3 13.0	33.0 10.2	35.8 7.5	38.5 4.7	41.3 2.0
1300	12.7 30.5	15.2 28.0	17.8 25.4	20.3 22.9	22.8 20.4	25.4 17.8	27.9 15.3	30.5 12.7	33.0 10.2	35.5 7.7	38.1 5.1
1400	11.8 31.4	14.1 29.1	16.5 26.7	18.9 24.3	21.2 22.0	23.6 19.6	25.9 17.3	28.3 14.9	30.6 12.6	33.0 10.2	35.4 7.8
1500	11.0 32.2	13.2 30.0	15.4 27.8	17.6 25.6	19.8 23.4	22.0 21.2	24.2 19.0	26.4 16.8	28.6 14.6	30.8 12.4	33.0 10.2
1600	10.3 32.9	12.4 30.8	14.4 28.8	16.5 26.7	18.6 24.6	20.6 22.6	22.7 20.5	24.8 18.5	26.8 16.4	28.9 14.3	30.9 12.3
1700	9.7 33.5	11.6 31.6	13.6 29.6	15.5 27.7	17.5 25.7	19.4 23.8	21.4 21.8	23.3 19.9	25.2 18.0	27.2 16.0	29.1 14.1
1800	9.2 34.0	11.0 32.2	12.8 30.4	14.7 28.5	16.5 26.7	18.3	20.2	22.0 21.2	23.8 19.4	25.7 17.5	27.5 15.7
1900	8.7 34.5	10.4	12.2	13.9	15.6 27.6	17.4	19.1	20.8	22.6	24.3 18.9	26.1 17.1
2000	8.3 35.0	9.9	11.6	13.2	14.9	16.5 26.7	18.2	19.8	21.5	23.1	24.8 18.5
	33.0	33.3	31.7	55.0	20.4	20.7	23.1	23.4	21.0	20.1	10.5

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 (500-10,000 cells)

Purple boxes: Optimal range of cell stock concentration to maximize the likelihood of achieving the desired cell recovery target (10,000-30,000 cells)

1.2 Load Chromium Next GEM Chip G



After removing chip from the sealed bag, use in \leq 24 h. When loading the chip, raising and depressing the pipette plunger should each take \sim 5 sec.

When dispensing, raise the pipette tips at the same rate as the liquid is rising, keeping the tips slightly submerged.



a. Add 50% glycerol solution to each unused well

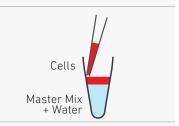
(if processing <8 samples/chip)

- 70 µl in each unused well in row labeled 1
- 50 µl in each unused well in row labeled 2
- 45 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.

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

Glycerol



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. Attach GEM Gasket

- Align the notch with the top left-hand corner.
- Ensure the gasket holes are aligned with the wells.
- · Avoid touching the smooth surface.

 $oldsymbol{\Lambda}$

Attach the gasket and run the chip in the Chromium Controller or X/iX immediately after loading the Partitioning Oil.



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



- 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.



- d. Check the volume in rows labeled 1-2. Abnormally high volume in any well indicates a clog.
- e. Slowly aspirate 100 μ l 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.
- 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.

Expose Wells at 45 Degrees







1.5 GEM-RT Incubation

Use a thermal cycler that can accommodate at least 100 μ l volume. A volume of 125 μ l 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



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

- **2.1** Post GEM-RT Cleanup Dynabeads
- 2.2 cDNA Amplification
- **2.3** cDNA Cleanup SPRIselect
- **2.4** cDNA QC & Quantification

2.0
Post GEM-RT Cleanup
& cDNA Amplification



Reducing Agent B 2000087 Thaw, vortex, verify no precipitate, centrifuge. -20°C	GET STARTE				
Feature cDNA Primers 3 Verify name & PN Dynabeads MyOne SILANE Beckman Coulter SPRIselect Reagent Agilent Bioanalyzer High Sensitivity Kit If used for QC and quantification Qubit dsDNA HS Assay Kit If used for QC and quantification Place on ice Place on ice Place on ice Place Sprimers 3 Vortex, centrifuge briefly. Vortex, centrifuge briefly. -20°C -20°C Vortex, centrifuge briefly. -20°C -20°C Vortex, centrifuge briefly. -20°C -20°C -20°C Vortex, centrifuge briefly. -20°C -20°C -20°C -20°C -20°C	Action	Item	10x PN	Preparation & Handling	Storage
Peature cDNA Primers 3 Verify name & PN Dynabeads MyOne SILANE Beckman Coulter - Manufacturer's recommendations. Reagent Agilent - Manufacturer's recommendations. Sensitivity Kit If used for QC and quantification Agailent - Manufacturer's recommendations. Agilent - Manufacturer's recommendations. Place on ice Amp Mix 2000047/ Vortex, centrifuge briefly20°C Thaw at 65°C Cleanup Buffer 2000088 Thaw for 10 min at 65°C at -20°C	oom	Reducing Agent B	2000087		-20°C
SILANE SILANE 230 sec) immediately before adding to the mix.	emperature	Primers 3	2000289	Vortex, centrifuge briefly.	-20°C
SPRIselect Reagent Agilent Bioanalyzer High Sensitivity Kit If used for QC and quantification Agilent TapeStation ScreenTape and Reagents If used for QC and quantification Qubit dsDNA HS Assay Kit If used for QC and quantification Place on ice Amp Mix Cleanup Buffer Amunfacturer's recommendations. Place on ice Amp Mix Cleanup Buffer Amunfacturer's recommendations. Place on ice Thaw at 65°C Cleanup Buffer Place on ice Thaw at 65°C and recommendations. Place on ice Thaw at 65°C and recommendations. Thaw at 65°C and recommendations.			2000048	(≥30 sec) immediately	4°C
Bioanalyzer High Sensitivity Kit If used for QC and quantification Agilent TapeStation ScreenTape and Reagents If used for QC and quantification Qubit dsDNA HS Assay Kit If used for QC and quantification Place on ice Amp Mix Cleanup Buffer Amanufacturer's recommendations. Amanufacturer's recommendations. Amp Mix 2000047/ 2000103 Thaw at 65°C Cleanup Buffer Crecommendations. Thaw for 10 min at 65°C at -20°C		SPRIselect	-		-
TapeStation recommendations. ScreenTape and Reagents If used for QC and quantification Qubit dsDNA HS - Manufacturer's recommendations. Assay Kit If used for QC and quantification Place on ice Amp Mix 2000047/ 2000103 Vortex, centrifuge briefly20°C Thaw at 65°C Cleanup Buffer 2000088 Thaw for 10 min at 65°C at -20°C		Bioanalyzer High Sensitivity Kit If used for QC and	-		-
Assay Kit If used for QC and quantification Place on ice Amp Mix 2000047/ 2000103 Vortex, centrifuge briefly20°C Thaw at 65°C Cleanup Buffer 2000088 Thaw for 10 min at 65°C at -20°C		TapeStation ScreenTape and Reagents If used for QC and	-		-
2000103 Thaw at 65°C Cleanup Buffer 2000088 Thaw for 10 min at 65°C at -20°C		Assay Kit If used for QC and	-		-
	lace on ice	Amp Mix		Vortex, centrifuge briefly.	-20°C
Verify no visible crystals. Cool to room temperature.	haw at 65°C	Cleanup Buffer	2000088	max speed on a thermomixer. Verify no visible crystals. Cool	-20°C
Obtain Recovery Agent 220016 - Ambien	btain	Recovery Agent	220016	-	Ambient
Qiagen Buffer EB - Manufacturer's - recommendations.		Qiagen Buffer EB	-		-
Bio-Rad 10% - Manufacturer's - Tween 20 recommendations.			-		-
10x Magnetic 230003 - Ambien Separator			230003	-	Ambient
Prepare 80% Ethanol Prepare 15 ml for 8 reactions.	_	Prepare 15 ml	-	-	-

2.1 Post GEM-RT Cleanup – Dynabeads



a. Add 125 µl Recovery Agent to each sample at room temperature. DO NOT pipette mix or vortex the biphasic mixture. Wait 2 min.

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



If biphasic separation is incomplete:

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



A smaller aqueous phase volume indicates a clog during GEM generation.



b. Slowly remove and discard 125 μl Recovery Agent/Partitioning Oil (pink) from the bottom of the tube. DO NOT aspirate any aqueous sample.



c. Prepare Dynabeads Cleanup Mix.

	Dynabeads Cleanup Mix Add reagents in the order listed	PN	1X (µl)	4Χ + 10% (μl)	8X + 10% (µl)
	Cleanup Buffer	2000088	182	801	1602
Resuspend clump	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



- **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)	10Χ (μl)
Buffer EB	-	98	980
10% Tween 20	-	1	10
Reducing Agent B	2000087	1	10
Total	-	100	1000



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 μ l 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.
- 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 $35 \mu l$ 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	1Χ (μl)	4X + 10% (μl)	8X + 10% (μl)
) Amp Mix	2000047	50	220	440
Feature cDNA Primers 3				
Verify name & PN Use indicated primer only	2000289	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 μ l). 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 tabl	e below for total # of cycles
6	72°C	00:01:00
7	4°C	Hold

Recommended starting point for cycle number optimization.

	Targeted Cell Recovery	Total Cycle
The optimal number of cycles is a trade-off between generating sufficient final mass for library construction	<500	13
and minimizing PCR amplification artifacts. The number of cDNA cycles should also be reduced if large numbers	500-6,000	12
of cells are sampled.	6,000-30,000	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



Step 2 cDNA Amplification & QC

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 μ l supernatant in a new tube strip without disturbing the pellet. Maintain at room temperature. DO NOT discard the transferred supernatant (cleanup for Cell Multiplexing library construction).
- 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 (pipette set to 35 μl).
- vii. Incubate 2 min at room temperature.
- viii. Place the tube strip on the magnet•High until the solution clears.
- ix. Transfer $40 \mu l$ sample to a new tube strip.



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 Multiplexing library)

- i. Vortex to resuspend the SPRIselect reagent. Add 70 μl SPRIselect reagent (2.0X) to 75 μl of the transferred supernatant and pipette mix 15x (pipette set to 130 µl).
- ii. Incubate for 5 min at room temperature.
- iii. Place on the magnet•High until the solution clears.
- iv. Remove supernatant.
- v. Add 300 µl 80% ethanol to the pellet. Wait 30 sec.
- vi. Remove the ethanol.
- vii. Repeat steps v and vi for a total of 2 washes.
- viii. Centrifuge briefly and place on the magnet•Low.
- ix. Remove any remaining ethanol. Air dry for 2 min. DO NOT exceed 2 min as this will decrease elution efficiency.
- x. Remove from the magnet. Add 40.5 µl Buffer EB. Pipette mix 15x (pipette set to 35 µl).
- xi. Incubate 2 min at room temperature.
- xii. Place the tube strip on the magnet•High until the solution clears.
- xiii. Transfer 40 µl sample to a new tube strip.



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 Multiplexing Library Construction.

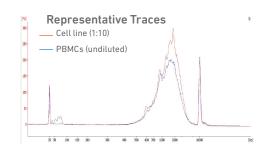
Step 2 cDNA Amplification & QC

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.

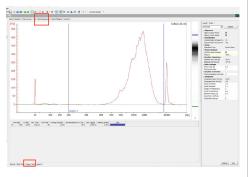
Lower molecular weight product (35 – 150 bp) may be present. This is normal and does not affect sequencing or application performance.



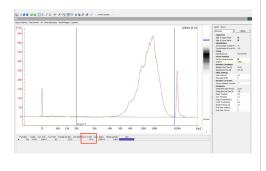
EXAMPLE CALCULATION

i. Select Region

Under the "Electropherogram" view choose the "Region Table". Manually select the region of $\sim 200 - \sim 9000$ bp



ii. Note Concentration [pg/µl]



iii. Calculate

Multiply the cDNA concentration [pg/ μ l] reported via the Agilent 2100 Expert Software by the elution volume (40 μ l) 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

- = Conc'n (pg/μl) x Elution Volume x Dilution Factor 1000 (pg/ng)
- = $\frac{1890.19 (pg/\mu l) \times 40 \times 10}{1000 (pg/ng)}$ = 756.08 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.

(If using Qubit Fluorometer and Qubit dsDNA HS Assay Kit, see Appendix)

Step 3

3' Gene Expression Library Construction

- 3.1 Fragmentation, End Repair & A-tailing
- 3.2 Post Fragmentation End Repair & A-tailing Double Sided Size Selection SPRIselect
- **3.3** Adaptor Ligation
- **3.4** Post Ligation Cleanup SPRIselect
- 3.5 Sample Index PCR
- 3.6 Post Sample Index PCR Double Sided Size Selection SPRIselect
- **3.7** Post Library Construction QC

3.0 3' Gene Expression Library Construction

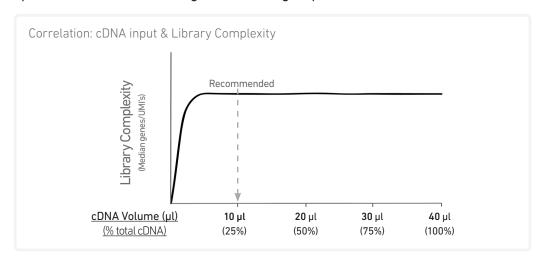


GET START	ED!				
Action		Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature	•	Fragmentation Buffer	2000091	Vortex, verify no precipitate, centrifuge briefly.	-20°C
		Adaptor Oligos	2000094	Vortex, centrifuge briefly.	-20°C
		Ligation Buffer	2000092	Vortex, verify no precipitate, centrifuge briefly.	-20°C
DUAL		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	C	Qiagen Buffer EB	-	-	Ambient
	1	0x Magnetic Separator	230003	See Tips & Best Practices.	Ambient
	P	Prepare 80% Ethanol Prepare 20 ml for 8 eactions	-	Prepare fresh.	Ambient

Step Overview (Step 3.1d)

Correlation between input & library complexity

A Single Cell 3' Gene Expression library is generated using a fixed proportion (10 μ l, 25%) of the total cDNA (40 μ l) 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 μ l, 75%) of the cDNA may be stored at 4°C for up to 72 h or at -20°C for longer-term storage (up to 4 weeks).



Note that irrespective of the total cDNA yield (ng), which may vary based on cell type, targeted cell recovery etc., this protocol has been optimized for a broad range of input mass (ng), as shown in the example below. The total number of SI PCR cycles (step 3.5e) should be optimized based on carrying forward a fixed proportion (10 μ l, 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 Vi		cDNA Input into Fragmentation		SI PCR Cycle
Cell Type	Cell Recovery	Yield – (ng)	Volume (µl)	Mass (ng)	Number
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	1Χ (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 μ l purified cDNA sample from Pellet Cleanup (step 2.3A-x) to a tube strip.

Note that only 10 μ l (25%) cDNA sample transfer is sufficient for generating 3' Gene Expression library.

The remaining 30 μ l (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 μ l) 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 μ l 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.
- 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** μ l Buffer EB to each sample. Pipette mix 15x (pipette set to 45 μ l).
- 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	1Χ (μl)	4Χ + 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 \mul** Adaptor Ligation Mix to **50 \mul** sample. Pipette mix 15x (pipette set to 90 μ l). 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 μ l SPRIselect Reagent (0.8X) to each sample. Pipette mix 15x (pipette set to 150 μ l).
- 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 (pipette set to 25 μl).
- k. Incubate 2 min at room temperature.
- I. Place on the magnet•Low until the solution clears.
- m. Transfer 30 μ l sample to a new tube strip.

INDE>

3.5 Sample Index PCR



- a. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x Sample Index name (PN-3000431 Dual Index Plate TT Set A well ID) used.
- **b.** Add **50 μl** Amp Mix (PN-2000047 or 2000103) to **30 μl** sample.
- c. Add 20 μ l of an individual Dual Index TT Set A to each sample and record the well ID used. Pipette mix 5x (pipette set to 90 μ l). 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 bel	low 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



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

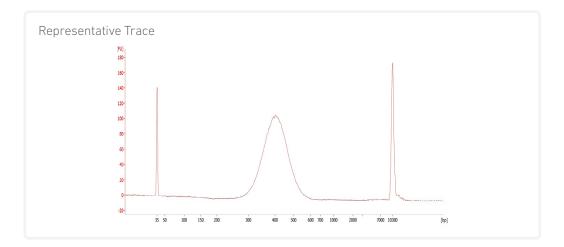
- a. Vortex to resuspend the SPRIselect reagent. Add 60 µl SPRIselect Reagent (0.6X) to each sample. Pipette mix 15x (pipette set to 150 µl).
- b. Incubate 5 min at room temperature.
- c. Place the magnet•High until the solution clears. DO NOT discard supernatant.
- d. Transfer 150 µl supernatant to 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 $165 \,\mu l$ 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.
- i. 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 (pipette set to 30 μl).
- n. Incubate 2 min at room temperature.
- o. Place on the magnet. Low until the solution clears.
- **p.** Transfer $35 \mu l$ to a new tube strip.



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

3.7
Post Library
Construction QC

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



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 μ l 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 Multiplexing 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 Multiplexing Library Construction



Storage -20°C
-20°C
-
-
-
-
-20°C
-
Ambient
Ambient
Ambient

INDE>

4.1 Sample Index PCR



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

	ample Index PCR Mix dd reagents in the order listed	PN	1Χ (μl)	4Χ + 10% (μl)	8X + 10% (μl)
O Ar	mp Mix	2000047	50	220	440
Bu	uffer EB	-	25	110	220
То	otal	-	75	330	660

c. Transfer ONLY 5 μ l 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 Multiplexing 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 Multiplexing libraries.

- **d.** Add **75 μl** Sample Index PCR Mix to each sample.
- e. Add 20 µl of an individual Dual Index NN Set A to each sample and record the well ID used. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.
- f. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 μl	~10-15 min
Step	Temperature	Time
1	98°C	00:00:45
2	98°C	00:00:20
3	54°C	00:00:30
4	72°C	00:00:20
5	Go to step 2, repeat 5X	for a total of 6 cycles
6	72°C	00:01:00
7	4°C	Hold

4.2 Post Sample Index PCR Size Selection – SPRIselect

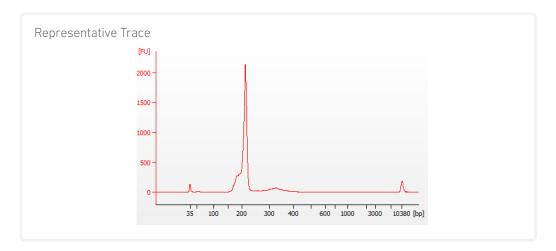
- a. Vortex to resuspend the SPRIselect reagent. Add 120 μ l SPRIselect Reagent (1.2X) to each sample. Pipette mix 15x (pipette set to 150 μ l).
- b. Incubate 5 min at room temperature.
- c. Place on the magnet•High until the solution clears.
- **d.** Remove the supernatant.
- e. Add 300 µl 80% ethanol 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. Air dry for 2 min.
- 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 sample at 1:20 dilution on an Agilent Bioanalyzer High Sensitivity chip.



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

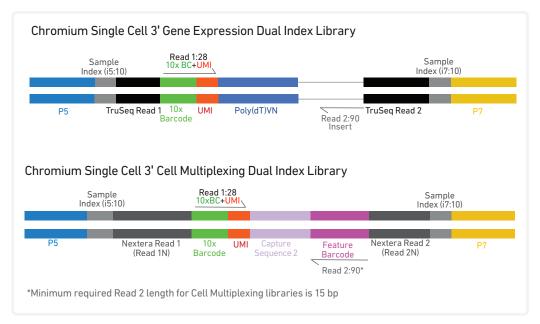
Sequencing



Step 5 Sequencing

Sequencing Libraries

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



Illumina Sequencer Compatibility

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

- MiSeq
- NextSeq 500/550
- NextSeq 1000/2000
- HiSeq 2500 (Rapid Run)
- HiSeq 3000/4000
- NovaSeq

Sample Indices

Each sample index in the Dual Index Kit TT Set A (PN-1000215) or Dual Index Kit NN Set A (PN-1000243), 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.

Step 5 Sequencing

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 i7 Index i5 Index Read 2	28 cycles 10 cycles 10 cycles 90 cycles

Cell Multiplexing Library Sequencing Depth & Run Parameters[†]

[†]Pooling Single Cell 3' Gene Expression & Cell Multiplexing 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 i7 Index i5 Index Read 2	28 cycles 10 cycles 10 cycles 90 cycles Minimum required Read 2 length for Cell Multiplexing libraries is 15 bp	

Library Loading

Once quantified and normalized, the 3' Gene Expression and Cell Multiplexing 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.

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

 $^{^{\}ast}$ Use 150pM loading concentration for Illumina XP workflow.

Library Pooling

The 3' Gene Expression and the Cell Multiplexing 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 Multiplexing library	5,000	1

Data Analysis and Visualization

Sequencing data may be analyzed using Cell Ranger or 10x Genomics Cloud Analysis and visualized using Loupe Browser. Key features for these tools are listed below. For detailed productspecific 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.

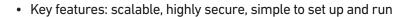


- Input: Base call (BCL) and FASTQ
- Output: BAM, MEX, CSV, HDF5, Web Summary, .cloupe/.loupe
- Operating System: Linux

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.



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.



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



Troubleshooting **





GEMs

STEP

NORMAL

REAGENT CLOGS & WETTING FAILURES

1.4 d After Chip G is removed from the Controller or X/iX and the wells are exposed

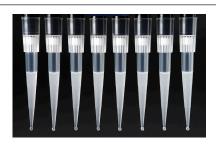


All 8 recovery wells are similar in volume and opacity.



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 tips C and E indicate a wetting failure. Pipette tip C contains partially emulsified GEMs. Emulsion is absent in pipette tip E. Pipette tip G indicates a reagent clog.

STEP

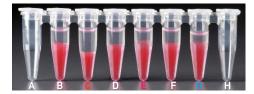
NORMAL

REAGENT CLOGS & WETTING FAILURES

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).



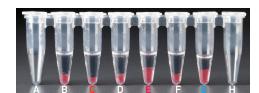
Tube G indicates a reagent clog has occurred. There is a decreased volume of aqueous layer (clear).

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



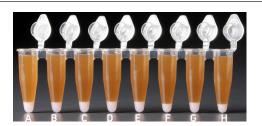
All liquid volumes are similar in the aqueous sample volume (clear) and residual Recovery Agent/Partitioning Oil (pink).



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



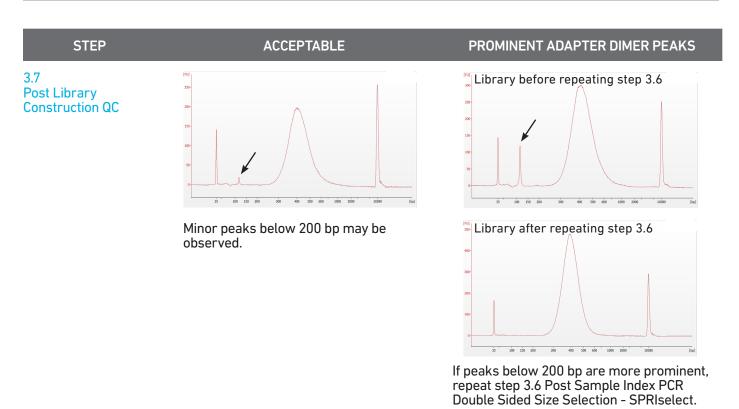
All liquid volumes are similar after addition of the Dynabeads Cleanup Mix.



Tube 6 indicates a reagent clog has occurred. There is an abnormal ratio of Dynabeads Cleanup Mix (brown) to Recovery Agent/Partitioning Oil (appears white).

Tube C and E indicate a wetting failure has occurred. There is an abnormal ratio of Dynabeads Cleanup Mix (brown) to Recovery Agent/Partitioning Oil (appears white).

If a channel clogs or wetting failure occurs during GEM generation, it is recommended that the sample be remade. If any of the listed issues occur, take a picture and send it to support@10xgenomics.com for further assistance.



Chromium 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 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 touch screen 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

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	1Χ (μl)
SYBR Fast Master Mix + Primer	12
Water	4
Total	16

- d. Dispense 16 μ l Quantification Master Mix for sample dilutions and DNA Standards into a 96 well PCR plate.
- e. Add $4 \mu l$ sample dilutions and $4 \mu l$ 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	00:00:30
	Read signal	
4	Go to Step 2, 29X	(Total 30 cycles)

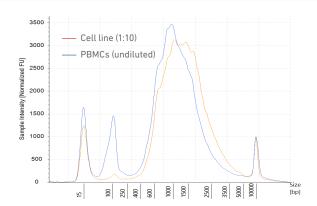
g. Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on insert size derived from the Bioanalyzer/TapeStation trace.

Agilent TapeStation Traces

Agilent TapeStation Traces

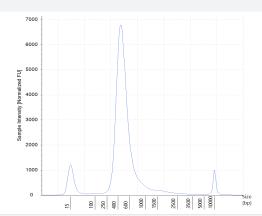
Agilent TapeStation High Sensitivity D5000 ScreenTape was used. Protocol steps correspond to the Chromium Next GEM Single Cell 3' (Dual Index) v3.1 User Guide with Feature Barcode technology for Cell Multiplexing (CG000388)

Protocol Step 2.4 - cDNA QC & Quantification



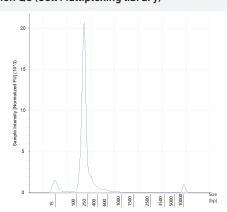
Run 2 μ l sample mixed with 2 μ l loading buffer. Ensure dilution factor is factored in when calculating cDNA yield.

Protocol Step 3.7 - Post Library Construction QC



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

Protocol Step 4.3 – Post Library Construction QC (Cell Multiplexing library)



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

All traces are representative.

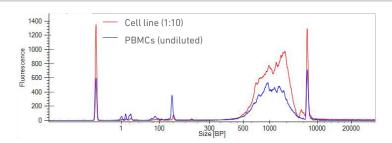
LabChip Traces

LabChip Traces

DNA High Sensitivity Reagent Kit was used.

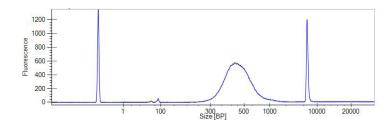
Protocol steps correspond to the Chromium Next GEM Single Cell 3' v3.1 (Dual Index) User Guide with Feature Barcode technology for Cell Multiplexing (CG000388)

Protocol Step 2.4 - cDNA QC & Quantification



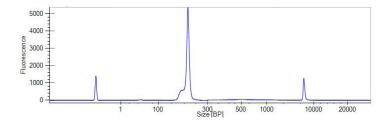
Run 10 μ l sample. cDNA yield calculation is same as Agilent Bioanalyzer traces.

Protocol Step 3.7 - Post Library Construction QC



Run 10 μ l diluted sample (1:10 dilution).

Protocol Step 4.3 – Post Library Construction QC (Cell Multiplexing library)



Run 10 μ l diluted sample (1:20 dilution).

All traces are representative.

Alternate QC Method:

Qubit Fluorometer and Qubit dsDNA HS Assay Kit

Multiply the cDNA concentration reported via the Qubit Fluorometer by the elution volume (40 μ l) to obtain the total cDNA yield in ng. To determine the equivalent range using the Agilent 2100 Expert Software, select the region encompassing 35-10,000 bp.

Oligonucleotide Sequences

Protocol steps correspond to the Chromium Next GEM Single Cell 3' (Dual Index) v3.1 User Guide with Feature Barcode technology for Cell Multiplexing (CG000388) Protocol Step 1.5 - GEM-RT Incubation Gel Bead **Primers** TruSeq Read 1 Barcode UMI 10x Nextera Read 1 UMI Capture Seq 2 (Read 1N) Barcode 5'-GTCAGATGTGTATAAGAGACAG-N16-N12-CCTTAGCCGCTAATAGGTGAGC-3' Template Switch Oligo 5'-AAGCAGTGGTATCAACGCAGAGTACATrGrGrG-3' PN-3000228 Lipid Cell Multiplexing Capture Seq 2 Feature Barcode Read 2N Oligo Feature Barcode 3'-GGAATCGGCGATTATCCACTCG-N15-GACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-5' cDNA from poly-adenylated mRNA GEM-RT **Products** Read 1 10x Barcode UMI Poly(dT)VN DNA from Cell Multiplexing Oligo Feature Barcode 10x Barcode UMI Capture Seq 2 Feature Barcode 5'-GTCAGATGTGTATAAGAGACAG-N16-N12-CCTTAGCCGCTAATAGGTGAGC-N15-CTGTCTCTTATACACATCTCCGAGCCCACGAGAC-3' Protocol Step 2.2 - cDNA Amplification Feature cDNA **Amplifies cDNA** Primers 3 PN-2000289 Forward Primer: Reverse Primer: Partial Read 1 Partial TSO 5'-CTACACGACGCTCTTCCGATCT-3' 5'-AAGCAGTGGTATCAACGCAGAG-3' Amplifies DNA from Cell Multiplexing Oligo Feature Barcode Forward Primer: Reverse primer: Partial Read 1N Partial Read 2N 5'-GCAGCGTCAGATGTGTATAAGAGACAG-3' 5'-GTCTCGTGGGCTCGGAGATGTGTATAA-3' Amplified cDNA from poly-adenylated mRNA Amplification **Products** 10x Barcode UMI Poly(dT)VN Read 1 Amplified DNA from Cell Multiplexing Oligo Feature Barcode Read 1N 10x Barcode UMI Capture Seg 2 Feature Barcode Read 2N 5'-GCAGCGTCAGATGTGTATAAGAGACAG-N16-N12-CCTTAGCCGCTAATAGGTGAGC-N15-CTGTCTCTTATACACATCTCCGAGCCCACGAGAC-3' 3'-CGTCGCAGTCTACACATATTCTCTGTC-N16-N12-GGAATCGGCGATTATCCACTCG-N15-GACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-5'

Protocol Step 3.3 - Adaptor Ligation (for 3' Gene Expression Library Construction) Adaptor Oligos PN -2000094 Partial Read 2 5'- GATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3' 3'-TCTAGCCTTCTCG-5' Ligation Product Read 1 10x Barcode UMI Poly(dT)VN Protocol Step 3.5 - Sample Index PCR (for 3' Gene Expression Library Construction) **Dual Index** Kit TT Set A Sample Partial Read 1 Sample Partial Read 2 P5 PN-1000215 Index (i5) 5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTC-3' 5'-CAAGCAGAAGACGGCATACGAGAT-N10-GTGACTGGAGTTCAGACGTGT-3' Sample Index (i5:10) Sample Index Sample Index (i7:10) **PCR Product** TruSeq Read 1 10x **UMI** Poly(dT)VN TruSeq Read 2 Barcode 5-AATGATACGGCGACCACCGAGATCTACAC-NID-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NI6-NI2-TTTTTTTTTTTTTTTTTTTTTTTTVN-cDNa_inseri-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-NID-ATCTCGTATGCCGTCTTCTCGCTTG-3' Protocol Step 4.1 - Sample Index PCR (for Cell Multiplexing Library Construction) Dual Index Kit NN Set A Sample Index (i5) Read 1N Sample Partial Read 2N PN-1000243 Index (i7) 5'-CAAGCAGAAGACGGCATACGAGAT-N10-GTCTCGTGGGCTCGG-3' 5'-AATGATACGGCGACCACCGAGATCTACAC-N10-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3' Sample Index PCR Product P5 Sample Nextera Read 1 10x Barcode UMI Capture Sequence 2 Nextera Read 2 Sample Feature (Read 2N) Index (i7:10) Index (i5:10) (Read 1N) Barcode 5'-AATGATACGGCGACCACCGAGATCTACAC-N10-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-N16-N12-CCTTAGCCGCTAATAC GC-N15-CTGTCTCTTATACACATCTCCGAGCCCACGAGAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3' 3'-TTACTATECCGCTGGTGGCTCTAGATGTG-N10-AGCAGCCGTCGCAGTCTACACATATTCTCTGTC-N16-N12-GGAATCGGCGATATCCACTCG-N15-GACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-N10-TAGAGCATACCGCAGAGAACAGC-S-