Chromium

Single Cell 3' Reagent Kits v2 User Guide

FOR USE WITH

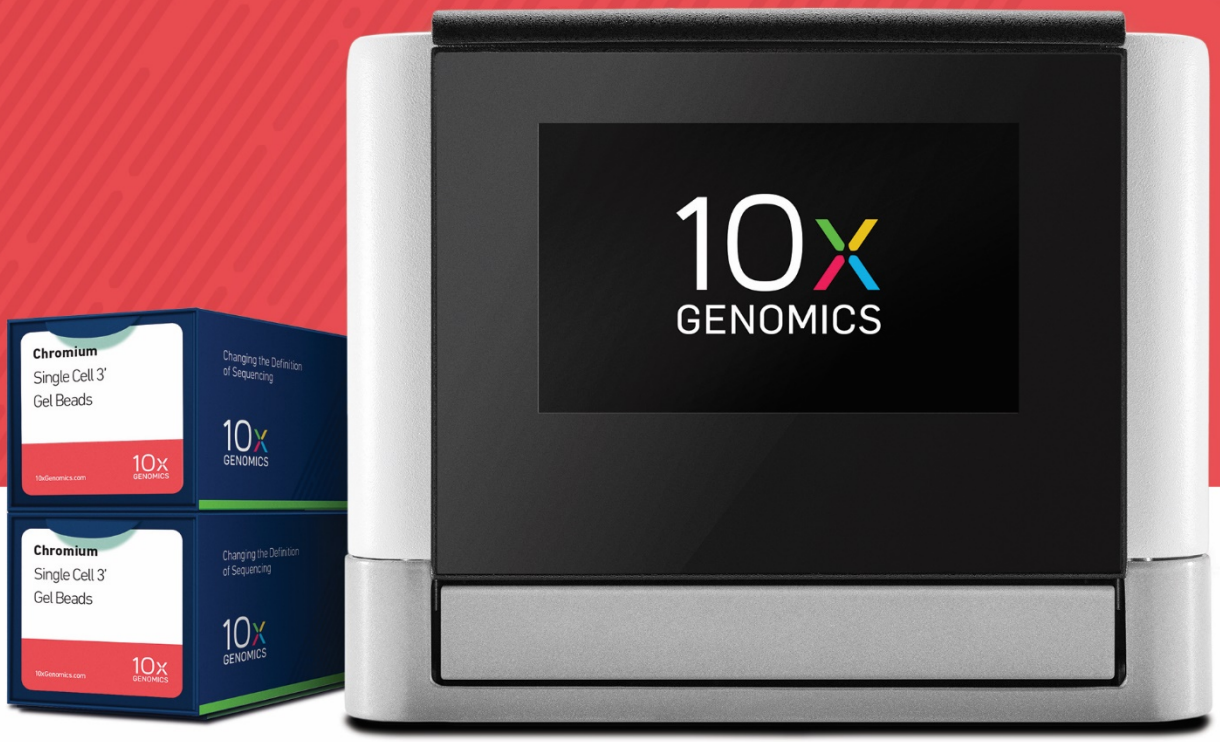
Chromium Single Cell 3' Library & Gel Bead Kit v2, 16 rxns PN-120237

Chromium Single Cell 3' Library & Gel Bead Kit v2, 4 rxns PN-120267

Chromium Single Cell A Chip Kit, 48 rxns PN-120236

Chromium Single Cell A Chip Kit, 16 rxns PN-1000009

Chromium i7 Multiplex Kit, 96 rxns PN-120262



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Support

Email: support@10xgenomics.com

10x Genomics

7068 Koll Center Parkway

Suite 401

Pleasanton, CA 94566 USA

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Introduction

Chromium Single Cell 3' Reagent Kits v2

Chromium Accessories

Additional Kits, Reagents & Equipment

Recommended Thermal Cyclers

Chromium Single Cell 3' Reagent Kits v2

CRITICAL!

*Parts from Chromium Single Cell 3' Reagents Kits v2 are **NOT** interchangable with parts from other Chromium Reagent Kits, despite the same or similar names, unless they also share the same Part Number.*

Chromium Single Cell 3' Library & Gel Bead Kit v2, 16 rxns PN-120237

Product	Components	#	Part Number
Chromium Single Cell 3' Library Kit v2, 16 rxns (store at –20°C)			120234
Reagents Module 1	RT Reagent Mix	1	220089
	RT Enzyme Mix	1	220079
	Additive A	1	220074
	RT Primer	2	310354
	Buffer Sample Clean Up 1	2	220020
	Amplification Master Mix	1	220125
	cDNA Primer Mix	1	220106
	cDNA Additive	1	220067
Reagents Module 2	Fragmentation Enzyme Blend	1	220107
	Fragmentation Buffer	1	220108
	Ligation Buffer	1	220109
	DNA Ligase	1	220110
	Adaptor Mix	1	220026
	SI-PCR Primer	1	220111
	Amplification Master Mix	1	220125
Chromium Single Cell 3' Gel Bead Kit v2, 16 rxns (store at –80°C)			120235
	Single Cell 3' Gel Beads	2	220104
Dynabeads™ MyOne™ SILANE, 16 rxns (store at 4°C)			2000048
	Dynabeads MyOne SILANE	1	2000048

Chromium Single Cell 3' Reagent Kits v2

CRITICAL!

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Chromium Single Cell 3' Library & Gel Bead Kit v2, 4 rxns PN-120267

Product	Components	#	Part Number
Chromium Single Cell 3' Library Kit v2, 4 rxns (store at –20°C)			120264
Reagents Module 1	RT Reagent Mix	1	220089
	RT Enzyme Mix	1	220127
	Additive A	1	220074
	RT Primer	1	310354
	Buffer Sample Clean Up 1	1	220020
	Amplification Master Mix	1	220129
	cDNA Primer Mix	1	220106
	cDNA Additive	1	220067
Reagents Module 2	Fragmentation Enzyme Blend	1	220130
	Fragmentation Buffer	1	220108
	Ligation Buffer	1	220109
	DNA Ligase	1	220131
	Adaptor Mix	1	220026
	SI-PCR Primer	1	220111
Chromium Single Cell 3' Gel Bead Kit v2, 4 rxns (store at –80°C)			120265
	Single Cell 3' Gel Beads (4 rxns)	1	220104
Dynabeads™ MyOne™ SILANE, 16 rxns (store at 4°C)			2000048
	Dynabeads MyOne SILANE	1	2000048

Chromium Single Cell 3' Reagent Kits v2

CRITICAL!

*Parts from Chromium Single Cell 3' Reagents Kits v2 are **NOT** interchangable with parts from other Chromium Reagent Kits, despite the same or similar names, unless they also share the same Part Number.*

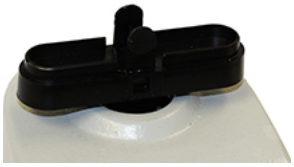
Chromium Single Cell A Chip Kits PN-120236 & PN-1000009

Product	Components	#	Part Number
Chromium Single Cell A Chip Kit, 48 rxns (store at ambient temperature)			120236
	Chip A Single Cell	6	230027
	Gaskets, 6-pack	1	370017
	Partitioning Oil	6	220088
	Recovery Agent	6	220016
Chromium Single Cell A Chip Kit, 16 rxns (store at ambient temperature)			1000009
	Chip A Single Cell	2	2000019
	Gasket, 2-pack	1	3000072
	Partitioning Oil	2	220088
	Recovery Agent	2	220016

Chromium Multiplex Kit, 96 rxns PN-120262

Product	Description	#	Part Number
Chromium i7 Multiplex Kit, 96 rxns (store at –20°C)			120262
	Chromium i7 Sample Index Plate	1	220103

Chromium Accessories

Product	Description	Part Number (Orderable)
10xVortex Adapter	The 10x Vortex Adapter attaches to the top of most standard laboratory vortexers and enables users to vortex Gel Bead Strips. 	120251
10x Chip Holder	The 10x Chip Holder encases the Chromium Chips and holds them in the correct position in the Chromium Controller. The 10x Gasket fits over the top of the 10x Chip Holder before inserting the assembly in the Chromium Controller. The 10x Chip Holder lid also conveniently flips over to become a stand, holding the Chromium Chip at the ideal 45° angle for removing GEMs from the Recovery Wells after a Chromium Controller run. Squeeze the black sliders on the back side of the 10x Chip Holder together to unlock the lid and return the 10x Chip Holder to a flat position. 	120252
10x Magnetic Separator	The 10x Magnetic Separator offers two positions of the magnets relative to the 8-tube strip inserted, depending on its orientation. Simply flip the 10x Magnetic Separator over to switch between the magnets being High or Low.	120250

Additional Kits, Reagents & Equipment

The items in the table below have been validated by 10x Genomics and are highly recommended for Single Cell 3' workflow, training, and system operations. USA Scientific, Eppendorf, and Thermo Fisher PCR 8-tube strips have been validated. Substituting materials may adversely affect system performance. Either Bioanalyzer, TapeStation, or Fragment Analyzer are needed for cDNA quantitation and quality control. A Qubit Flurometer may also be used for cDNA quantitation.

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 *(alternate to Eppendorf or Thermo Fisher Scientific product)*	1402-4700
Thermo Fisher Scientific	MicroAmp 8-Tube Strip, 0.2 ml *(alternate to Eppendorf or USA Scientific product)* 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
Sigma	Ethanol, Pure (200 Proof, anhydrous)	E7023-500ML
Beckman Coulter	SPRIselect Reagent Kit	B23318
Bio-Rad	10% Tween 20	1610781
Ricca Chemical Company	Glycerin (glycerol), 50% (v/v) Aqueous Solution	3290-32
Equipment		
Rainin	Tips LTS 200UL Filter RT-L200FLR Pipet-Lite Multi Pipette L8-50XLS+ Pipet-Lite Multi Pipette L8-200XLS+	17007961 17013804 17013805
VWR	Vortex Mixer Divided Polystyrene Reservoirs	10153-838 41428-958
	Table continued in next page	

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Quantification & Quality Control		
Agilent	2100 Bioanalyzer Laptop Bundle	G2943CA
	High Sensitivity DNA Kit	5067-4626
	4200 TapeStation	G2291aa
	High Sensitivity D1000 ScreenTape	5067-5584
	High Sensitivity D1000 Reagents	5067-5585
	High Sensitivity D5000 ScreenTape	5067-5592
	High Sensitivity D5000 Reagents	5067-5593
Thermo Fisher Scientific	Qubit 3.0 Fluorometer	Q33216
	Qubit dsDNA HS Assay Kit	Q32854
Advanced Analytical	Fragment Analyzer Automated CE System – 12 cap	FSv2-CE2F
	Fragment Analyzer Automated CE System – 48/96 cap	FSv2-CE10F
	High Sensitivity NGS Fragment Analysis Kit	DNF-474
KAPA Biosystems	Illumina Library Quantification Kit	KK4824

Not all of these instruments and reagents are required.

Choose among Bioanalyzer, TapeStation, Qubit, and Fragment Analyzer based on availability and preferences.

Additional Kits, Reagents & Equipment

The following are additional and alternative kits, reagents, and equipment recommended by 10x Genomics and contribute to optimal system performance. If using plates, Eppendorf twin.tec brand PCR plates are recommended to ensure stability of GEM emulsions, and the specific model should be selected based on compatibility with thermal cycler in use.

Supplier	Description	Part Number (US)
Plastics		
Eppendorf	Heat Sealing Foil, PCR clean *(alternate to Bio-Rad product)*	0030127854
	twin.tec 96-Well PCR Plate Semi-skirted (*Choose specific plate based on thermal cycler compatibility*)	0030129326
	twin.tec 96-Well PCR Plate Divisible, Unskirted	2231000209
	twin.tec 96-Well PCR Plate Unskirted	0030133390
Bio-Rad	Optical Flat 8-Cap Strips	TCS0803
	Microseal 'B' Adhesive Seals	MSB1001
	Pierceable Foil Heat Seal *(if PCR plates used)*	1814040
Equipment		
Eppendorf	ThermoMixer C	5382000015
	SmartBlock 1.5 ml, Thermoblock for 24 Reaction Vessels *(alternatively, use a temperature-controlled Heat Block)*	5360000038
Bio-Rad	PX1 PCR Plate Sealer *(if PCR plates used)*	1814000
Rainin	Tips LTS 20UL Filter RT-L10FLR	30389240
	Tips LTS 1ML Filter RT-L1000FLR	30389213
	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
	Pipet-Lite Multi Pipette L8-10XLS+	17013802
	Pipet-Lite Multi Pipette L8-20XLS+	17013803
-	qPCR instrument and compatible consumables	

Recommended Thermal Cyclers

Thermal cyclers used with the Single Cell 3' Protocol must support uniform heating of 100 µl emulsion volumes. Thermal cyclers recommended for use with the Single Cell 3' Protocol are:

- Bio-Rad C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (PN-1851197)
- Eppendorf MasterCycler Pro (PN North America 950030010, International 6321 000.019)
- Thermo Fisher Veriti 96-Well Thermal Cycler (PN-4375786)

The Single Cell 3' Reagent Kit Protocol

Stepwise Objectives

Steps & Timing

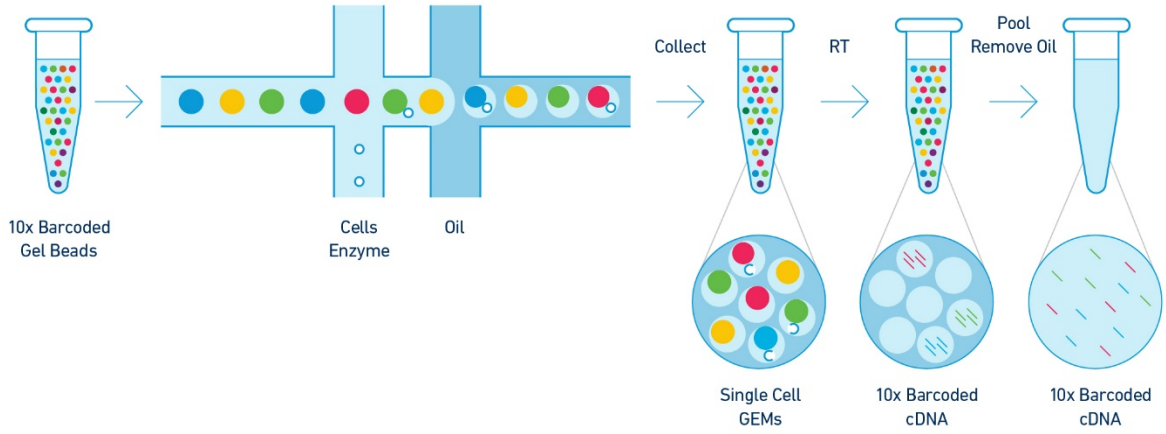
The Single Cell 3' Reagent Kit Protocol – Stepwise Objectives

Step 1 – GEM Generation & Barcoding

The Single Cell 3' Protocol upgrades short read sequencers to deliver a scalable microfluidic platform for 3' digital gene expression profiling of up to 10,000 individual cells per sample. The 10x GemCode Technology samples a pool of ~ 750000 barcodes to separately index each cell's transcriptome. It does so by partitioning thousands of cells into nanoliter-scale Gel Bead-In-EMulsions (GEMs), where all generated cDNA share a common 10x Barcode. Libraries are generated and sequenced from the cDNA and the 10x Barcodes are used to associate individual reads back to the individual partitions.

To achieve single cell resolution, the cells are delivered at a limiting dilution, such that the majority (~90-99%) of generated GEMs contains no cell, while the remainder largely contain a single cell.

Upon dissolution of the Single Cell 3' Gel Bead in a GEM, primers containing (i) an Illumina R1 sequence (read 1 sequencing primer), (ii) a 16 nt 10x Barcode, (iii) a 10 nt Unique Molecular Identifier (UMI), and (iv) a poly-dT primer sequence are released and mixed with cell lysate and Master Mix. Incubation of the GEMs then produces barcoded, full-length cDNA from poly-adenylated mRNA. After incubation, the GEMs are broken and the pooled fractions are recovered.



Step 2 – Post GEM-RT Cleanup & cDNA Amplification

Silane magnetic beads are used to remove leftover biochemical reagents and primers from the post GEM reaction mixture. Full-length, barcoded cDNA is then amplified by PCR to generate sufficient mass for library construction.

Step 3 – Library Construction

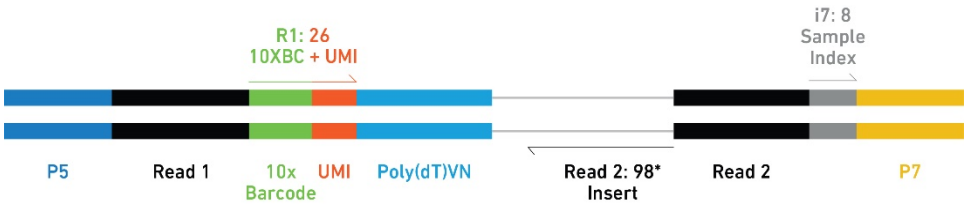
Enzymatic Fragmentation and Size Selection are used to optimize the cDNA amplicon size prior to library construction. R1 (read 1 primer sequence) are added to the molecules during GEM incubation. P5, P7, a sample index, and R2 (read 2 primer sequence) are added during library construction via End Repair, A-tailing, Adaptor Ligation, and PCR. The final libraries contain the P5 and P7 primers used in Illumina bridge amplification.

Step 4 – Sequencing Libraries

The Single Cell 3' Protocol produces Illumina-ready sequencing libraries. A Single Cell 3' Library comprises standard Illumina paired-end constructs which begin and end with P5 and P7. The Single Cell 3' 16 bp 10x Barcode and 10 bp UMI are encoded in Read 1, while Read 2 is used to sequence the cDNA fragment. Sample

index sequences are incorporated as the i7 index read. Read 1 and Read 2 are standard Illumina sequencing primer sites used in paired-end sequencing.

Final Library Structure:



Single Cell 3' Library Analysis

Sequencing a Single Cell 3' Library produces a standard Illumina BCL data output folder. The BCL data will include the paired-end Read 1 (containing the 16 bp 10x Barcode and 10 bp UMI) and Read 2 and the sample index in the i7 index read.

The Cell Ranger analysis pipelines perform secondary analysis and visualization. In addition to performing standard analysis steps such as demultiplexing, alignment, and gene counting, Cell Ranger leverages the 10x Barcodes to generate expression data with single-cell resolution. This data type enables applications including cell clustering, cell type classification, and differential gene expression at a scale of hundreds to millions of cells.

Chromium Single Cell 3' How-to Video Series Available at 10x Genomics Support

- Getting Started
- Protocol Step 1.1 – Preparing Single Cell Master Mix
- Protocol Step 1.2 – Assembling the Chip
- Protocol Step 1.2 – Combining Single Cell Master Mix, Water & Cells
- Protocol Step 1.2 – Adding Master Mix + Cells, Gel Beads and Partitioning Oil to the Chip
- Protocol Step 1.3 – Running the Chromium Controller
- Protocol Step 1.4 – Transferring GEMs
- Protocol Step 2.1 – Breaking the GEMs with Recovery Agent

The Single Cell 3' Reagent Kit Protocol – Steps & Timing

	Bench Time	Instrumentation Time	Stop & Store Options
	Cell Preparation Sample Dependent 1 - 1.5 h		
1 h	Reagent Prep - 20 min Loading Single Cell A Chip - 10 min Transferring GEMs - 3 min	GEM Generation - 6.5 min	
2 h		GEM-RT Incubation - 55 min	STOP 4°C ≤72 h or –20°C ≤1 week
	Post GEM-RT Recovery - 10 min Cleanup - Silane Beads - 35 min cDNA Amplification Prep - 5 min		
3 h		cDNA Amplification - 30 - 45 min	STOP 4°C ≤72 h
	Cleanup - SPRIselect - 20 min		STOP 4°C ≤72 h or –20°C ≤1 week
4 h		QC & Quantification - 50 min	
5 h	Frag, End Repair & A-tailing Prep - 10 min	Fragmentation, End Repair & A-tailing Incubation - 40 min	
6 h	Double Sided SPRIselect Sizing - 25 min Adaptor Ligation Prep - 10 min		
		Adaptor Ligaton Incubation - 15 min	
	Cleanup - SPRIselect - 20 min Sample Index PCR Prep - 10 min		
7 h		Sample Index PCR - 20 - 40 min	STOP 4°C ≤72 h
	Double Sided SPRIselect Sizing - 25 min		STOP 4°C ≤72 h or –20°C long-term
8 h		QC - 50 min	
		qPCR Quantification*	

**qPCR Quantification (~ 1 h total) time not included.*

Protocol Step 1

GEM Generation & Barcoding

Partition input cells across tens of thousands of GEMs for lysis and barcoding

1

1. GEM Generation & Barcoding

Tips

Importance of Emulsion-safe Plastic Consumables

Some plastics can interact with and destabilize GEMs. It is therefore critical to use validated emulsion-safe plastic consumables when handling GEMs. 10x Genomics has validated Eppendorf twin.tec PCR plates and Rainin LTS low retention pipette tips as GEM-compatible plastics. USA Scientific, Eppendorf, and Thermo Fisher PCR 8-tube strips have also been validated. Substituting these materials can adversely affect performance.

Importance of Loading Cell Concentration

The recommended starting point for a new sample type is to load ~1700 cells into each reaction, recovering approximately 1000 cells, to achieve an expected multiplet rate of approximately 0.8%. Loading fewer cells per reaction will result in a lower multiplet rate while loading more cells per reaction will increase the multiplet rate (see table below). To maximize the likelihood of achieving the desired recovery target, the optimal input cell concentration is 700 – 1200 cells/µl. The presence of dead cells in the suspension may reduce the observed recovery rate. Consult the Single Cell Protocols Cell Preparation Guide and Guidelines for Optimal Sample Preparation flowchart (Documents CG00053 and CG000126 respectively) for more information on preparing cells for use in this Protocol.

Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~0.4%	~870	~500
~0.8%	~1700	~1000
~1.6%	~3500	~2000
~2.3%	~5300	~3000
~3.1%	~7000	~4000
~3.9%	~8700	~5000
~4.6%	~10500	~6000
~5.4%	~12200	~7000
~6.1%	~14000	~8000
~6.9%	~15700	~9000
~7.6%	~17400	~10000

Best Practices for Handling Chip A Single Cell

The generation of GEMs occurs in channels that are narrower than the typical human hair (i.e. <100 µm). Care should be taken to avoid introduction of particles, fibers or clumped cells into these channels. Prepare reagents and load the chips in a positive-pressure laminar flow hood, and filter the single cell suspension before addition to the Master Mix whenever possible. Minimize exposure of reagents, chips, and gaskets to sources of particles and fibers, such as open reagent reservoirs, laboratory wipes, frequently opened flip-cap tubes, clothing that easily sheds fibers, and dusty surfaces. The presence of excess Partitioning Oil in

recovered GEMs from the Chip A Single Cell after running the Chromium Controller may indicate that a clog occurred. See Practical Tips & Troubleshooting for more information (Section 5).

Pay particular attention to the timing of loading and running chips. Steps should be executed successively without pauses or delays. When multiple chips are to be used, load and run the chips in series, collecting the GEMs from the one chip before loading the next.

All input wells (rows 1, 2, and 3) of unused channels on a chip should be filled with a 50% volume/volume aqueous solution of glycerol before loading the used wells with reagents. See Practical Tips & Troubleshooting (Section 5) for information on purchasing or preparing a 50% glycerol solution.

When removing a chip from the box and inserting it into a Chip Holder, avoid contacting the bottom surface of the chip with gloved hands and other surfaces. Frictional charging can interfere with correct priming of the channels, potentially leading to either clogs or wetting failures. See Practical Tips & Troubleshooting (Section 5) for the definition of these failures.

Minimize the distance that a loaded chip has to be moved to reach the Chromium Controller. When transferring to the Chromium Controller, 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.

Best Practices – Preparing & Handling Reagents & Master Mixes

- Cover Partitioning Oil tubes and reservoirs to minimize evaporation.
- Ensure that reagents are fully thawed and thoroughly mixed before use. Keep all enzyme components and Master Mixes on ice during setup and promptly move reagents back to the recommended storage temperature when possible.
- Assemble Master Mix on ice and keep cold until Chip A Single Cell loading.
- For tips on processing fewer than 8 reactions, see Practical Tips & Troubleshooting (Section 5).

Best Practices – Handling Gel Beads

- Equilibrate the Single Cell 3' Gel Beads Strip to room temperature before use.
- Store any unused Single Cell 3' Gel Beads at –80°C and avoid more than 10 freeze-thaw cycles.
- Never store Single Cell 3' Gel Beads at –20°C.
- Pierce the Gel Bead Strip foil seals with pipette tips without engaging the plunger.
- Upon initial Gel Bead Strip foil seal puncture, the pipette tips should extend no more than 2 mm below the seal. Then, raise the tips above the foil seal and depress the plunger. Lower the tips to the bottom of the wells and widen the opening by gently rocking the tips back and forth, keeping the plunger engaged. This technique will maximize recovery of Gel Beads for optimal performance.

Best Practices – Loading the Chip A Single Cell

- Wait >30 sec between loading the Master Mix and loading the Gel Beads to ensure proper priming of the channels. Vortex the Single Cell 3' Gel Bead Strip for 30 sec AFTER loading the Master Mix to ensure that the correct time has passed before loading the Gel Beads. Do not exceed 120 sec between loading Master Mix and Gel Beads.
- When aspirating Gel Beads from the Gel Bead Strip or emulsion from the Recovery Wells, pipet slowly to avoid introducing air bubbles and leave the pipette tips in the wells for an additional 5 sec after the aspiration stops to allow pressure to equilibrate.
- When dispensing Gel Beads into the Chip A Single Cell, wait for the remainder to drain into the bottom of the pipette tips and dispense again to ensure complete transfer.

- Start GEM generation immediately after Chip A Single Cellloading. Do not exceed 120 sec between loading the chip and starting the run.

Best Practices – GEM Recovery

- Retrieve GEMs immediately after the completion of a run.
- When dispensing GEMs into the PCR 8-tube strip or the PCR plate, wait for the remainder to drain into the bottom of the pipette tips and dispense again to ensure complete transfer.
- Immediately place the plate of recovered GEMs on a chilled metal block resting on ice.

Getting Started!

Equilibrate to room temperature before use:

Item	Part Number	Storage Location
Single Cell 3' Gel Beads *Equilibrate to room temperature 30 min before loading the Chip A*	220104	**–80°C**
RT Reagent Mix *Vortex and verify no precipitate*	220089	–20°C
RT Primer *Provided as lyophilized oligos; after resuspension, store unused primers at –80°C*	310354	–20°C
Additive A *Vortex, verify no precipitate, centrifuge briefly*	220074	–20°C

Resuspend:

Briefly centrifuge the lyophilized RT Primer and then resuspend the oligo by adding 40 µl of low TE. Vortex 15 sec at full speed, centrifuge briefly, and leave at room temperature for ≥ 30 min

50% glycerol solution:

If processing fewer than 8 reactions, see Practical Tips & Troubleshooting (Section 5) for information on purchasing or generating 50% glycerol solution

Place on ice:

Item	Part Number	Storage Location
RT Enzyme Mix *Maintain on ice, centrifuge briefly before adding to Master Mix*	220079 or 220127	–20°C
Chilled Metal Block	-	-

Obtain:

Item	Part Number	Storage Location
Partitioning Oil	220088	Ambient temperature
Chip(s) A Single Cell	230027 or 2000019	Ambient temperature
10x Gasket(s)	370017 or 3000072	Ambient temperature
10x Chip Holder	120252	Ambient temperature

Plate sealer:

If PCR plates used, set the Bio-Rad PX1 PCR Plate Sealer to seal at 185°C for 6 sec. Keep heat block external to sealer until plate sealing step

Cell Suspension Volume Calculator Table

CRITICAL!

Consult the Single Cell Protocols Cell Preparation Guide and Guidelines for Optimal Sample Preparation flowchart (Documents CG00053 and CG000126) for more information on preparing cells for use in this Protocol.

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

Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery										
	500 cells	1000 cells	2000 cells	3000 cells	4000 cells	5000 cells	6000 cells	7000 cells	8000 cells	9000 cells	10000 cells
100	8.7 25.1	17.4 16.4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
200	4.4 29.5	8.7 25.1	17.4 16.4	26.1 7.7	n/a	n/a	n/a	n/a	n/a	n/a	n/a
300	2.9 30.9	5.8 28.0	11.6 22.2	17.4 16.4	23.2 10.6	29.0 4.8	n/a	n/a	n/a	n/a	n/a
400	2.2 31.6	4.4 29.5	8.7 25.1	13.1 20.8	17.4 16.4	21.8 12.1	26.1 7.7	30.5 3.4	n/a	n/a	n/a
500	1.7 32.1	3.5 30.3	7.0 26.8	10.4 23.4	13.9 19.9	17.4 16.4	20.9 12.9	24.4 9.4	27.8 6.0	31.3 2.5	n/a
600	1.5 32.4	2.9 30.9	5.8 28.0	8.7 25.1	11.6 22.2	14.5 19.3	17.4 16.4	20.3 13.5	23.2 10.6	26.1 7.7	29.0 4.8
700	1.2 32.6	2.5 31.3	5.0 28.8	7.5 26.3	9.9 23.9	12.4 21.4	14.9 18.9	17.4 16.4	19.9 13.9	22.4 11.4	24.9 8.9
800	1.1 32.7	2.2 31.6	4.4 29.5	6.5 27.3	8.7 25.1	10.9 22.9	13.1 20.8	15.2 18.6	17.4 16.4	19.6 14.2	21.8 12.0
900	1.0 32.8	1.9 31.9	3.9 29.9	5.8 28.0	7.7 26.1	9.7 24.1	11.6 22.2	13.5 20.3	15.5 18.3	17.4 16.4	19.3 14.5
1000	0.9 32.9	1.7 32.1	3.5 30.3	5.2 28.6	7.0 26.8	8.7 25.1	10.4 23.4	12.2 21.6	13.9 19.9	15.7 18.1	17.4 16.4
1100	0.8 33.0	1.6 32.2	3.2 30.6	4.7 29.1	6.3 27.5	7.9 25.9	9.5 24.3	11.1 22.7	12.7 21.1	14.2 19.6	15.8 18.0
1200	0.7 33.1	1.5 32.4	2.9 30.9	4.4 29.5	5.8 28.0	7.3 26.6	8.7 25.1	10.2 23.7	11.6 22.2	13.1 20.8	14.5 19.3
1300	0.7 33.1	1.3 32.5	2.7 31.1	4.0 29.8	5.4 28.4	6.7 27.1	8.0 25.8	9.4 24.4	10.7 23.1	12.0 21.8	13.4 20.4
1400	0.6 33.2	1.2 32.6	2.5 31.3	3.7 30.1	5.0 28.8	6.2 27.6	7.5 26.3	8.7 25.1	9.9 23.9	11.2 22.6	12.4 21.4
1500	0.6 33.2	1.2 32.6	2.3 31.5	3.5 30.3	4.6 29.2	5.8 28.0	7.0 26.8	8.1 25.7	9.3 24.5	10.4 23.4	11.6 22.2
1600	0.5 33.3	1.1 32.7	2.2 31.6	3.3 30.5	4.4 29.5	5.4 28.4	6.5 27.3	7.6 26.2	8.7 25.1	9.8 24.0	10.9 22.9
1700	0.5 33.3	1.0 32.8	2.0 31.8	3.1 30.7	4.1 29.7	5.1 28.7	6.1 27.7	7.2 26.6	8.2 25.6	9.2 24.6	10.2 23.7
1800	0.5 33.3	1.0 32.8	1.9 31.9	2.9 30.9	3.9 29.9	4.8 29.0	5.8 28.0	6.8 27.0	7.7 26.1	8.7 25.1	9.7 24.1
1900	0.5 33.3	0.9 32.9	1.8 32.0	2.7 31.1	3.7 30.1	4.6 29.2	5.5 28.3	6.4 27.4	7.3 26.6	8.2 25.6	9.2 24.6
2000	0.4 33.4	0.9 32.9	1.7 32.1	2.6 31.2	3.5 30.3	4.4 29.5	5.2 28.6	6.1 27.7	7.0 26.8	7.8 26.0	8.7 25.1

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

GEM Generation & Barcoding

1.1. Preparing Single Cell Master Mix

NOTE

Volumes for 4 and 8 reactions are listed in all reagent tables and include 10% excess (i.e. 4.4X and 8.8X, respectively).

To set up a different number of reactions (n), multiply the indicated 1 reaction volumes by n.n (for example, multiply by 2.2 to set up 2 reactions with 10% excess).

a) Prepare Master Mix on ice. Add reagents in the order shown below. Pipette mix 15 times and centrifuge briefly. **Do not add Single Cell Suspension at this point.**

Master Mix	PN	1 rxn (µl)	4 rxns + 10% xs (µl)	8 rxns + 10% xs (µl)
RT Reagent Mix	220089	50.0	220.0	440.0
RT Primer	310354	3.8	16.7	33.4
Additive A	220074	2.4	10.6	21.1
RT Enzyme Mix	220079 or 220127	10.0	44.0	88.0
Total	-	**66.2**	**291.3**	**582.5**

b) Place the Master Mix on a chilled metal block resting on ice.

c) Dispense **66.2 µl** Master Mix into each well of a PCR 8-tube strip on a chilled metal block resting on ice.

1.2. Loading the Chip A Single Cell

a) Place a Chip A Single Cell in a 10x Chip Holder. Handle the chip by its edges, taking care to avoid touching its bottom surface. See Practical Tips & Troubleshooting (Section 5) for tips on assembly.

CRITICAL!

The order in which the wells of Chip A Single Cell are loaded is critical for optimal performance. Always load the rows in the labeled order: 1 followed by 2, then 3.

b) If processing fewer than 8 samples per Chip A Single Cell, first add the following volumes of 50% glycerol solution to each unused well:

i. **90 µl** in the row labeled 1

ii. **40 µl** in the row labeled 2

iii. **270 µl** in the row labeled 3

CRITICAL!

Do not add 50% glycerol solution to Recovery Wells (row labeled ◀). Do not use Partitioning Oil or any other solution as a substitute for 50% glycerol solution.

c) Add the appropriate volume of **nuclease-free water** (determined from the Cell Suspension Volume Calculator Table) into each well containing Master Mix.

CRITICAL!

The next step is critical to recovering the maximum number of cells. It is important to ensure that all the cells in the sample are suspended and that the sample is homogenous when adding the cell suspension volume to the Master Mix. To do this, estimate the volume of the cell suspension and set the pipette at half that volume for pipette mixing. When withdrawing the volume of cell suspension from the tube, place the pipette tip near the center of the suspension volume. Cells settle at different rates, so it is important to mix directly before taking the sample.

d) Gently pipette mix the tube containing the washed and diluted cells. Add the appropriate **volume (µl) of single cell suspension** (determined from the Cell Suspension Volume Calculator Table) to each well of the tube strip containing the Master Mix and **nuclease-free water**.

NOTE

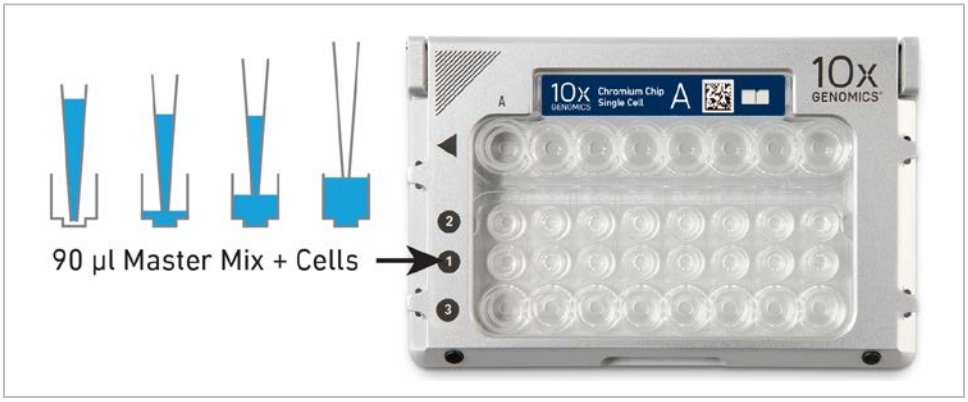
The total combined volume of cells, Master Mix, and nuclease-free water is 100 µl in each well.

e) With a pipette set to 90 µl, gently pipette mix the combined cells, Master Mix, and nuclease-free water (from here onwards referred to as Master Mix containing cells) 5 times while keeping the tube strip on a chilled metal block resting on ice.

f) Without discarding the pipette tips, transfer **90 µl** Master Mix containing cells to the wells in the **row labeled 1**, taking care not to introduce bubbles. To do this, place the tips into the bottom center of the wells and raise the tips slightly above the bottom before slowly dispensing the Master Mix containing cells.

CRITICAL!

Pipette slowly. Raising and depressing the pipette plunger should each take 2 sec. Raise the pipette tips at the same rate as the liquid level is rising in the well, keeping the tip slightly submerged.



g) Snap the Single Cell 3' Gel Bead Strip into a 10x Vortex Adapter and vortex for **30 sec**.

CRITICAL!

A 30 sec wait while vortexing the Single Cell 3' Gel Bead Strip is required to ensure proper priming of the Master Mix containing cells in the Chip A Single Cell. Then, immediately load the Single Cell 3' Gel Beads.

h) Remove the Single Cell 3' Gel Bead Strip and flick in a sharp, downward motion to ensure maximum recovery. Confirm that there are no bubbles at the bottom of the tube and that liquid levels are uniform.

NOTE

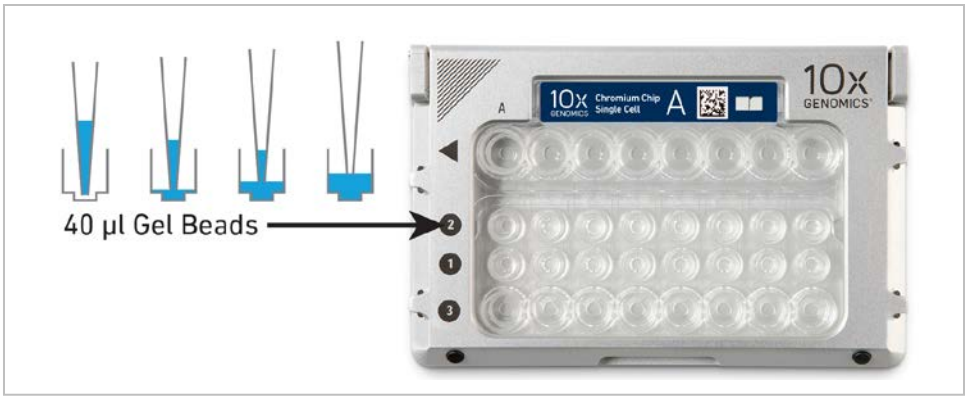
Pipette Single Cell 3' Gel Beads slowly as they have a viscosity similar to high-concentration glycerol.

i) Carefully puncture the foil seal and slowly aspirate **40 µl** Single Cell 3' Gel Beads, taking care not to introduce air bubbles.

NOTE

Only puncture the foil of a number of wells in the Single Cell 3' Gel Bead Strip equal to the number of samples that will be processed.

j) Slowly dispense the Single Cell 3' Gel Bead suspension **into the bottom of the wells in the row labeled 2**, taking care not to introduce bubbles. To do this, place the tips into the bottom center of the wells and raise the tips slightly above the bottom before slowly dispensing the Gel Beads.



CRITICAL!

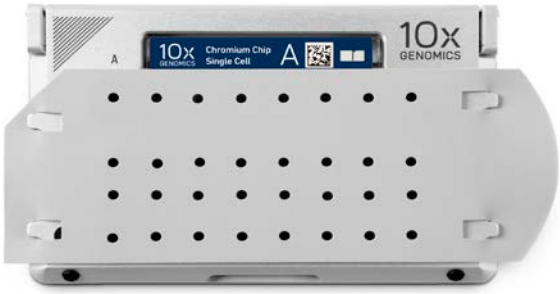
Raise the pipette tips at the same rate as the liquid level is rising in the wells, keeping the tip slightly submerged. Confirm that the pipette tips do not contain leftover Gel Beads. If necessary, wait for the remaining Gel Beads to drain into the bottom of the pipette tips and dispense into the wells without introducing bubbles.

k) Pipette a total volume of **270 µl** of Partitioning Oil into the wells in the **row labeled 3 by pipetting two aliquots of 135 µl** from a reagent reservoir. Do not add Partitioning Oil to any unused input wells that already contain 50% glycerol solution.

CRITICAL!

Failure to add Partitioning Oil can damage the Chromium Controller or the Chromium Single Cell Controller.

l) Attach the 10x Gasket. The notched cut should be at the top left corner. Ensure the 10x Gasket holes are aligned with the wells. Avoid touching the smooth side of the 10x Gasket and do not press down on the top of the 10x Gasket after attachment. Keep the assembly horizontal to avoid wetting the 10x Gasket with Partitioning Oil.



1.3. Running the Chromium Controller

NOTE

The same instructions apply to the Chromium Single Cell Controller.

a) Press the button on the touchscreen of the Chromium Controller to eject the tray.

b) Place the assembled Chip, 10x Chip Holder and 10x Gasket on the tray.

c) Press the button on the touchscreen again to retract the tray. Confirm the Chromium Chip A Single Cell program shows on screen and press the play button to begin the run.

d) At the completion of the run (**~6.5 min**), the Chromium Controller will chime. Proceed immediately to the next step.

Place the assembled Chip, 10x Chip Holder and 10x Gasket in the tray and press the button on the touchscreen to retract the tray

Confirm the Chromium Chip A Single Cell program shows on the screen and press the play button to start the run

1.4. Transferring GEMs

a) Maintain an emulsion-safe PCR 8-tube strip or PCR plate for GEM transfer on a chilled metal block resting on ice. (See Tips section for more information on emulsion-safe plastic consumables).

b) Press the eject button to eject the tray and remove the Chip A Single Cell. Remove and discard the 10x Gasket. Press the button to retract the empty tray (or the tray will automatically close after 40 sec).

c) Open the 10x Chip Holder and fold the lid back until it clicks to expose the wells at a 45-degree angle.

d) Check for volume uniformity in the Gel Bead, Sample, and Partitioning Oil wells remaining in the Chip A Single Cell.

NOTE

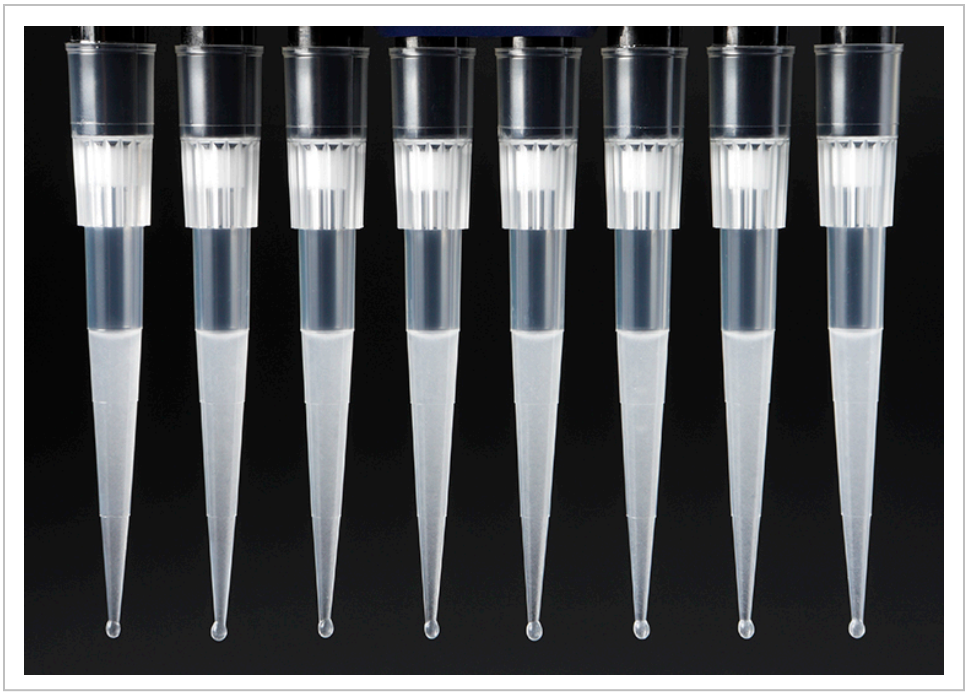
Abnormally high volume in any of the wells may indicate that a clog occurred during GEM generation.

e) Slowly aspirate **100 µl** GEMs from the lowest points of the Recovery Wells (**row labeled ◀**) without creating a seal between the tips and the bottom of the wells. Avoid introducing air bubbles.

NOTE

Pipette GEMs slowly as they have a high viscosity. If a tip aspirates excessive air the sample may be compromised.

f) Withdraw pipette tips from the wells and verify that there is no air in the tips. GEMs should appear opaque and uniform across all channels.



NOTE

The presence of excess Partitioning Oil (clear) in the pipette tips indicates a potential clog during GEM generation.

g) Over the course of ~20 sec, dispense the GEMs into the emulsion-safe tube strip or plate on a chilled metal block resting on ice with the pipette tips **against the sidewalls of the wells**. (See Practical Tips & Troubleshooting, Section 5). Keep the tips above the liquid level to minimize GEMs lost on the outside of the tips.

NOTE

Check the volume uniformity of the GEMs and the Partitioning Oil in the tube strip or plate. A clog occurred if the Partitioning Oil volume in one or more wells is increased compared to other wells. See Practical Tips & Troubleshooting for more information (Section 5).

h) If multiple Chip A Single Cell are run back-to-back, cap the GEM containing tube strip or seal the plate wells with a cap strip and maintain on ice before proceeding to load reagents into the next chip. Avoid storing the GEMs on ice for more than 1 h.

i) Discard the used Chip A Single Cell. Push the black sliders on the back of the 10x Chip Holder toward the middle to release the lock and close the lid.

1.5. GEM-RT Incubation

a) If GEMs have been dispensed into a tube strip, continue with step c. If using a plate, remove the cap strips from the plate. Check that the Plate Sealer plate block is at room temperature.

b) Seal the plate with pierceable foil heat seal at **185°C** for **6 sec** and promptly remove.

c) Load the capped tube strip or sealed plate into a thermal cycler that can accommodate at least 100 µl reaction volume and proceed with the following incubation protocol.

NOTE

A reaction volume of 125 µl is the preferred setting on the Bio-Rad C1000 Touch Thermal Cycler. If using an alternate thermal cycler, the highest reaction volume setting should be used.

Lid Temperature	Reaction Volume	Run Time
53°C	125 µl	~55 min
Step	**Temperature**	**Time**
1	53°C	45:00
2	85°C	5:00
3	4°C	Hold

STOP

d) Store in the tube strip or in the plate at **4°C** for up to **72 h** or at **–20°C** for up to a **week**, or proceed directly to Post GEM-RT Cleanup.

Protocol Step 2

Post GEM-RT Cleanup & cDNA Amplification

Isolate and amplify cDNA for library construction

2

2. Post GEM-RT Cleanup & cDNA Amplification

Tips

Best Practices

Ensure that the reagents are fully thawed and thoroughly mixed before use. During the bead-based cleanup steps, ensure that the samples are thoroughly mixed with the Silane beads or the SPRIselect Reagent to achieve optimal recovery. Always use fresh preparations of 80% Ethanol.

Best Practices – Pipette Calibration & Maintenance

Pipettes are sensitive instruments. In order to maintain pipette accuracy, the manufacturer's calibration and preventative maintenance schedules should be followed. Pipette accuracy is particularly important in SPRIselect Cleanups.

Best Practices – SPRIselect Cleanups

After aspirating the desired volume of SPRIselect Reagent, examine the pipette tip(s) before dispensing to ensure that the correct volume is being transferred. Pipette mix thoroughly as insufficient mixing of sample and SPRIselect Reagent will lead to inconsistent results. Always use fresh preparations of 80% Ethanol. See Practical Tips & Troubleshooting (Section 5) for more information.

Best Practices – Post cDNA Amplification Reaction QC

Agilent Bioanalyzer analysis is the recommended method for ensuring successful cDNA amplification before proceeding into library construction. Accurate quantification of cDNA at this step is necessary to determine Total Sample Index Cycles for the Sample Index PCR (step 3.5).

Getting Started!

Equilibrate to room temperature before use:

Item	Part Number	Storage Location
Dynabeads MyOne SILANE *Vortex thoroughly to resuspend beads immediately before use*	2000048	4°C
Additive A *Vortex, verify no precipitate, centrifuge briefly*	220074	–20°C
cDNA Additive *Vortex, centrifuge briefly*	220067	–20°C
cDNA Primer Mix *Vortex, centrifuge briefly*	220106	–20°C
Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendation
Agilent Bioanalyzer High Sensitivity Kit *If used for QC and quantification*	-	Manufacturer's recommendation
Agilent TapeStation ScreenTape and Reagents *If used for QC and quantification*	-	Manufacturer's recommendation
Qubit dsDNA HS Assay Kit *If used for quantification*	-	Manufacturer's recommendation

Place on ice:

Item	Part Number	Storage Location
Amplification Master Mix *Maintain on ice, Centrifuge briefly before adding to Master Mix*	220125 or 220129	–20°C

Obtain:

Item	Part Number	Storage Location
Recovery Agent	220016	Ambient temperature
Qiagen Buffer EB	-	Manufacturer's recommendation
Bio-Rad 10% Tween 20	-	Manufacturer's recommendation
10x Magnetic Separator *Ensure that a pellet forms after placing the tube strip into a 10x Magnetic Separator in the Low position. If not, place in the High position.*	120250	Ambient temperature

Thaw at 65°C:

Item	Part Number	Storage Location
Buffer Sample Clean Up 1 *Thaw for 10 min at 65°C at max speed on a thermomixer. Verify there are no visible crystals. Let cool to room temperature. Centrifuge briefly*	220020	–20°C

Prepare 80% Ethanol (15 ml for 8 samples)

Post GEM-RT Cleanup & cDNA Amplification

2.1. Post GEM-RT Cleanup – Silane Dynabeads

NOTE

If stored at -20°C, thaw the PCR 8-tube strip or the PCR plate at room temperature for 10 min (this will result in a translucent rather than clear aqueous phase), before proceeding directly into Post GEM-RT Cleanup.

a) Add **125 µl** Recovery Agent to each well of the PCR 8-tube strip containing post incubation GEMs. Wait **60 sec.** Do not pipette mix or vortex the biphasic mixture.

If using a PCR plate, remove the foil seal, add **125 µl** Recovery Agent to each well containing post incubation GEMs. Do not pipette mix or vortex the biphasic mixture. Wait **60 sec** and then transfer the entire volume to a tube strip.

NOTE

If using a plate, after transferring the initial volume to a tube strip, aqueous phase recovery can be maximized by lightly sealing the plate with a Microseal® 'B' Adhesive Seal and spinning in a plate centrifuge at 1200 rpm for 30 sec. The collected volumes can be combined with those previously transferred into the tube strip.

b) The recovered biphasic mixture contains distinct Recovery Agent/Partitioning Oil (pink) and aqueous phases (clear), with no persisting emulsion (opaque).

NOTE

A decrease in the aqueous phase indicates that a clog occurred during GEM generation.

c) Slowly remove **125 µl** Recovery Agent/Partitioning Oil (pink) from the bottom of the tubes and discard. Be careful not to aspirate any of the clear aqueous sample.

A small volume of Recovery Agent/Partitioning Oil will remain.

NOTE

Do not aspirate the aqueous solution during Recovery Agent/Partitioning Oil removal. Should aspiration of the aqueous solution occur, return the solution to the tube strip, reduce removal volume by 5 µl, and reattempt removal.

d) Vortex Dynabeads until fully resuspended. Prepare Dynabeads Cleanup Mix by adding reagents in the order shown below. Vortex mix thoroughly.

Dynabeads Cleanup Mix	PN	1X (µl)	4 rxns + 10% xs (µl)	8 rxns + 10% xs (µl)
Nuclease-free Water	-	9	39	79
Buffer Sample Clean Up 1	220020	182	801	1602
Dynabeads MyOne SILANE	2000048	4	18	35
Additive A	220074	5	22	44
Total	-	**200**	**880**	**1760**

e) Add **200 µl** Dynabeads Cleanup Mix to each sample. Pipette mix 5 times (pipette set to 200 µl) and incubate at room temperature for **10 min**.

f) Prepare Elution Solution I by adding reagents in the order shown below. Vortex mix thoroughly and centrifuge briefly.

Elution Solution I	PN	1 rxn (µl)	10 rxns (µl)
Buffer EB	-	98	980
10% Tween 20	-	1	10
Additive A	220074	1	10
Total	-	**100**	**1000**

g) After the 10 min incubation step is complete, place the tube strip into a 10x Magnetic Separator in the **High** position until the supernatant is clear.

NOTE

A white interface may appear between the aqueous solution and Recovery Agent layers. This is normal.

h) Carefully remove and discard the supernatant.

i) Add **150 µl** freshly prepared 80% ethanol **twice** to the pellet while on the magnet for a total volume of 300 µl and stand for **30 sec.**

j) Carefully remove and discard the ethanol wash.

k) Add **200 µl** 80% ethanol to the pellet and stand for **30 sec**.

l) Carefully remove and discard the ethanol wash.

m) Centrifuge the tube strip briefly and return it to a 10x Magnetic Separator in the **Low** position.

n) Remove and discard any remaining ethanol and allow the samples to air dry for **1 min**.

o) Remove the tube strip from the magnet and add **35.5 µl** Elution Solution I. Pipette mix thoroughly until beads are fully resuspended (pipette set to 30 µl to avoid introducing air bubbles).

NOTE

Silane Dynabeads can be difficult to resuspend due to residual reagents from the GEM reaction. To aid resuspension, the tube strip can be capped, vortexed for 5 sec and then centrifuged briefly prior to incubation.

p) Incubate at room temperature for **1 min**.

q) Place the tube strip in a 10x Magnetic Separator in the **Low** position until the solution is clear.

r) Transfer **35 µl** of purified GEM-RT product to a new tube strip.

2.2. cDNA Amplification Reaction

a) Prepare cDNA Amplification Reaction Mix on ice. Add reagents in the order shown below. Mix thoroughly and centrifuge briefly. **Do not add Purified GEM-RT Product at this point.**

cDNA Amplification Reaction Mix	PN	1 rxn (µl)	4 rxns + 10% xs (µl)	8 rxns + 10% xs (µl)
Nuclease-free Water	-	8	35	70
Amplification Master Mix	220125 or 220129	50	220	440
cDNA Additive	220067	5	22	44
cDNA Primer Mix	220106	2	9	18
Total	**-**	**65**	**286**	**572**

b) Add **65 µl** cDNA Amplification Reaction Mix to each tube containing **35 µl** of purified GEM-RT product.

c) Pipette mix 15 times (pipette setting 90 µl) and centrifuge briefly.

d) Cap and load the tube strip into a thermal cycler that can accommodate at least 100 µl reaction volume and proceed with the following incubation protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~30-45 min
Step	**Temperature**	**Time**
1	98°C	3:00
2	98°C	0:15
3	67°C	0:20
4	72°C	1:00
5	Go to Step 2, see table below for # of cycles	
6	72°C	1:00
7	4°C	Hold

NOTE

The optimal number of cycles for the cDNA amplification reaction is a trade-off between generating sufficient mass for the subsequent library construction steps and minimizing PCR amplification artifacts. If large numbers of cells are sampled, the total number of cDNA amplification cycles should be reduced. The following table is a recommended starting point for optimization.

Targeted Cell Recovery	Total cDNA Amplification Cycles
<2000	14
2000 – 6000	12
6000 – 10000	10
>10000	8

STOP

e) Store the samples at **4°C** in a tube strip for up to **72 h** or proceed directly to SPRIselect Cleanup.

2.3. Post cDNA Amplification Reaction Cleanup – SPRIselect

NOTE

See Practical Tips & Troubleshooting (Section 5) for more information on calculating SPRIselect Reagent ratios.

a) Vortex the SPRIselect Reagent until fully resuspended. Add **60 µl** SPRIselect Reagent (**0.6X**) to each sample in the tube strip and pipette mix 15 times (pipette set to 150 µl).

b) Incubate the tube strip at room temperature for **5 min**.

c) Place the tube strip in a 10x Magnetic Separator in the **High** position until the solution is clear.

d) Carefully remove and discard the supernatant.

e) Add **200 µl** 80% ethanol to the pellet and stand for **30 sec**.

f) Carefully remove and discard the ethanol wash.

Repeat

g) **Repeat** steps e and f for a total of 2 washes.

h) Centrifuge the tube strip briefly and return it to a 10x Magnetic Separator in the **Low** position.

i) Remove and discard any remaining ethanol and allow the samples to air dry for **2 min**. **Do not exceed 2 min** as this will lead to decreased elution efficiency.

j) Remove the tube strip from the 10x Magnetic Separator and add **40.5 µl** Buffer EB.

k) Pipette mix 15 times and incubate at room temperature for **2 min**.

l) Place the tube strip in a 10x Magnetic Separator in the **High** position until the solution is clear.

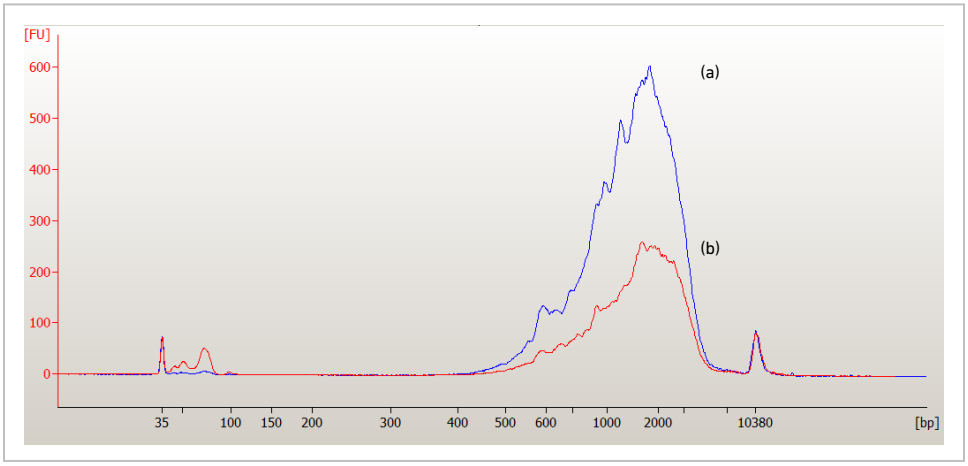
m) Transfer **40 µl** of sample to a new tube strip and cap the sample wells.

STOP

n) Store the samples at **4°C** in a tube strip for up to **72 h** or at **–20°C** for up to a **week**, or proceed directly to Post cDNA Amplification QC & Quantification.

2.4. Post cDNA Amplification Reaction QC & Quantification

a) Run **1 µl** of sample at a dilution of 1 part sample:5 parts nuclease-free water on the Agilent Bioanalyzer D5000 ScreenTape High Sensitivity chip for qualitative analysis (trace a). Traces should resemble the overall shape of the sample electropherograms shown below.



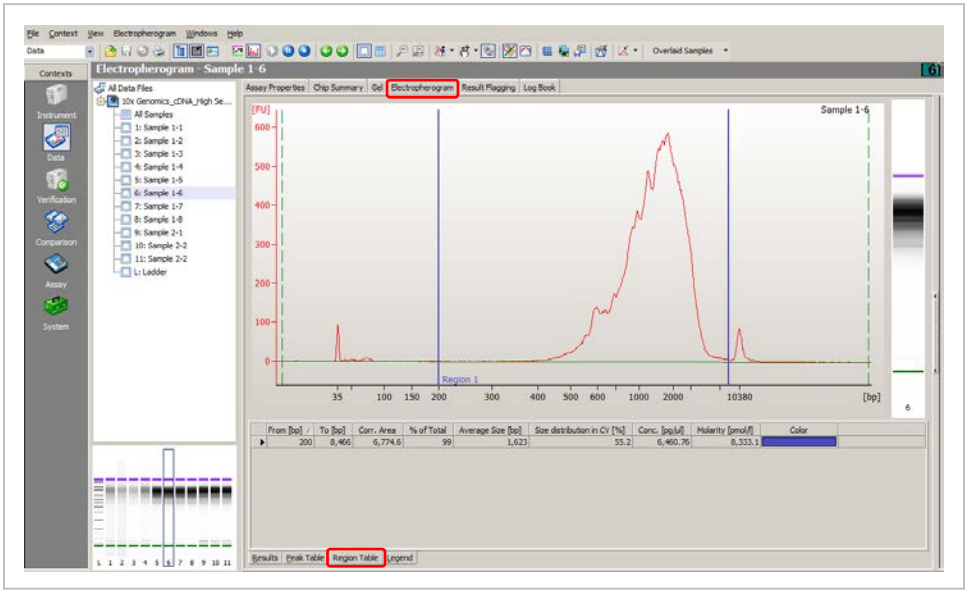
NOTE

If the input cells are particularly RNA-poor (<1pg total RNA/cell), it may be necessary to run 1 µl of undiluted product (trace b). Lower molecular weight product (35 – 150 bp) may be present. This is normal and does not affect sequencing or application performance.

See Practical Tips & Troubleshooting (Section 5) for more information on using the Agilent TapeStation for cDNA quantification.

b) To determine the cDNA yield per sample, under the "Electropherogram" view choose the "Region Table" tab on the Agilent 2100 Expert Software.

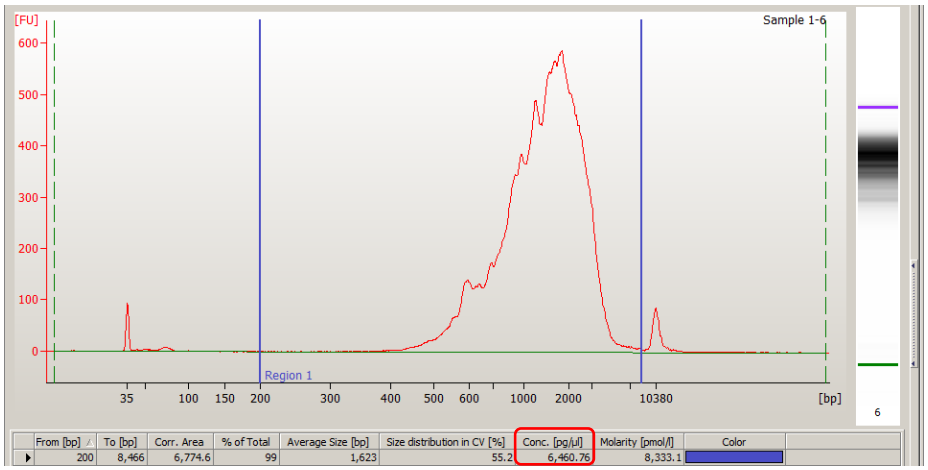
c) Manually select the region encompassing ~200 – ~9000 bp.



d) 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.

NOTE

This mass will be used in step 3.5 to determine the appropriate number of Sample Index PCR cycles to generate sufficient concentration of final library, while minimizing PCR amplification artifacts.



Example Calculation of cDNA Total Yield:

Agilent 2100 Expert Concentration: 6460.76 [pg/µl]

Dilution Factor used to run the Agilent 2100 Bioanalyzer: 6

Post cDNA Amplification Clean Up Elution Volume: 40 µl

Total cDNA Yield:

$$= \frac{\text{Concentration}\left(\frac{\text{pg}}{\mu\text{l}}\right) \times \text{Elution Volume } (\mu\text{l}) \times \text{Dilution Factor}}{1000\ \left(\frac{\text{pg}}{\text{ng}}\right)}$$

$$= \frac{6460.76\left(\frac{\text{pg}}{\mu\text{l}}\right) \times 40\ (\mu\text{l}) \times 6}{1000\ \left(\frac{\text{pg}}{\text{ng}}\right)}$$

= 1550 ng

Refer to step 3.5 for the appropriate number of Sample Index PCR cycles.

Calculation of cDNA Total Yield Using a Qubit 3.0 Fluorometer:

cDNA Total Yield may also be calculated using the Qubit Fluorometer and Qubit dsDNA HS Assay Kit. Quantification using this method includes all material within the sample. Multiply the cDNA concentration [ng/µl] reported via the Qubit Fluorometer by the elution volume (40 µl) of the Post cDNA Amplification Reaction Clean Up sample to obtain the total cDNA yield in ng. To determine the equivalent range using the Agilent 2100 Expert Software, select the region encompassing 35 – 10000 bp in step c.

Refer to step 3.5 for the appropriate number of Sample Index PCR cycles.

Protocol Step 3

Library Construction

Insert P5, P7, Read2, and Sample Index to prepare for sequencing

3

3. Library Construction

Tips

General

The final Single Cell 3' Libraries contain the P5 and P7 primers used in Illumina bridge amplification PCR. The 10x Barcode and Read 1 (primer site for sequencing read 1) is added to the molecules during the GEM-RT incubation. The P5 primer, Read 2 (primer site for sequencing read 2), Sample Index and P7 primer will be added during library construction. The Protocol is designed to support library construction from a wide range of cDNA amplification yields spanning at least 2 ng to >2 µg without modification.

Best Practices – Reagents

Ensure that the reagents are fully thawed and thoroughly mixed before use. Keep all enzyme components and Master Mixes on ice during setup and promptly move back to the recommended storage temperature when possible.

Best Practices – Enzymatic Fragmentation

Ensure that Enzymatic Fragmentation reactions are prepared on a chilled metal block resting 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.

Best Practices – Pipette Calibration & Maintenance

Pipettes are sensitive instruments. In order to maintain pipette accuracy, the manufacturer's calibration and preventative maintenance schedules should be followed. Pipette accuracy is particularly important in SPRIselect Double Sided Size Selections and Cleanups.

Best Practices – SPRIselect Cleanups & Double Sided Size Selections

After aspirating the desired volume of SPRIselect Reagent, examine the pipette tip(s) before dispensing to ensure that the correct volume is being transferred. Pipette mix thoroughly as insufficient mixing of sample and SPRIselect Reagent will lead to inconsistent results. Always use fresh preparations of 80% Ethanol. See Practical Tips & Troubleshooting (Section 5) for more information.

Best Practices – Sample Index PCR

Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Each sample index set is base-balanced to avoid monochromatic signal issues when it is the sole sample loaded on an Illumina sequencer. The sample index sets can therefore be used in any combination.

Getting Started!

Equilibrate to room temperature before use:

Item	Part Number	Storage Location
Fragmentation Buffer *Vortex, verify no precipitate, centrifuge briefly*	220108	–20°C
Ligation Buffer *Vortex, verify no precipitate, centrifuge briefly*	220109	–20°C
Adaptor Mix	220026	–20°C
SI-PCR Primer	220111	–20°C
Chromium i7 Sample Index Plate	220103	–20°C
Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendation
Agilent Bioanalyzer High Sensitivity Kit *If used for QC*	-	Manufacturer's recommendation
Agilent TapeStation ScreenTape and Reagents *If used for QC*	-	Manufacturer's recommendation

Obtain:

Item	Part Number	Storage Location
Qiagen Buffer EB	-	Manufacturer's recommendation
10x Magnetic Separator *Ensure that a pellet forms after placing the tube strip into a 10x Magnetic Separator in the Low position. If not, place in the High position.*	120250	Ambient temperature

Place on ice:

Item	Part Number	Storage Location
Fragmentation Enzyme Blend *Maintain on ice, centrifuge briefly before adding to Fragmentation Mix*	220107 or 220130	–20°C
DNA Ligase *Maintain on ice, centrifuge briefly before adding to Adaptor Ligation Mix*	220110 or 220131	–20°C
Amplification Master Mix *Maintain on ice, centrifuge briefly before adding to Sample Index PCR mix*	220125 or 220129	–20°C
Chilled Metal Block	-	-
KAPA DNA Quantification Kit for Illumina Platforms	-	Manufacturer's recommendation

Prepare 80% Ethanol (20 ml for 8 samples)

Library Construction

3.1. Fragmentation, End Repair & A-tailing

NOTE

*It is important to ensure Enzymatic Fragmentation reactions are prepared on a chilled metal block resting on ice and are inserted into a pre-cooled (**4°C**) thermal cycler.*

a) Prepare a thermal cycler with the following incubation protocol and initiate the **4°C** pre-cool block step prior to assembling the Fragmentation Mix.

Lid Temperature	Reaction Volume	Run Time
65°C	50 µl	~35 min
Step	**Temperature**	**Time**
Pre-cool block	4°C	Hold
Fragmentation	32°C	5:00
End Repair & A-tailing	65°C	30:00
Hold	4°C	Hold

b) Vortex the Fragmentation Buffer. Verify there is no precipitate before proceeding.

c) Prepare the Fragmentation Mix on ice. Add the reagents in the order shown below. Mix thoroughly and centrifuge briefly.

Fragmentation Mix	PN	1 rxn (µl)	4 rxns + 10% xs (µl)	8 rxns + 10% xs (µl)
○ Fragmentation Buffer	220108	5	22	44
● Fragmentation Enzyme Blend	220107 or 220130	10	44	88
Total	-	**15**	**66**	**132**

d) Dispense **15 µl** Fragmentation Mix into each well of a PCR 8-tube strip on a chilled metal block resting on ice.

e) Add **35 µl** purified cDNA to each well of the tube strip containing the Fragmentation Mix. Pipette mix 15 times (pipette set to 35 µl) and centrifuge briefly before returning the tube strip to a chilled metal block resting on ice.

f) Transfer the chilled tube strip into the pre-cooled thermal cycler (**4°C**) and press "SKIP" to initiate the Fragmentation protocol.

g) After the Fragmentation protocol is complete, proceed directly to step 3.2.

3.2. Post Fragmentation, End Repair & A-tailing Double Sided Size Selection – SPRIselect

See Practical Tips & Troubleshooting (Section 5.8) for more information on calculating SPRIselect Reagent ratios.

NOTE

After aspirating the desired volume of SPRIselect Reagent, examine the pipette tip(s) before dispensing to ensure that the correct volume is being transferred. Pipette mix thoroughly as insufficient mixing of sample and SPRIselect Reagent will lead to inconsistent size selection results. See.

a) Vortex the SPRIselect Reagent until fully resuspended. Add **30 µl** SPRIselect Reagent (**0.6X**) to each sample in the tube strip and pipette mix 15 times (pipette set to 75 µl).

b) Incubate the tube strip at room temperature for **5 min**.

c) Place the tube strip in a 10x Magnetic Separator in the **High** position until the solution is clear.

CRITICAL!

DO NOT discard supernatant.

d) Transfer **75 µl** supernatant to a new tube strip and discard the previous tube strip.

e) Add **10 µl** SPRIselect Reagent (**0.8X**) to each sample in the tube strip and pipette mix 15 times (pipette set to 75 µl).

f) Incubate the tube strip at room temperature for **5 min**.

g) Place the tube strip in a 10x Magnetic Separator in the **High** position until the solution is clear.

h) Carefully remove and discard **80 µl** supernatant.

NOTE

Due to the low volume of the SPRIselect Reagent used in this step it is crucial to not discard any of the beads with the supernatant. To ensure that beads are not discarded 5 µl of supernatant is left in each well of the tube strip. This will not impact the effectiveness of the size selection.

i) With the tube strip still in a 10x Magnetic Separator, add **125 µl** 80% ethanol to the pellet and stand for **30 sec**.

j) Carefully remove and discard the ethanol wash.

Repeat

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

l) Briefly centrifuge the tube strip and return it to the 10x Magnetic Separator in the **Low** position. Carefully remove and discard the remaining ethanol wash.

NOTE

Due to the low volume of SPRIselect Reagent used in this size selection it is crucial not to over-dry the beads. Proceed directly into resuspension with Buffer EB without waiting for the beads to dry to ensure maximum elution efficiency.

m) Remove the tube strip from the 10x Magnetic Separator and add **50.5 µl** Buffer EB. Pipette mix 15 times.

n) Incubate the tube strip at room temperature for **2 min**.

o) Place the tube strip in a 10x

p) Magnetic Separator in the **High** position until the solution is clear.

q) Transfer **50 µl** of sample to a new tube strip and cap the sample wells.

3.3. Adaptor Ligation

a) Prepare Adaptor Ligation Mix by adding the reagents in the order shown below. Mix thoroughly and centrifuge briefly.

Adaptor Ligation Mix	PN	1 rxn (µl)	4 rxns + 10% xs (µl)	8 rxns + 10% xs (µl)
Nuclease-free Water	-	17.5	77	154
Ligation Buffer	220109	20	88	176
DNA Ligase	220110 or 220131	10	44	88
Adaptor Mix	220026	2.5	11	22
Total	-	**50**	**220**	**440**

b) Add **50 µl** Adaptor Ligation Mix to each tube containing **50 µl** sample from the Post Fragmentation, End Repair, and A-tailing Size Selection. Pipette mix 15 times (pipette set to 50 µl) and 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	15:00

d) Proceed immediately to the next step.

3.4. Post Ligation Cleanup – SPRIselect

NOTE

See Practical Tips & Troubleshooting (Section 5) for more information for more information on calculating SPRIselect Reagent ratios.

a) Vortex the SPRIselect Reagent until fully resuspended. Add **80 µl** SPRIselect Reagent (**0.8X**) to each sample in the tube strip and pipette mix 15 times (pipette set to 150 µl).

b) Incubate the tube strip at room temperature for **5 min**.

c) Place the tube strip in a 10x

d) Magnetic Separator in the **High** position until the solution is clear.

e) Carefully remove and discard the supernatant.

f) Add **200 µl** 80% ethanol to the pellet and stand for **30 sec**.

g) Carefully remove and discard the ethanol wash.

Repeat

h) **Repeat** steps e and f for a total of 2 washes.

i) Centrifuge the tube strip briefly and return it to a 10x Magnetic Separator in the **Low** position.

j) Remove and discard any remaining ethanol and allow the samples to air dry for **2 min**. Do not exceed 2 min as this will lead to decreased elution efficiency.

k) Remove the tube strip from the 10x Magnetic Separator and add **30.5 µl** Buffer EB. Pipette mix 15 times.

l) Incubate the tube strip at room temperature for **2 min**.

m) Place the tube strip in a 10x Magnetic Separator in the **Low** position until the solution is clear.

n) Transfer **30 µl** of sample to a new tube strip.

3.5. Sample Index PCR

NOTE

Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run.

CRITICAL!

Record the 10x Sample Index name (PN-220103 Chromium i7 Sample Index Plate well ID) used, especially if running more than one sample.

a) Prepare Sample Index PCR Mix by adding the reagents in the order shown below. Mix thoroughly and centrifuge briefly.

Sample Index PCR Mix	PN	1 rxn (µl)	4 rxns + 10% xs (µl)	8 rxns + 10% xs (µl)
Nuclease-free Water	-	8	35	70
Amplification Master Mix	220125 or 220129	50	220	440
SI-PCR Primer	220111	2	9	18
Total	-	**60**	**264**	**528**

b) Add **60 µl** Sample Index PCR Mix to each tube containing **30 µl** purified Post Ligation sample.

c) Add **10 µl** of an individual Chromium i7 Sample Index to each well and record their assignment. Pipette mix 15 times (pipette set to 90 µl) and centrifuge briefly.

d) Index the library DNA 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	0:45
2	98°C	0:20
3	54°C	0:30
4	72°C	0:20
5	Go to step 2, see table below for # of cycles	
6	72°C	1:00
7	4°C	Hold

NOTE

The optimal number of cycles for the Sample Index PCR reaction is a trade-off between generating sufficient final mass for sequencing and minimizing PCR amplification artifacts.

The following table is a recommended starting point for optimization. Input into Library Construction refers to the cDNA quantification result from step 2.4.

Input into Library Construction	Total Sample Index Cycles
1 – 25 ng	14 - 16
25 – 150 ng	12 - 14
150 – 500 ng	10 - 12
500 – 1000 ng	8 - 10
1000 – 1500 ng	6 - 8
>1500 ng	5

e) Store the tube strip at **4°C** for up to **72 h** or proceed directly to Post Sample Index PCR Double Sided Size Selection.

3.6. Post Sample Index PCR Double Sided Size Selection – SPRIselect

NOTE

See Practical Tips & Troubleshooting (Section 5.8) for more information on calculating SPRIselect Reagent ratios.

After aspirating the desired volume of SPRIselect Reagent, examine the pipette tip(s) before dispensing to ensure that the correct volume is being transferred. Pipette mix thoroughly as insufficient mixing of sample and SPRIselect Reagent will lead to inconsistent size selection results.

a) Vortex the SPRIselect Reagent until fully resuspended. Add **60 µl** SPRIselect Reagent (**0.6X**) to each sample in the tube strip and pipette mix 15 times (pipette set to 150 µl).

b) Incubate the tube strip at room temperature for **5 min**.

c) Place the tube strip in a 10x Magnetic Separator in the **High** position until the solution is clear.

CRITICAL!

DO NOT discard supernatant.

d) Transfer **150 µl** supernatant to a new tube strip and discard the previous tube strip.

e) Add **20 µl** SPRIselect Reagent (**0.8X**) to each sample in the tube strip and pipette mix 15 times (pipette set to 150 µl).

f) Incubate the tube strip at room temperature for **5 min**.

g) Place the tube strip in a 10x Magnetic Separator in the **High** position until the solution is clear.

h) Carefully remove and discard **165 µl** supernatant.

NOTE

Due to the low volume of the SPRIselect Reagent used in this step it is crucial to not discard any of the beads with the supernatant. To ensure that beads are not discarded 5 µl of supernatant is left in each well of the tube strip. This will not impact the effectiveness of the size selection.

i) With the tube strip still in a 10x Magnetic Separator, add **200 µl** 80% ethanol to the pellet and stand for **30 sec**.

j) Carefully remove and discard the ethanol wash.

Repeat

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

l) Briefly centrifuge the tube strip and return it to the 10x Magnetic Separator in the **Low** position. Carefully remove and discard the remaining ethanol wash.

NOTE

Due to the low volume of SPRIselect Reagent used in this size selection it is crucial not to over-dry the beads. Proceed directly into resuspension with Buffer EB without waiting for the beads to dry to ensure maximum elution efficiency.

m) Remove the tube strip from the 10x Magnetic Separator and add **35.5 µl** Buffer EB. Pipette mix 15 times.

n) Incubate the tube strip at room temperature for **2 min**.

o) Place the tube strip in a 10x Magnetic Separator in the **Low** position until the solution is clear.

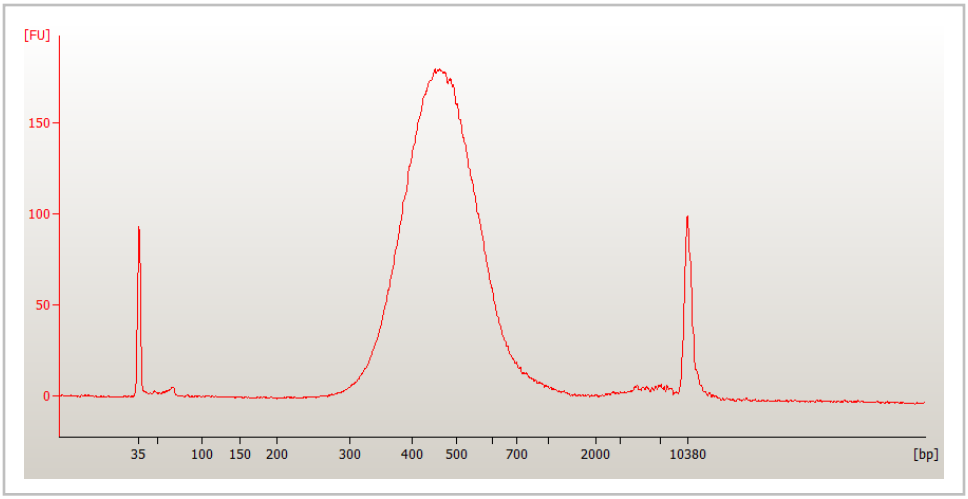
p) Transfer **35 µl** of sample to a new tube strip and cap the sample wells.

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

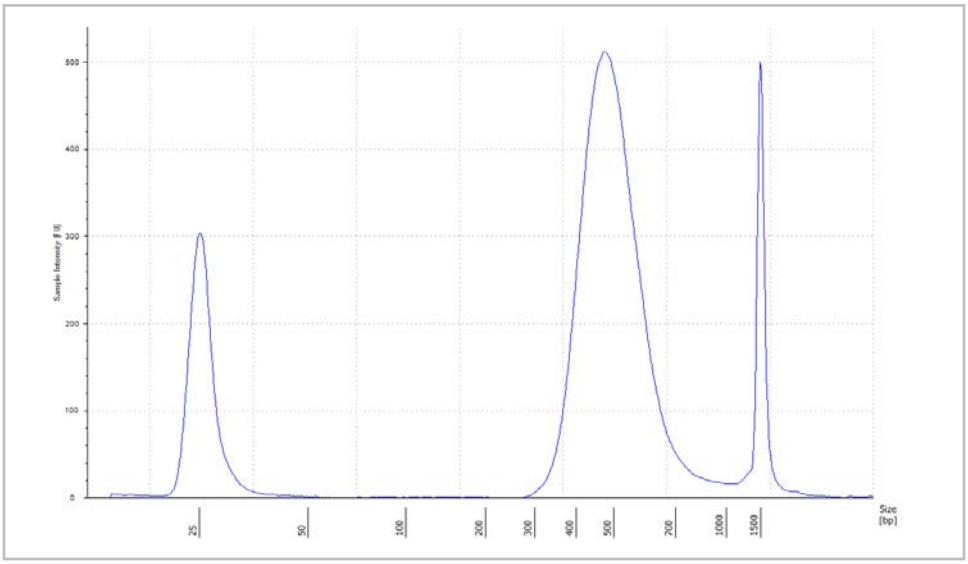
3.7. Post Library Construction QC

a) **EITHER** Run **1 µl** of sample at 1:10 dilution on the Agilent Bioanalyzer High Sensitivity chip for qualitative analysis. Traces should resemble the overall shape of the sample electropherogram shown below.

A 1:10 dilution ratio is typically sufficient to avoid over-loading the High Sensitivity DNA Chip. For samples of particularly RNA-rich cells, additional dilution may be required to QC the library.



b) **OR** Run **1 µl** of sample at 1:10 dilution on the Agilent TapeStation High Sensitivity D1000 ScreenTape for qualitative analysis. Traces should resemble the overall shape of the sample electropherogram shown below.



c) Determine the average fragment size from the Bioanalyzer/TapeStation trace. This value will be used as the insert size for accurate library quantification in qPCR (step 3.8).

3.8. Post Library Construction Quantification

NOTE

Typically a series of 1:40,000, 1:200,000, 1:1,000,000 and 1:5,000,000 of the completed Single Cell 3' library is required to fall within the dynamic range of the assay.

a) Thaw KAPA DNA Quantification Kit for Illumina Platforms.

b) Dilute **1 µl** of sample with nuclease-free water to appropriate dilutions that fall within the linear detection range of the KAPA DNA Quantification Kit.

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	**1 rxn (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** of sample dilutions and **4 µl** DNA Standards to appropriate wells. Centrifuge the PCR plate briefly.

f) Run DNA Quantification Cycling Protocol with data acquisition at Step 3.

Step	**Temperature**	**Time**
1	95°C	3:00
2	95°C	0:05
3	67°C	0:30
4	Go to Step 2, 29X (for 30 cycles in total)	

g) Follow the manufacturer's recommendations for qPCR analysis. The average fragment size derived from the Bioanalyzer/TapeStation trace from step 3.7 is used as the insert size for accurate library quantification in qPCR.

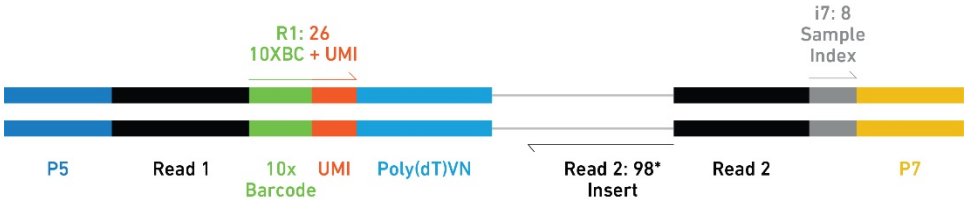
Protocol Step 4

Sequencing

Sequencing prepared libraries

4. Sequencing Libraries

A Single Cell 3' Library comprises standard Illumina paired-end constructs which begin and end with P5 and P7. The Single Cell 3' v2 16 bp 10x Barcodes are encoded at the start of Read 1, while sample index sequences are incorporated as the i7 index read. Read 1 and Read 2 are standard Illumina sequencing primer sites used in paired-end sequencing. Read 1 is used to sequence the 16 bp 10x Barcode and 10 bp UMI, while Read 2 is used to sequence the cDNA fragment.



Each sample index provided in the Chromium i7 Sample Index Kit combines 4 different sequences in order to balance across all 4 nucleotides.

4.1. Sequencing Depth Recommendations

a) The technical performance of Single Cell 3' libraries is driven by sequencing coverage per cell. 50000 raw reads per cell is recommended.

b) Adjust loading concentrations according to Illumina specifications.

c) The following are supported sequencing platforms for Single Cell 3' libraries.

Platform
MiSeq
NextSeq 500/550
HiSeq 2500 (Rapid Run and High Output)
HiSeq 3000/4000
Nova Seq

NOTE

Consult Chromium Single Cell 3' v2 Libraries – Sequencing Metrics for Illumina Sequencers (Document CG000089), Chromium Single Cell 3' v2 Libraries – Sequencing Performance on Illumina NextSeq 500 Flow Cells (Document CG000085) and Chromium Single Cell 3' v2 Libraries – Sequencing Metrics for Illumina NovaSeq (Document CG000120) for more information.

4.2. Sequencing Run Parameters

a) Single Cell 3' libraries use standard Illumina sequencing primers for both sequencing and index reads, and require no custom primers.

b) Single Cell libraries must be run using paired-end sequencing with single indexing. The dual-index configuration on the HiSeq 3000/4000 platform is not recommended for sequencing Single Cell 3' v2 libraries. The supported number of cycles for each read is shown below.

Sequencing Read	Recommended Number of Cycles
Read 1	26 cycles
i7 Index	8 cycles
i5 Index	0 cycles
Read 2	98 cycles

4.3. Sample Indices

a) Sample Indices are a mix of four oligos. The 10x Sample Index sequence is not needed for the sample sheet (required for generating FASTQs with cellranger mkfastq), but the 10x Sample Index name (Chromium i7 Sample Index plate well ID) is needed if running more than one sample.

4.4. Loading Single Cell 3' Libraries

Once quantified and normalized, Single Cell 3' Libraries should be denatured and diluted as recommended for Illumina sequencing platforms. Refer to Illumina documentation for Denaturing and Diluting Libraries.

NOTE

Consult Chromium Single Cell 3' v2 Libraries - Sequencing Metrics for Illumina Sequencers (Document CG000089) for more information.

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Practical Tips & Troubleshooting

- Processing Fewer than 8 Reactions
- Assembling a Chip, 10x Chip Holder & 10x Gasket
- Pipetting Gel Beads
- Pipetting GEMs
- 50% Glycerol Solution
- Post cDNA Amplification Reaction QC & Quantification with the Agilent TapeStation
- Post cDNA Amplification Reaction & Post Library Construction QC with the Fragment Analyzer
- SPRIselect Cleanups & Size Double Sided Selections
- Failure Modes during GEM Generation
- Chromium Controller Errors
- Glossary of Terms

5. Practical Tips & Troubleshooting

5.1. Processing Fewer than 8 Reactions

a) Puncture foil seals in the Gel Bead Strip as needed for a run.

b) Store any unused Gel Beads at –80°C and avoid more than 10 freeze-thaw cycles.

c) Never store Gel Beads at –20°C.

d) Reagent volumes should be calculated with a 10% excess of 1 rxn values quoted in the protocol. e.g. For 3 samples, multiply the 1 rxn volume quoted in the protocol by 3.3 to determine the suitable volume to prepare. Using larger reagent excesses may reduce the total number of reactions that can be run using one kit.

e) Store any unused RT Primer at –80°C for future use.

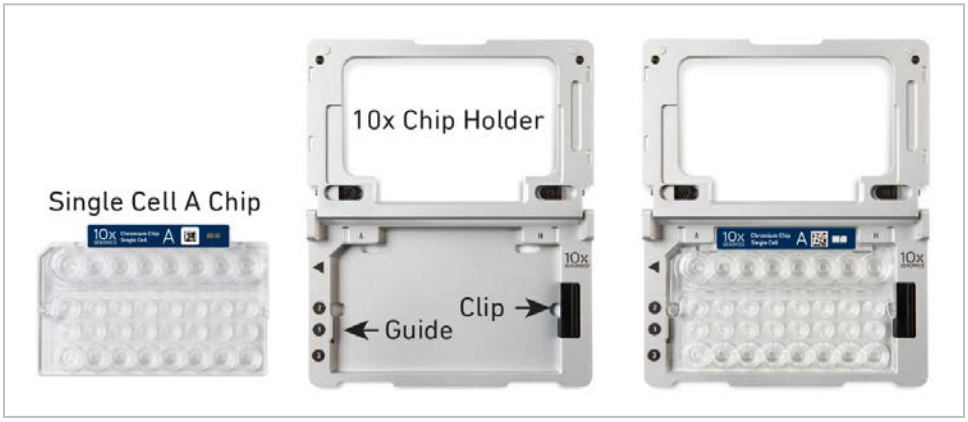
5.2. Assembling a Chip, 10x Chip Holder & 10x Gasket

NOTE

Always handle the Chromium Chip by its edges and avoid touching its bottom surface. Once the chip is in the holder, keep the assembly horizontal at all times to avoid wetting the 10x Gasket with Partitioning Oil.

a) Align the notch on the upper left corner of the Chromium Chip with the notch on the 10x Chip Holder and insert the left-hand side of the Chromium Chip under the guide.

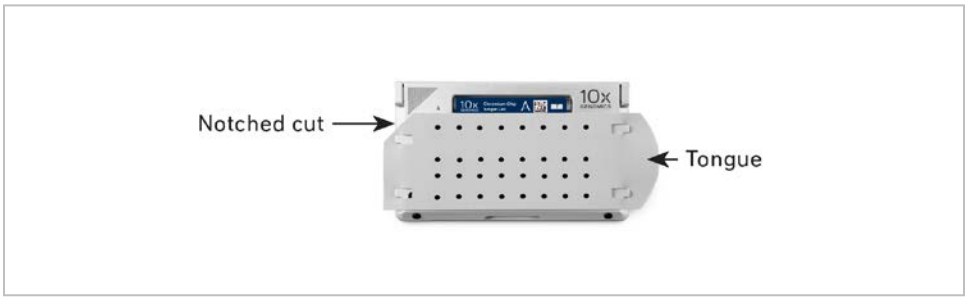
a) Depress the right-hand side of the Chromium Chip until the spring-loaded clip engages the Chromium Chip.



b) Close the hinged lid of the 10x Chip Holder. After loading the Chromium Chip, the 10x Chip Holder should lay flat on the bench top with the lid closed.

c) Position the assembly so that the Partitioning Oil wells (row labeled 3) are toward you and identify the rows labeled 1, 2 and 3 for correct addition of the reagents.

d) After the reagents have been added, attach a 10x Gasket by holding the tongue (curved end, to the right) and hook it on the left-hand tabs of the 10x Chip Holder. Gently pull the 10x Gasket toward the right and hook it on the two right-hand tabs. Avoid touching the smooth side of the 10x Gasket and do not press down on the top of the 10x Gasket after attachment. Keep the assembly horizontal to avoid wetting the 10x Gasket with Partitioning Oil.



Click back to Loading the Chip A Single Cell

5.3. Pipetting Gel Beads

a) After vortexing, remove the Gel Bead Strip from the 10x Vortex Adapter and flick the Gel Bead Strip in a sharp, downward motion to ensure maximum Gel Bead recovery. Confirm that there are no bubbles at the bottom of the tube.

b) Best practices for recovering adequate volume of Gel Beads from the Gel Bead Strip include the following:

   i. Set a pipette to the volume being pipetted and, without engaging the plunger, puncture the foil seal on the Gel Bead Strip. The pipette tips should extend no more than 2 mm below the seal.

   ii. Once the holes are formed, raise the pipette tips above the seal and engage the plunger.

   iii. Lower the tips to the bottom of the wells and widen the opening by gently rocking the tips back and forth, keeping the plunger engaged. Widening the foil seal opening allows the pipette tips to reach the bottom of the Gel Bead Strip wells. This is important for recovering the full volume of Gel Beads required for optimal performance.

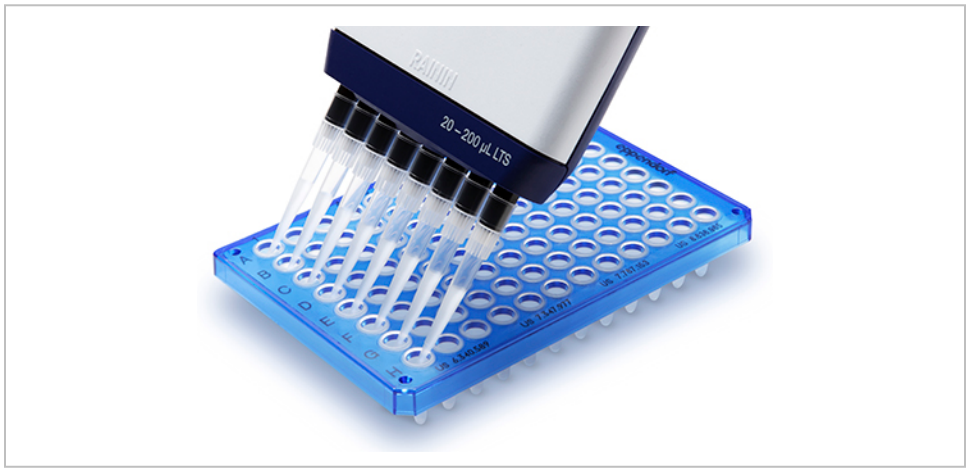
   iv. With the pipette tips still in the Gel Bead Strip, very slowly aspirate the required volume of Gel Beads. After aspiration stops, leave the pipette tips in the wells for an additional 5 sec to allow pressure to equilibrate.

c) If the required volume of beads cannot be recovered, place the pipette tips against the sidewalls of the Gel Bead Strip wells and slowly dispense the Gel Beads back into the strip. Take care not to introduce bubbles into the wells and verify that the pipette tips contain no leftover Gel Beads. Attempt to withdraw the full volume of beads again by pipetting slowly.

5.4. Pipetting GEMs

a) After the completion of a Chromium™ Controller run, the hinged lid of the 10x Chip Holder is folded back to expose the wells at a 45-degree angle. The GEMs should be aspirated from the lowest points of the Recovery Wells (row labeled ◀) without creating a seal between the tips and the bottom of the wells. Avoid introducing air bubbles.

b) When transferring the GEMs from the Chip A Single Cell after the Chromium Controller run into the tube strip or plate (on a chilled metal block resting on ice), the pipette tips should be positioned against the side walls of the wells as shown for a plate below.



Click back to Loading the Chip A Single Cell

5.5. 50% Glycerol Solution

It is critical to add glycerol in a ~50% volume/volume aqueous solution in all unused wells in Rows labeled 1, 2 and 3 of the Chip A Single Cell prior to running the Chromium Controller or the Chromium Single Cell Controller. 50% glycerol solution can be purchased: Ricca Chemical Company, Glycerin (glycerol), 50% (v/v) Aqueous Solution, PN-3290-32

Alternatively, 50% glycerol solution can be made from a stock solution of glycerol as follows:

a) Mix an equal volume of water and 99% Glycerol, Molecular Biology Grade.

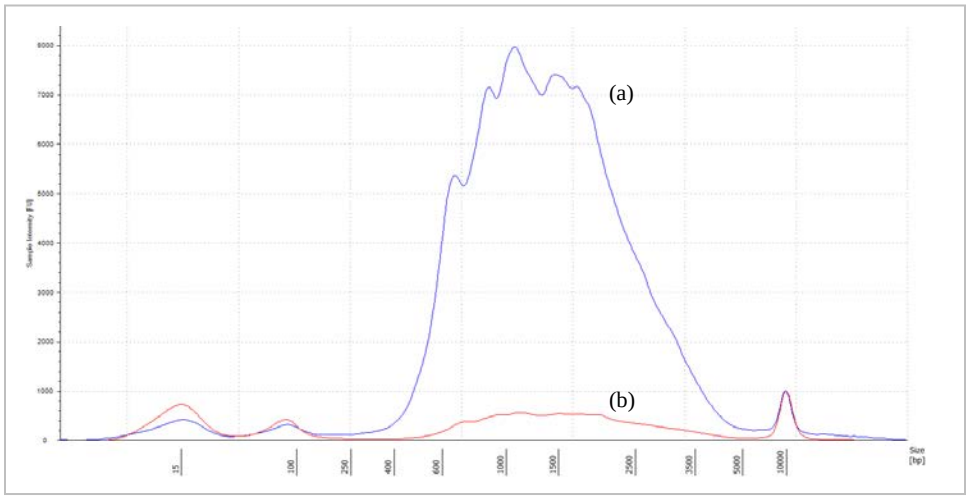
b) Filter through a 0.2 µm filter.

c) Store at –20°C in 1 ml LoBind tubes.

d) 50% glycerol solution should be equilibrated to room temperature before use.

5.6. Post cDNA Amplification Reaction QC & Quantification with the Agilent TapeStation

a) Run **1 µl** of undiluted sample on the Agilent TapeStation High Sensitivity D5000 ScreenTape for qualitative analysis. Traces should resemble the overall shape of the sample electropherogram shown below (traces a and b).

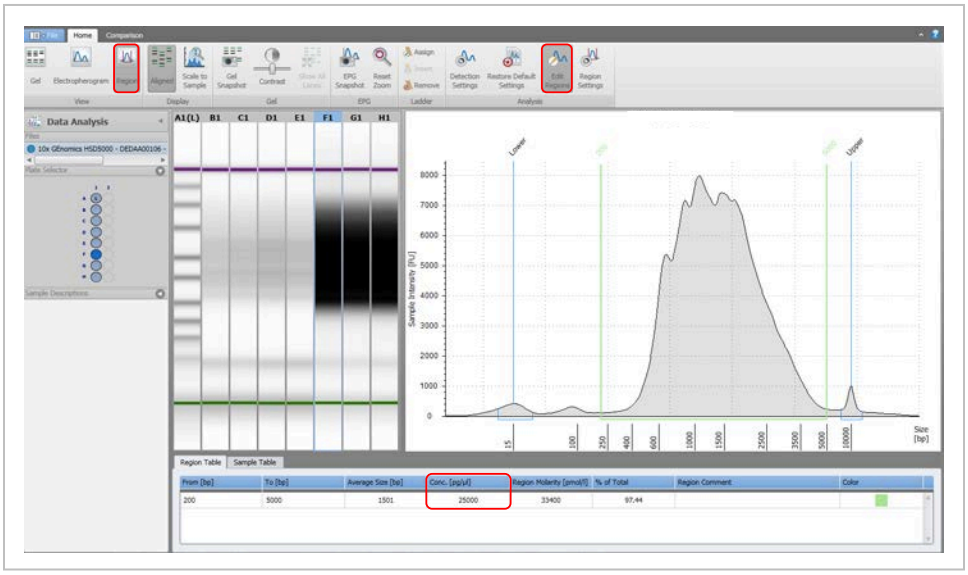


NOTE

If the input cells are particularly RNA-rich (>15pg total RNA/cell), it may be necessary dilute the sample prior to analysis. Lower molecular weight product (35 – 150 bp) may be present. This is normal and does not affect sequencing or application performance.

b) To determine the cDNA yield per sample, under the "Region" view choose the "Edit Regions" tab on the TapeStation Analysis Software.

c) Manually select the region encompassing ~200 – ~5000 bp.



d) Multiply the cDNA concentration [pg/µl] reported via the TapeStation Analysis 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.

NOTE

This mass will be used in step 3.5 to determine the appropriate number of Sample Index PCR cycles to generate sufficient concentration of final library, while minimizing PCR amplification artifacts.

Example Calculation of cDNA Total Yield:

TapeStation Analysis Concentration:	25000 [pg/µl]
Dilution Factor used to run the Agilent TapeStation:	1
Post cDNA Amplification Clean Up Elution Volume:	40 µl

Total cDNA Yield:

$$= \frac{\text{Concentration}\left(\frac{\text{pg}}{\mu\text{l}}\right) \times \text{Elution Volume (µl)} \times \text{Dilution Factor}}{1000\ \left(\frac{\text{pg}}{\text{ng}}\right)}$$

$$= \frac{25000\left(\frac{\text{pg}}{\mu\text{l}}\right) \times 40\ (\mu\text{l}) \times 1}{1000\ \left(\frac{\text{pg}}{\text{ng}}\right)}$$

$$= 1000\ \text{ng}$$

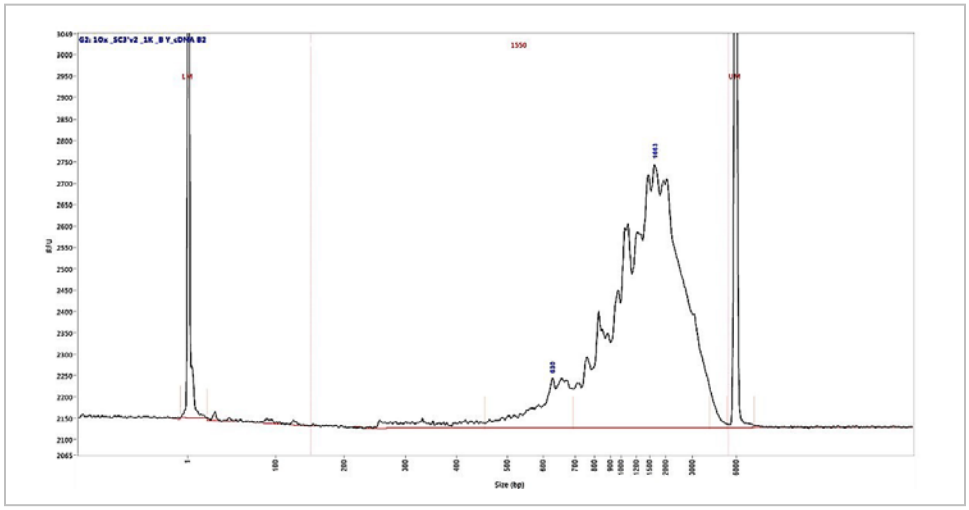
Refer to step 3.5 for the appropriate number of Sample Index PCR cycles.

5.7. Post cDNA Amplification Reaction & Post Library Construction QC & Quantification with the Fragment Analyzer

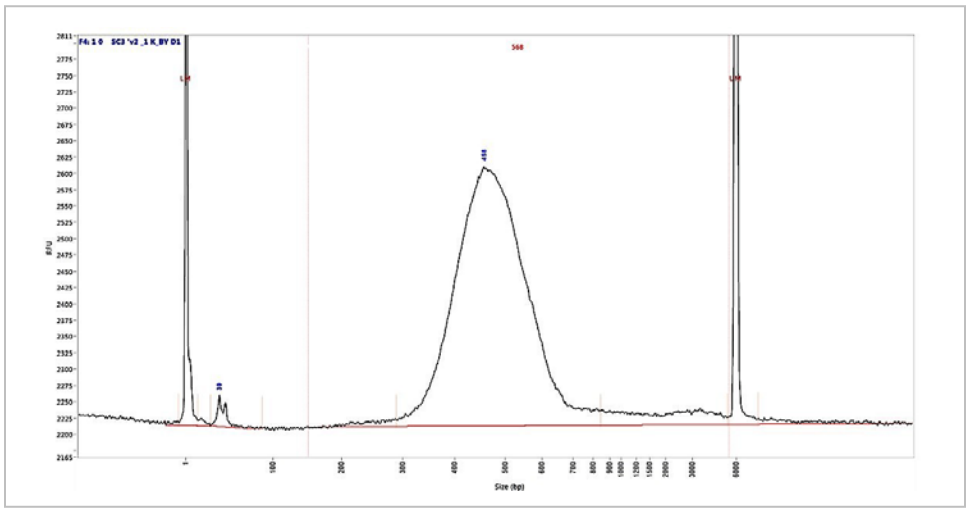
NOTE

If the concentration of the input sample is >5 ng/µl, dilute the sample before analyzing in the Fragment Analyzer.

a) Run **2 µl of** Post cDNA Amplification Reaction Cleanup sample on the Fragment Analyzer Automated CE system for qualitative and quantitative analysis. Traces should resemble the overall shape of the sample electropherogram shown below.

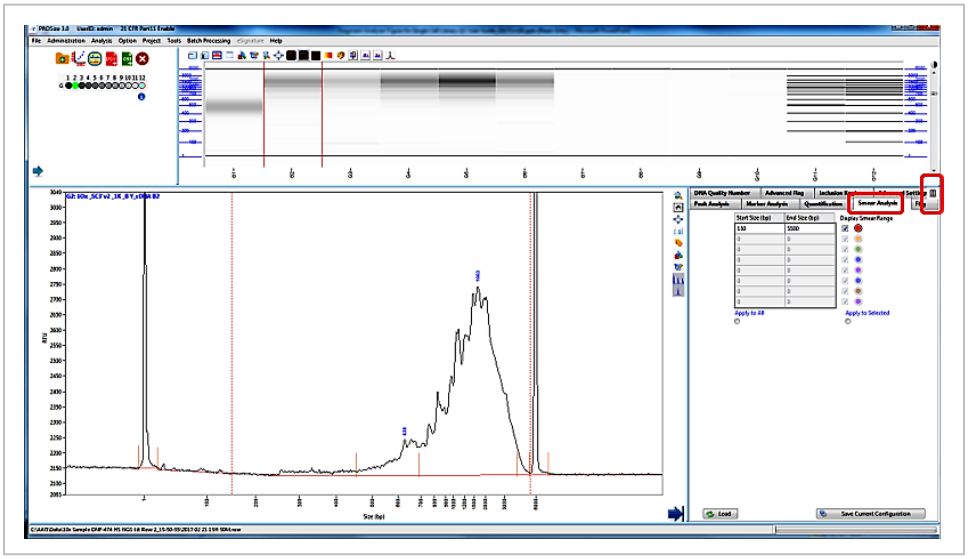


b) Run **2 µl** of Post Library Construction sample on the Fragment Analyzer Automated CE system for qualitative and quantitative analysis. Traces should resemble the overall shape of the sample electropherogram shown below.



c) To determine the concentration of the sample, open the data file in the PROSize® Data Analysis software and select the Smear Analysis tab on the main screen, as shown below for post cDNA amplification reaction sample.

d) Enter the Start Size and the End Size to select the region to be analyzed (encompassing ~150 – ~5500 bp).



e) Toggle the Show Results icon on the upper right side of the screen and select Smear Analysis tab to display the concentration and the average size of the analyzed sample.

5.8. SPRIselect Cleanups & Double Sided Size Selections

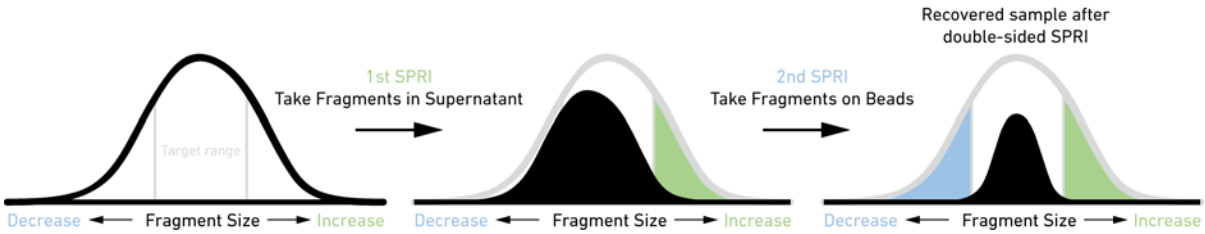
Solid Phase Reversible Immobilization (SPRI) beads selectively bind DNA fragments according to the ratio of SPRIselect Reagent (beads) to DNA solution. SPRIselect Reagent:DNA sample ratios indicated throughout the Protocol in SPRIselect Reagent steps are calculated as follows.

Example from Section 2.3 Post cDNA Amplification Reaction Cleanup

Step 2.3a: Add **60 µl** SPRIselect Reagent (**0.6X**) to each sample in the tube strip and pipette mix 15 times (pipette set to 150 µl).

$$\text{Ratio} = \frac{\text{Volume of SPRIselect Reagent added to the sample}}{\text{Volume of DNA sample (from cDNA Amplification Reaction)}} = \frac{60\ \mu l}{100\ \mu l} = \mathbf{0.6X}$$

Schematic Overview 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). Consult Technical Note SPRIselect: DNA Ratios Affect the Size Range of Library Fragments (Document CG000061) for more information on the use of SPRIselect Reagents.

Example from Section 3.2 Post Fragmentation, End Repair & A-tailing Double Sided Size Selection

Step 3.2a: Add **30 µl** SPRIselect Reagent (**0.6X**) to each sample in the tube strip and pipette mix 15 times (pipette set to 75 µl).

$$\text{Ratio} = \frac{\text{Volume of SPRIselect Reagent added to the sample}}{\text{Volume of DNA sample (from Fragmentation, End Repair \& A-tailing)}} = \frac{30\ \mu l}{50\ \mu l} = \mathbf{0.6X}$$

Continued Example from Section 3.2 Post Fragmentation, End Repair & A-tailing Double Sided Size Selection

Step 3.2e: Add **10 µl** SPRIselect Reagent (**0.8X**) to each sample in the tube strip and pipette mix 15 times (pipette set to 75 µl).

$$\text{Ratio} = \frac{\textbf{Total}\text{ volume of SPRIselect Reagent added to the sample (steps 3.2a + 3.2e)}}{\textbf{Original}\text{ volume of DNA sample (from Fragmentation, End Repair \& A-tailing)}} = \frac{30\ \mu l + 10\ \mu l}{50\ \mu l} = \mathbf{0.8X}$$

5.9. Failure Modes during GEM Generation

Reagent Clogs

GEM reagents are manufactured in a cleanroom environment to minimize the level of particles and fibers that could clog microfluidic channels during GEM generation and therefore reduce technical performance.

To avoid clogs, it is also important for users to minimize exposure of reagents, chips, and gaskets to sources of particles and fibers such as open reagent reservoirs, laboratory wipes, frequently opened flip-cap tubes, clothing that easily sheds fibers, and dusty surfaces.

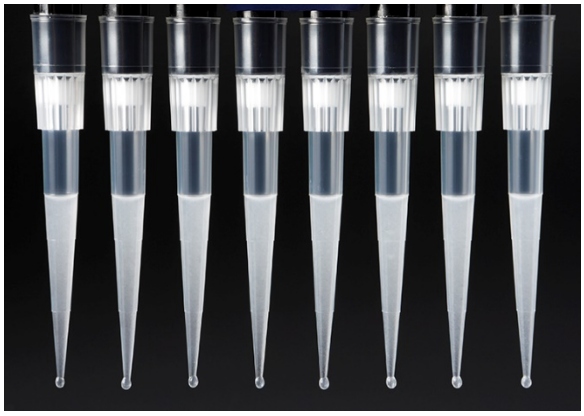
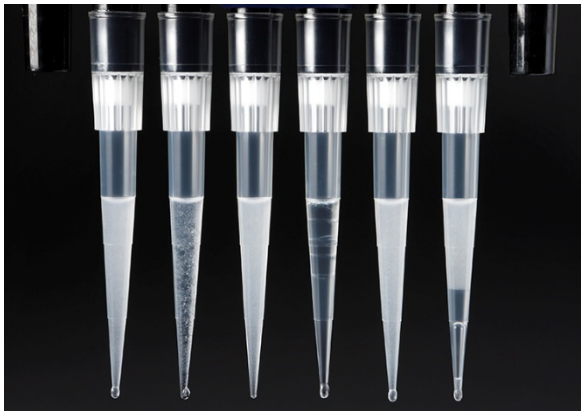
There are several ways to identify if a clog has occurred as outlined below. If any of the following occur, take a picture and send it to support@10xgenomics.com for further assistance. If a channel clogs during GEM generation, it is recommended that the sample be remade.

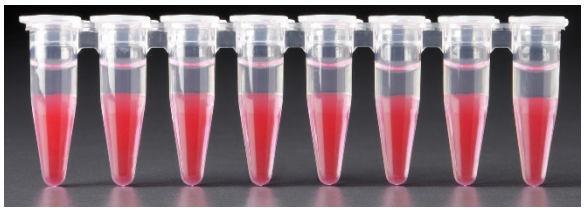
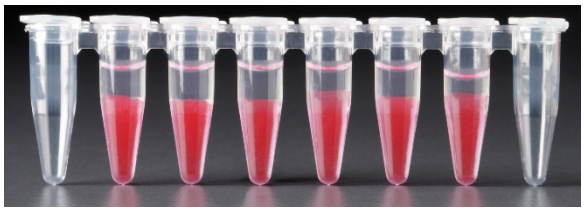
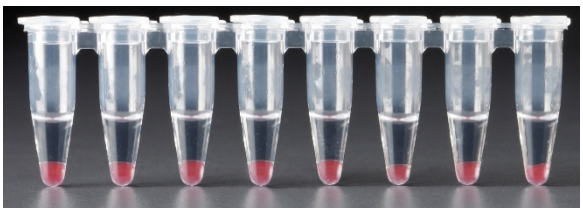
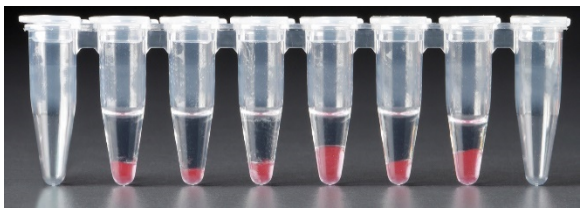
Wetting Failures

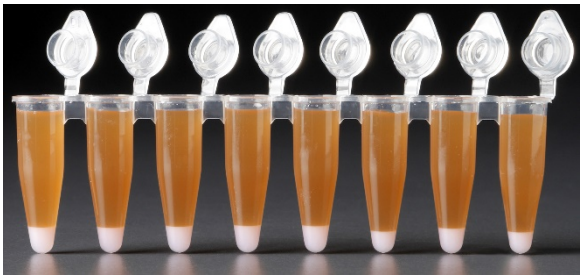
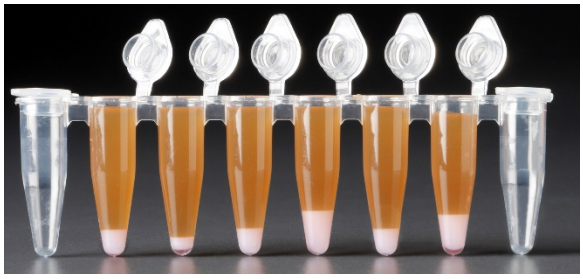
Once reagents are added to the Chromium Chip wells, they immediately flow into and prime the microfluidic channels on the chip. Incorrect priming can result in wetting failures, in which polydisperse, millimeter-scale droplets are formed instead of a uniform GEM.

To minimize the occurrence of wetting failures, it is critical to add reagents in the stipulated order and to wait 30 sec between addition of Master Mix and addition of Gel Beads.

There are several ways to identify if a wetting failure has occurred as outlined below. If any of the following occur, take a picture and send it to support@10xgenomics.com for further assistance. If a wetting failure occurs during GEM generation, it is recommended that the sample be remade.

Normal Operations	Reagent Clogs or Wetting Failures
After the Chromium Chip A Single Cell is removed from the Chromium Controller and the wells exposed: All Recovery Well levels are similar in volume and opacity.	**After the Chromium Chip A Single Cell is removed from the Chromium Controller and the wells exposed:** Recovery Well G indicates a reagent clog has occurred in this channel. Recovery Wells C and E indicate a wetting failure has occurred in these channels. There is an absence of emulsion. Recovery Wells B, D, and F are normal. Note 50 % glycerol solution was entered into wells A and H.
 A B C D E F G H	 A B C D E F G H
After aspirating the GEMs from the Chromium Chip A Single Cell Recovery Wells: All liquid levels are similar in volume and opacity. There is also no air in the pipette tips.	**After aspirating the GEMs from the Chromium Chip A Single Cell Recovery Wells:** Pipette tip G indicates a reagent clog has occurred in this channel. There is excess Partitioning Oil (clear) and air in the pipette tip. In some reagent clog cases, there is only 5-10 µl excess Partitioning Oil (and no air) in the pipette tip. Pipette tips C and E indicate indicate a wetting failure has occurred in these channels. Pipette tip C contains partially emulsified GEMs. Emulsion is absent in pipette tip E. Pipette tips B, D, and F are normal.

	Pipette tips A and H are missing.
Normal Operations	**Reagent Clogs or Wetting Failures**
 A B C D E F G H **After transfer of the GEMs + Recovery Agent to a tube strip:** All liquid levels are similar in the aqueous sample volume (clear) and Recovery Agent/Partitioning Oil (pink).	 A B C D E F G H **After transfer of the GEMs + Recovery Agent to a tube strip:** Tube G indicates a reagent clog has occurred in this channel. There is a decreased volume of aqueous sample (clear) when compared to normal channels B, D, and F. Tubes C and E indicate a wetting failure has ocurred in these channels. There can be an abnormal volume of Recovery Agent/Partitioning Oil (pink) when compared to normal channels B, D, and F. Tubes A and H are empty.
 A B C D E F G H **After removing the designated volume of Recovery Agent/Partitioning Oil:** All liquid volumes are similar in the aqueous sample volume (clear) and residual Recovery Agent/Partitioning Oil (pink).	 A B C D E F G H **After removing the designated volume of Recovery Agent/Partitioning Oil:** Tube G indicates a reagent clog has occurred in this channel. There is a decreased volume of aqueous sample (clear). There is also a greater residual volume of Recovery Agent/Partitioning Oil (pink) when compared to normal channels B, D, and F. Tubes C and E indicate a wetting failure has ocurred in these channels. There is an abnormal residual volume of Recovery Agent/Partitioning Oil (pink) when compared to normal channels B, D, and F. Tubes A and H are empty.

Normal Operations	Reagent Clogs or Wetting Failures
 A B C D E F G H	 A B C D E F G H
After addition of Dynabeads Cleanup Mix: All liquid volumes are similar after addition of the Dynabeads Cleanup Mix.	**After addition of Dynabeads Cleanup Mix:** Tube G indicates a reagent clog has occurred in this channel. There is an abnormal ratio of Dynabeads Cleanup Mix (brown) to Recovery Agent/Partitioning Oil (appears white) when compared to normal channels B, D, and F. Tubes C and E indicate a wetting failure has ocurred in these channels. There is an abnormal ratio of Dynabeads Cleanup Mix (brown) to Recovery Agent/Partitioning Oil (appears white) when compared to normal channels B, D, and F. Tubes A and H are empty.
After removing the Chromium Chip A Single Cell from the Chromium Controller: The image opposite illustrates clogs have occurred in the Gel Bead line (orange arrow) and the Sample line (yellow arrow) as evidenced by higher than usual residual volumes in the input wells.	

5.10. Chromium Controller Errors

If the Chromium Controller or 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 10x Chip 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 there is a 10x Gasket on the Chromium Chip. In the case the 10x Gasket installation was forgotten, install and try again. In the case a 10x Gasket was already installed, remove, reapply, and try again. If the error message is still received after trying either of these more than twice, contact support@10xgenomics.com for further assistance.

c) Pressure not at Setpoint:

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 Chip must be discarded. **Do not try running this Chromium Chip again as this may damage the Chromium Controller**.

d) **CAUTION: Chip Holder not Present**: Eject the tray by pressing the eject button to check there is a 10x Chip Holder encasing the Chromium Chip. In the case the 10x Chip Holder was forgotten, install with a 10x Gasket in place, and try again. If the error message is still received after a 10x Chip Holder is confirmed as in place, contact support@10xgenomics.com for further assistance.

e) **Invalid Chip CRC Value**: This indicates the Chromium Chip has encountered an error, should not be run, and must be discarded. Contact support@10xgenomics.com for further assistance.

Click back to Running the Chromium Controller

5.11. Glossary of Terms

10x Barcode

Defined DNA sequences that are added to each cDNA generated in a GEM so they can be distinguished and sorted during data analysis.

Chromium Chip A Single Cell

The Chromium Chip A Single Cell is a microfluidic chip specifically designed to run the Single Cell 3' Protocol in the Chromium Controller. The Chip A Single Cell is indicated by a red label at the top of the chip. Other chips used with the Chromium System include the Chromium Genome Chip.

Gel Beads

Gel Beads are the foundation of 10x Genomics technology, and are beads functionalized with millions of copies of a 10x Barcoded primer. Gel Beads are provided in 8-reaction Gel Bead Strips.

GEM

GEM is an abbreviation of Gel Bead-In-EMulsion. In the Chip A Single Cell, a library of Single Cell 3' Gel Beads is combined with cells and a reverse transcriptase (RT) Master Mix to create single nanoliter reaction volumes partitioned by oil.

GemCode Technology

The GemCode Technology is the microfluidic chip-based technology that partitions cells across tens of thousands of GEMs. Upon isothermal incubation, the cDNA produced in each GEM contains a 10x Barcode that identifies them as having originated from the same sample partition.