Chromium™

Single Cell 3' Reagent Kits User Guide

FOR USE WITH

Chromium™ Single Cell 3' Library, Gel Bead & Multiplex Kit, 16 rxns PN-120233

Chromium™ Single Cell 3' Chip Kit PN-120232

10x GENOMICS®

10xGenomics.com

Notices

Manual Part Number

CG00026 Rev B

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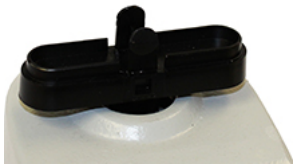
Chromium™ Single Cell 3' Reagent Kits – Components

Product	Description	#	Part Number
Chromium™ Single Cell 3' Library Kit (store at –20°C)			120230
Reagents Module 1	RT Reagent Mix	1	220071
	RT Enzyme	1	220070
	RNase Inhibitor	1	220065
	Additive A	1	220074
	RT Primer	2	310354
	Buffer for Sample Cleanup 1	2	220020
	cDNA Primer Mix	1	220066
	cDNA Additive	1	220067
Reagents Module 2	Amplification Master Mix	2	220073
	End Repair and A-tailing Buffer	1	220046
	End Repair and A-tailing Enzyme	1	220047
	Ligation Buffer	1	220048
	DNA Ligase	1	220049
	R1 Adaptor Mix	1	220064
	SI-PCR Primer	1	220068
	Surrogate Fluid	1	220021
	Single Cell 3' Sample Index Plate	1	210136
Chromium™ Single Cell 3' Gel Bead Kit (store at –80°C)			120231
	Single Cell 3' Gel Beads	2	220069
Chromium™ Single Cell 3' Chip Kit (store at ambient temperature)			120232
	Single Cell 3' Chips	6	230008
	Gaskets	6	370017
	Partitioning Oil	5	220017
	Recovery Agent	6	220016

Chromium™ Accessories

Product	Description	Part Number
10x™ Vortex Adapter	The 10x Vortex Adapter attaches to the top of a standard laboratory vortexer and enables users to vortex Gel Bead Strips. 	330002
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.  	330019
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.	230003

Additional Kits, Reagents & Equipment

Supplier	Description	Part Number (US)
Qiagen	Buffer EB	19086
Thermo Fisher	DynaBeads® MyOne™ Silane Beads*	37002D
	Nuclease-Free Water	AM9937
	Low TE Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA)	12090-015
	Phosphate Buffered Saline (PBS), pH 7.4	10010023
	Countess® II Automated Cell Counter‡	AMQAX1000
	Countess® II Automated Cell Counting Chamber Slides‡	C10228
	UltraPure™ BSA (50 mg/mL) (alternate to Sigma product)	AM2616
Sigma	Ethanol, Pure (200 Proof, anhydrous)	459836-500ML
	Bovine Serum Albumin (alternate to Thermo Fisher product)	B6917
Beckman Coulter	SPRIselect Reagent Kit*	B23318
USA Scientific	TempAssure PCR 8-tube strip* (alternate to Eppendorf product)	14024700
Eppendorf	twin.tec® 96-Well PCR Plate* Semi-skirted§	951020362
	twin.tec® 96-Well PCR Plate* Divisible, unskirted§	0030133374
	twin.tec® 96-Well PCR Plate* Unskirted§	0030133390
	ThermoMixer C®	5382000015
	SmartBlock 1.5 mL, Thermoblock for 24 Reaction Vessels	5360000038
	DNA LoBind Tube Microcentrifuge Tube, 0.5 mL*	022431005
	DNA LoBind Tube Microcentrifuge Tube, 1.5 mL*	022431021
	DNA LoBind Tube Microcentrifuge Tube, 2.0 mL*	022431048
	PCR Tubes 0.2 mL 8-tube strips* (alternate to USA Scientific product)	951010022
Bio-Rad	PX1 PCR Plate Sealer**	1814000
	Optical Flat 8-Cap Strips	TCS0803
	Pierceable Foil Heat Seal**	1814040
	10% Tween 20	1610781
	Microseal 'B' Adhesive Seals	MSB1001
KAPA Biosystems	Illumina Library Quantification Kit	KK4824
-	qPCR instrument and compatible consumables	-
VWR	Vortex Mixer*	10153-838
	Divided Polystyrene Reservoirs**	41428-958
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
Covaris	M220 Focused Ultrasonicator™***	500295
	M220 Holder microTUBE***	500301
	microTUBE AFA Fiber Screw-Cap 6 x 16 mm***	520096
Ricca Chemical Company	Glycerin (glycerol), 50% (v/v) Aqueous Solution (additional Surrogate Fluid, if needed)	3290-32

Supplier	Description	Part Number US
Rainin	Tips LTS 20UL Filter RT-L10FLR	17007957
	Tips LTS 200UL Filter RT-L200FLR*	17007961
	Tips LTS 1ML Filter RT-L1000FLR	17007954
	Tips LTS W-O 1MLUL Fltr RT-L1000WFLR	17014297
	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
	Pipet-Lite Multi Pipette L8-50XLS+	17013804
	Pipet-Lite Multi Pipette L8-200XLS+	17013805
Bel-Art	Flowmi™ Cell Strainer, 40 µm	H13680-0040
Miltenyi Biotec	MACS SmartStrainers, 30 µm	130-098-458
iNCYTO	Hemocytometer C chip†	DHC-N01
EMD Millipore	Scepter™ 2.0 Handheld Automated Cell Counter†	PHCC20060
	Scepter™ Cell Counter Sensors, 60 µm†	PHCC60050
	Scepter™ Cell Counter Sensors, 40 µm†	PHCC40050

*No substitutions are allowed. Items have been validated by 10x Genomics® and are required for Single Cell 3' workflow, training and system operations. §Eppendorf twin.tec® brand PCR plates are required to ensure stability of GEM emulsions, but the specific model should be selected based on compatibility with thermal cycler in use. **Substituting materials may adversely affect system performance and are not supported. †10x Genomics® recommends the Countess II Cell Counter for most applications, however users should determine the best system for specific sample types. ‡Either Bioanalyzer or TapeStation needed for quality control. ***Models LE220 and S2 have also been validated for use with the manufacturer's recommended tubes.

PCR 8-tube strips

USA Scientific TempAssure PCR 8-tube strip and Eppendorf PCR Tubes 0.2 mL 8-tube strips have been validated by 10x Genomics®.

If USA Scientific or Eppendorf 8-tube strips are not available in your region, alternatives are MicroAmp® and BIOplastics 8-tube strips and caps.

Recommended Thermal Cyclers

Thermal cyclers used with the Single Cell 3' Protocol must support uniform heating of 105 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 (# 1851197)
- Eppendorf MasterCycler® Pro (# North America 950030010, International 6321 000.019)
- Thermo Fisher Veriti© 96-Well Thermal Cycler (# 4375786)

The Single Cell 3' Reagent Kit Protocol

Stepwise Objectives

Steps & Timing

The Single Cell 3' Reagent Kit Protocol – Stepwise Objectives

Step 1 – Cell Preparation

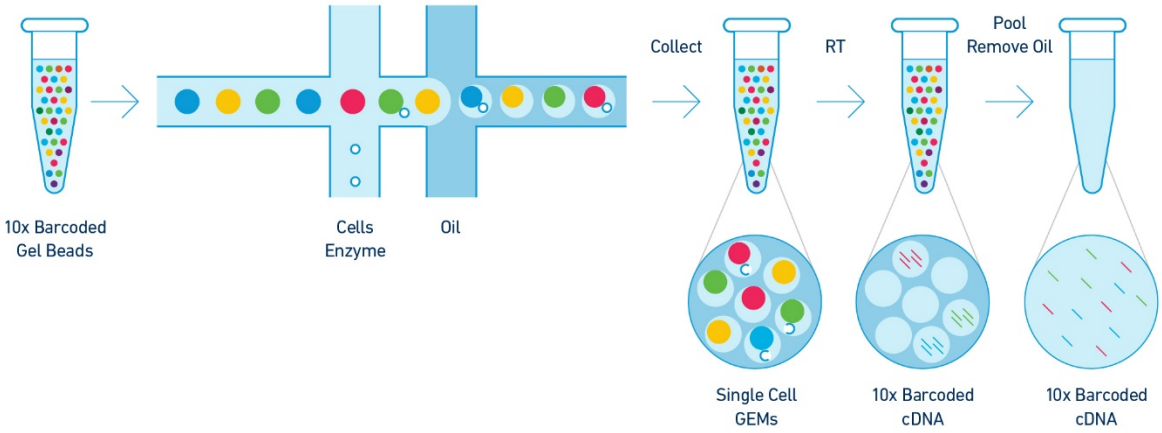
The Single Cell 3' Protocol enables transcriptional profiling of thousands to tens of thousands of individual cells in a single experiment for sensitive and accurate detection of rare cell types. Cells are first washed to remove contaminants and then prepared in an ideal concentration to go forward to the next step.

Step 2 – 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 1000 – 6000 individual cells per sample. The 10x™ GemCode™ Technology samples a pool of ~ 750,000 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 vast majority (~93-99%) of generated GEMs contains no cell, while the remainder largely contains a single cell.

Upon dissolution of the Single Cell 3' Gel Bead in a GEM, primers containing (i) an Illumina P7 and R2 sequence (read 2 sequencing primer), (ii) a 14 bp 10x Barcode, (iii) a 10 bp randomer 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 3 – Post GEM-RT Cleanup & cDNA Amplification

Silane magnetic beads and Solid Phase Reversible Immobilization (SPRI) 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.

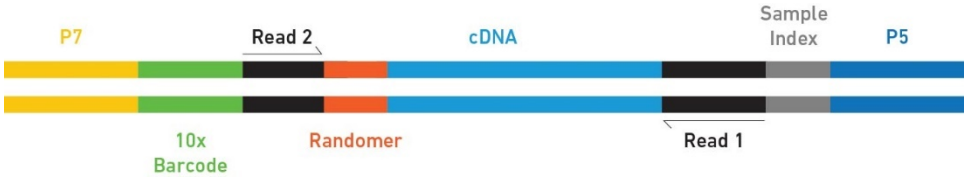
Step 4 – Library Construction

A shearing step optimizes the cDNA amplicon size prior to library construction. P7 and R2 (read 2 primer sequence) are added to the molecules during the GEM incubation. P5, a sample index and R1 (read 1 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 5 – 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' 14 bp 10x™ Barcode is encoded in the i7 index read, while sample index sequences are incorporated as the i5 index read.

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 and Read 2, the sample index in the i5 read, and the 14 bp 10x Barcode 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 an unprecedented scale of thousands of cells.

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

Day 1 – Steps		Hands-on
Cell Preparation		Sample dependent
Reagent Preparation		20 min
GEM Generation		15 min
RT Incubation (2 h)	STOP	-
Silane Bead & SPRIselect Cleanup	STOP	1 h
cDNA Amplification	STOP	5 min
SPRIselect Cleanup	STOP	10 min
Total Hands-on Time		**1 h 50 min**
Total Turnaround Time		**6.5 h**

Day 2 – Steps		Hands-on
Shearing		10 min
SPRIselect Size Selection		10 min
End Repair & A-tailing		5 min
Adaptor Ligation, SPRIselect Cleanups		20 min
Sample Index PCR, SPRIselect Cleanups	STOP	20 min
Total Hands-on Time		**1h 5 min**
Total Turnaround Time		**5.5 h**

Library Quantification Sequencing

Single Cell 3' Library Analysis

STOP - Potential stopping points

Protocol Step 1

Cell Preparation

Preparing viable single cell suspensions from cultured cell lines

1

1. Cell Preparation

Tips

Single Cell Suspensions from Cultured Cell Lines

The Chromium™ Controller requires a suspension of viable single cells as input. Minimizing the presence of cellular aggregates, dead cells, non-cellular nucleic acids and potential inhibitors of reverse transcription is critical to obtaining high quality data.

This protocol describes best practices for preparing single cell suspensions from cultured cell lines. Optimal preparation of single cell suspensions from other sample types, such as primary cell cultures, blood or solid tissues may require additional dissociation, straining and washing steps not covered here.

Cell Concentration for Optimal Performance

The recommended preparation for each cultured cell line sample run on the Chromium Controller is at least 20 µl of single cells suspended in 1X PBS containing 0.04% weight/volume BSA (400 µg/ml) at a concentration of 1000 cells/µl. BSA is added to minimize losses and aggregation during the wash steps. For recommendations on working with higher or lower cell concentrations, refer to the Cell Suspension Volume Calculator Table in Protocol Step 2.

Primary cells, stem cell lines and other sensitive sample types may require washing and suspension in alternative buffers to maintain optimal viability. If necessary, PBS can be replaced with most common cell culture buffers and media without significant loss of performance.

Best Practices for Handling Samples

Best practices for handling any sample type include following all applicable biohazard safety regulations, using sterile technique, nuclease-free reagents and consumables, minimizing pipetting steps, and using wide-bore pipette tips where possible to minimize cell damage.

Getting Started!

Prepare:

- Warm ~30 ml of culture medium and, if harvesting adherent cells (section 1.2), ~ 8 ml of 0.25% Trypsin-EDTA solution in a 37°C incubator.
- 1X PBS containing 0.04% BSA (400 µg/ml).

Cell Preparation

1.1. Cell Harvesting – Suspension Cell Lines

a) Determine the cell concentration using Countess® II Automated Cell Counter.

b) Ideally, cells will be 3 x 10^5 to 1 x 10^6 /ml with >90% alive. If the cell concentration is above 6 x 10^5 /ml, transfer 1.5 ml of the cell suspension to a 2 ml Eppendorf tube. If the cell concentration is below 6 x 10^5 /ml, transfer 1.5 ml to two 2 ml Eppendorf tubes for a total of 3 ml of cell suspension.

1.2. Cell Harvesting – Adherent Cell Lines

NOTE

This protocol is written for a 75 cm^2 flask. Adjust the reagent volume for other flasks or dishes proportionally.

Prepare warm ~30 ml of culture medium and ~ 8 ml of 0.25% Trypsin-EDTA solution by incubating in a 37°C incubator.

a) Using a 10 ml serological pipette, remove and discard culture medium.

b) Using a 10 ml serological pipette, add 1.5 ml of 0.25% Trypsin-EDTA solution to flask and briefly rinse the cell layer to eliminate residual serum. Immediately discard the spent trypsin solution.

c) Using a 10 ml serological pipette, add 5 - 6 ml of 0.25% Trypsin-EDTA solution to cover the cell layer and incubate flask at 37°C for 5- 15 min until the cells dissociate from the flask surface.

NOTE

Periodically, check the cell dissociation under a microscope if available. Over-incubation may damage cells. Avoid shaking or hitting the flask to minimize clumping.

d) Using a 10 ml serological pipette, add 10 ml of medium to stop digestion.

e) Transfer the cell suspension to a 50 ml conical tube.

f) Rinse the flask with 2 ml of medium using a 10 ml serological pipette and transfer the wash solution to the conical tube containing the cells.

g) Centrifuge cells at 250 rcf for 5 min.

NOTE

The optimal centrifugation speed and time may vary depending on the cell type. Smaller cells will generally require higher speeds.

CRITICAL!

Depending on the rotor type, the cell pellet forms on the side or on the bottom of the conical tube. Know the expected position of the pellet, especially when working with small or limited numbers of cells, as the pellet can be invisible to naked eye.

h) Using a 10 ml serological pipette, discard supernatant without disrupting cell pellet.

i) Resuspend the pelleted cells in medium and determine the cell concentration using Countess® II Automated Cell Counter.

j) If the cell concentration is above 6 x 10^5 /ml, transfer 1.5 ml of the cell suspension to a 2 ml Eppendorf tube. If the cell concentration is below 6 x 10^5 /ml, transfer 1.5 ml to two 2 ml Eppendorf tubes for a total of 3 ml of cell suspension.

1.3. Cell Washing

This section assumes 1.5-3 ml cell suspensions have been prepared according to sections 1.1 or 1.2. If the number of available cells are limiting, it may be necessary to skip one or more wash steps to minimize cell losses.

NOTE

The optimal centrifugation speed and time may vary depending on the cell type. Smaller cells will generally require higher speeds.

a) Centrifuge cells in a 2 ml Eppendorf tube at 150 rcf for 3 min.

CRITICAL!

Depending on the rotor type, the cell pellet forms on the side or on the bottom of the conical tube. Know the expected position of the pellet, especially when working with small or limited cells, as the pellet can be invisible to naked eye.

b) Remove supernatant without disrupting cell pellet.

c) Using a wide bore tip, add 1 ml of 1X PBS with 0.04% BSA to each tube and gently pipette mix 5 times and invert tubes to resuspend cell pellet. Pool the tubes if necessary.

d) Centrifuge cells at 150 rcf for 3 min.

e) Remove supernatant without disrupting cell pellet.

f) Using a wide bore tip, add 1 ml of 1X PBS with 0.04% BSA to the tube. Gently pipette mix 5 times and invert tubes to resuspend cell pellet.

g) Centrifuge cells at 150 rcf for 3 min.

h) Remove supernatant without disrupting cell pellet.

i) Add 500 µl 1X PBS with 0.04% BSA or appropriate volume of 1X PBS with 0.04% BSA such that cell concentration is above 1 x 10^6 /ml. Gently pipette 10 - 15 times or until cells are completely suspended.

NOTE

Do not invert the tube in this step, as cells can stick to the sides of the tube, changing the cell concentration.

OPTIONAL

Use a cell strainer to remove cell debris and large clumps. For low volume cell suspensions, a Flowmi™ Tip Strainer is recommended for minimal loss of additional sample volume.

j) Determine the cell concentration using Countess® II Automated Cell Counter.

k) Ideally, the cells will be at 1 x 10^6 /ml (1000/µl). If the cells are too concentrated, adjust volume accordingly. Re-count.

l) Once cells at 1 x 10^6 /ml (1000/µl) are obtained, place the cells on ice and proceed to the next step.

Protocol Step 2

GEM Generation & Barcoding

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

2

2. 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. Substituting these materials can adversely affect performance.

Importance of Loading Cell Concentration

The recommended starting point for a new sample type is to load ~2600 cells into each reaction, recovering approximately 1200 cells, to achieve an expected multiplet rate of approximately 1.1%. Loading fewer cells per reaction will result in a lower multiplet rate, but may affect application performance if the number of recovered cells is less than 1000. Loading more cells per reaction will elevate the doublet rate, which may also impact application performance (see table below).

Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~1.1%	~2600	~1200
~1.8%	~4300	~2000
~2.7%	~6400	~3000
~3.6%	~8500	~4000
~4.5%	~10700	~5000
~5.3%	~12800	~6000

Best Practices for Handling Single Cell 3' Chips

Single Cell 3' Chips are microfluidic chips, which can clog due to the introduction of particulates. Therefore, it is important to minimize exposure of reagents, chips, and gaskets to sources of fibers such as reagent reservoirs, KimWipes, repeat-usage of flip-cap tubes, and the general laboratory environment.

The presence of excess Partitioning Oil in recovered GEMs from the Single Cell 3' Chip after running the Chromium™ Controller may indicate that a clog occurred. See Practical Tips & Troubleshooting for more information.

It is critical all unused wells in Rows labeled 1, 2 and 3 of the Single Cell 3' Chip contain Surrogate Fluid prior to running the Chromium™ Controller. Surrogate Fluid for up to 4 Single Cell 3' Chip channels is provided in the Library Kit. See Practical Tips & Troubleshooting for information on purchasing or generating more Surrogate Fluid, if required.

Best Practices for Preparing & Handling Reagents & Master Mixes

- Covering Partitioning Oil tubes and holders to minimize evaporation.
- Ensuring the 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.

- Assembling Master Mix on ice and keeping cold until Single Cell 3' Chip loading.
- For tips on processing fewer than 8 reactions, see Practical Tips & Troubleshooting.

Best Practices for Handling Gel Beads

- Equilibrating the Single Cell 3' Gel Beads Strip to room temperature before use.
- Storing any unused Single Cell 3' Gel Beads at –80°C and avoiding more than 4 freeze-thaw cycles.
- Never storing Single Cell 3' Gel Beads at –20°C.
- Piercing the Gel Bead Strip foils 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 will maximize recovery of Gel Beads for optimal performance.

Best Practices for Loading the Single Cell 3' Chip

- Waiting 30 sec between loading the Master Mix and loading the Gel Beads into the Single Cell 3' Chip to ensure proper priming of the channels.
- When aspirating Gel Beads from the Gel Bead Strip or 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 Single Cell 3' Chip, wait for remainder to collect into the pipette tips and dispense again to ensure complete transfer.
- Starting GEM generation immediately after Single Cell 3' Chip loading.

Best Practices for GEM Recovery

- Unloading GEMs immediately after the completion of GEM generation.
- When dispensing GEMs into the PCR plate, wait for remainder to collect into the pipette tips and dispense again to ensure complete transfer.
- Keeping the GEMs on a chilled metal block resting on ice immediately after recovery.

Getting Started!

Equilibrate to room temperature before use:

- Single Cell 3' Gel Beads (equilibrate to room temperature for 30 min before loading the Single Cell 3' Chip)
- RT Reagent Mix (vortex and verify no precipitate)
- RT Primer (provided as lyophilized oligos; after resuspension, store unused primers at –80°C)
- Additive A (vortex and verify no precipitate)
- Surrogate Fluid (if processing fewer than 8 samples)

Resuspend:

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

Place on ice:

- RT Enzyme (keep on ice, centrifuge briefly before adding to Master Mix)
- RNase Inhibitor (keep on ice, centrifuge briefly before adding to Master Mix)
- Chilled metal block resting on ice

Obtain:

- Partitioning Oil
- Single Cell 3' Chip(s), 10x™ Gasket(s) and 10x Chip Holder

Plate sealer:

- Set the Bio-Rad® PX1 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

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

Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery					
	1200 cells	2000 cells	3000 cells	4000 cells	5000 cells	6000 cells
100	25.6 | 11.8	n/a	n/a	n/a	n/a	n/a
200	12.8 | 24.6	21.3 | 16.1	32.0 | 5.4	n/a	n/a	n/a
300	8.5 | 28.9	14.2 | 23.2	21.3 | 16.1	28.4 | 9.0	35.6 | 1.8	n/a
400	6.4 | 31.0	10.7 | 26.7	16.0 | 21.4	21.3 | 16.1	26.7 | 10.7	32.0 | 5.4
500	5.1 | 32.3	8.5 | 28.9	12.8 | 24.6	17.1 | 20.3	21.3 | 16.1	25.6 | 11.8
600	4.3 | 33.1	7.1 | 30.3	10.7 | 26.7	14.2 | 23.2	17.8 | 19.6	21.3 | 16.1
700	3.7 | 33.7	6.1 | 31.3	9.1 | 28.3	12.2 | 25.2	15.2 | 22.2	18.3 | 19.1
800	3.2 | 34.2	5.3 | 32.1	8.0 | 29.4	10.7 | 26.7	13.3 | 24.1	16.0 | 21.4
900	2.8 | 34.6	4.7 | 32.7	7.1 | 30.3	9.5 | 27.9	11.9 | 25.5	14.2 | 23.2
1000	2.6 | 34.8	4.3 | 33.1	6.4 | 31.0	8.5 | 28.9	10.7 | 26.7	12.8 | 24.6
1100	2.3 | 35.1	3.9 | 33.5	5.8 | 31.6	7.8 | 29.6	9.7 | 27.7	11.6 | 25.8
1200	2.1 | 35.3	3.6 | 33.8	5.3 | 32.1	7.1 | 30.3	8.9 | 28.5	10.7 | 26.7
1300	2.0 | 35.4	3.3 | 34.1	4.9 | 32.5	6.6 | 30.8	8.2 | 29.2	9.8 | 27.6
1400	1.8 | 35.6	3.0 | 34.4	4.6 | 32.8	6.1 | 31.3	7.6 | 29.8	9.1 | 28.3
1500	1.7 | 35.7	2.8 | 34.6	4.3 | 33.1	5.7 | 31.7	7.1 | 30.3	8.5 | 28.9
1600	1.6 | 35.8	2.7 | 34.7	4.0 | 33.4	5.3 | 32.1	6.7 | 30.7	8.0 | 29.4
1700	1.5 | 35.9	2.5 | 34.9	3.8 | 33.6	5.0 | 32.4	6.3 | 31.1	7.5 | 29.9
1800	1.4 | 36.0	2.4 | 35.0	3.6 | 33.8	4.7 | 32.7	5.9 | 31.5	7.1 | 30.3
1900	1.3 | 36.1	2.2 | 35.2	3.4 | 34.0	4.5 | 32.9	5.6 | 31.8	6.7 | 30.7
2000	1.3 | 36.1	2.1 | 35.3	3.2 | 34.2	4.3 | 33.1	5.3 | 32.1	6.4 | 31.0

Grey boxes: Volumes that would exceed the allowable water volume in each reaction

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

GEM Generation & Barcoding

2.1. Preparing Single Cell Master Mix

NOTE

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

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	1X (µl)	8.8X (µl)
Nuclease-Free Water	From Table	Calculate
RT Reagent Mix	50.0	440.0
RT Primer	4.0	35.2
RNase Inhibitor	1.5	13.2
Additive A	2.5	22.0
RT Enzyme	4.6	40.5
Total	Calculate	Calculate

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

2.2. Loading the Single Cell 3' Chip

a) Place a Single Cell 3' Chip in a 10x™ Chip Holder. See Practical Tips & Troubleshooting for tips on assembly.

CRITICAL!

The order in which the wells of Single Cell 3' Chips 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 Single Cell 3' Chip, add Surrogate Fluid 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 Surrogate Fluid to Recovery Wells (row labeled ◀)

c) Snap the Single Cell 3' Gel Bead Strip into a 10x Vortex Adaptor and vortex for 25 sec.

d) 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 well levels look even. Place the Gel Bead Strip aside at room temperature.

e) Dispense the **calculated total volume (µl) of Master Mix** into each well of an 8-tube strip on a chilled metal block resting on ice.

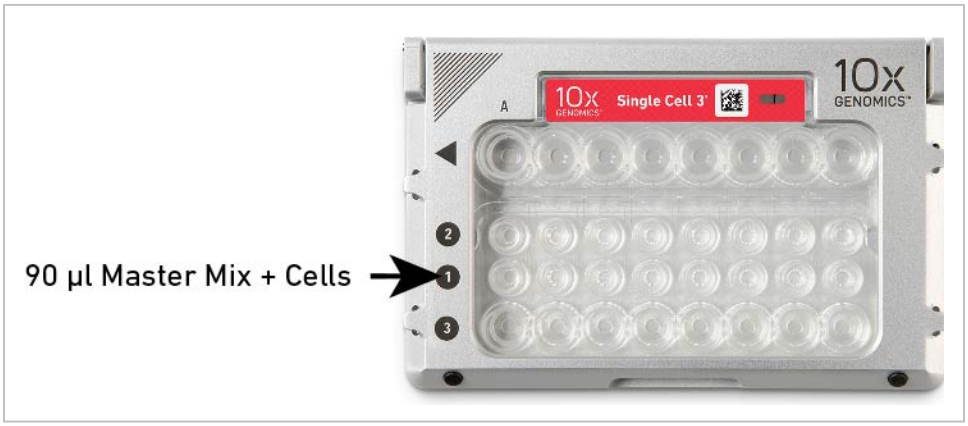
f) Gently pipette mix the tube containing the washed and diluted cells. Add the appropriate **volume (µl) of single cell suspension** (from the Cell Suspension Volume Calculator Table) to each well of the tube strip containing the Master Mix.

g) With a pipette set to 90 µl gently pipette mix the combined cells and Master Mix 5 times while keeping the tube strip on a chilled metal block resting on ice.

h) Without discarding the pipette tips, transfer 90 µl Master Mix containing cells to the wells in the **row labeled 1**. 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. Wait 30 sec, then immediately load the Single Cell 3' Gel Beads.

CRITICAL!

Pipette slowly. Raising and depressing the pipette plunger should each take 2 sec. A 30 second wait is required to ensure proper priming of the Single Cell 3' Chip.



NOTE

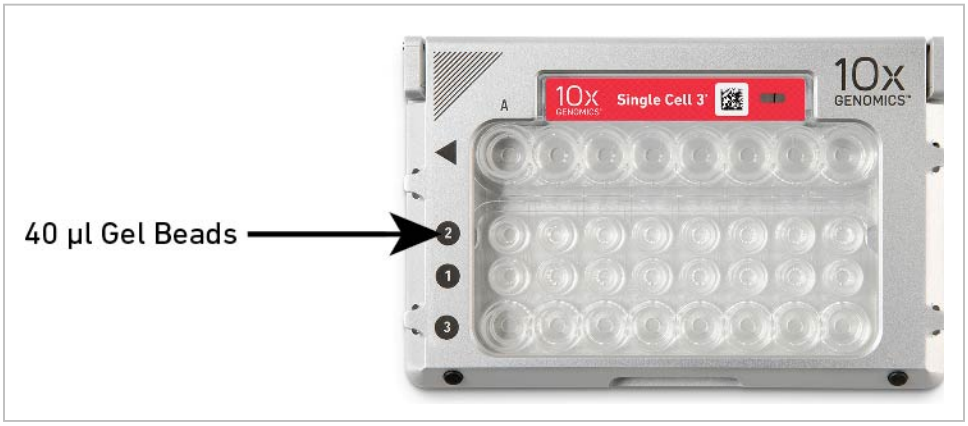
Pipette Single Cell 3' Gel Beads slowly as they have a viscosity similar to high-concentration glycerol.

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

NOTE

If processing fewer than 8 samples, only puncture sufficient wells in the Single Cell 3' Gel Bead Strip.

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!

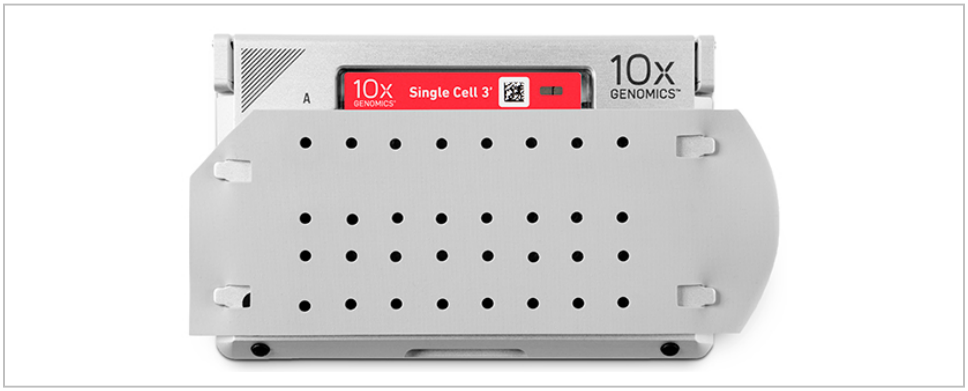
Confirm that the pipette tips do not contain leftover Gel Beads. If necessary, wait for the remaining Gel Beads to collect into the bottom of the pipette tips and dispense into the wells.

k) Pipette 135 µl Partitioning Oil from a reagent reservoir into the wells in the **row labeled 3** twice for a total of 270 µl.

CRITICAL!

Failure to add Partitioning Oil can damage the Chromium™ 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.



2.3. Running the Chromium™ 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 Single Cell 3' program shows on screen and press the play button to begin the run.

d) At the completion of the run (~6 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 Single Cell 3' program shows on the screen and press the play button to start the run

2.4. Transferring GEMs

a) Maintain an Eppendorf twin.tec® 96-well PCR plate for GEM transfer on a chilled metal block resting on ice.

b) Press the eject button to eject the tray and remove the Single Cell 3' Chip. Remove and discard the 10x Gasket. Press the button to retract the empty tray.

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 Single Cell 3' Chip.

NOTE

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

e) Slowly aspirate 105 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) indicates a potential clog during GEM generation.

g) Over the course of ~20 sec, dispense the GEMs into the Eppendorf® twin-tec 96-well PCR plate (on a chilled metal block resting on ice) with the pipette tips **against the sidewalls of the wells**. (See Practical Tips & Troubleshooting). 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 PCR 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.

h) If multiple Single Cell 3' Chips are run back-to-back, keep plate containing recovered GEMs on ice and seal the plate wells containing GEMs with Strip Caps before proceeding to generate GEMs for the next set of samples.

i) Discard the used Single Cell 3' Chip. Push the black sliders on the back of the 10x™ Chip Holder toward the middle to release the lock and close the lid.

2.5. GEM-RT Incubation

a) If necessary, remove the strip caps from the PCR plate with recovered GEMs. 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 sealed PCR plate into a thermal cycler that can accommodate at least 105 µl reaction volume and proceed with the following incubation protocol. The run will take ~2 h 10 min.

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**	
55°C	125 µl	
Step	**Temperature**	**Time**
1	55˚C	2:00:00
2	85˚C	5:00
3	4˚C	Hold

d) Place PCR plate on a chilled metal block resting on ice after completion of the thermal cycler program.

STOP

e) Store in the PCR plate at 4°C for up to 72 h before proceeding to Post GEM-RT Cleanup.

Protocol Step 3

Post GEM-RT Cleanup & cDNA Amplification

Isolate and amplify cDNA for library construction

3

3. 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.

Best Practices – Post cDNA Amplification Reaction QC

Agilent Bioanalyzer is the recommended method for library QC after GEM cleanup and amplification to ensure successful cDNA amplification, before proceeding with library construction.

Getting Started!

Equilibrate to room temperature before use:

- Additive A (vortex and verify no precipitate)
- DynaBeads® MyOne™ Silane beads
- Beckman Coulter SPRIselect Reagent
- Agilent Bioanalyzer High Sensitivity Kit (if used for QC)
- Amplification Master Mix
- cDNA Primer Mix
- cDNA Additive

Obtain:

- Recovery Agent
- Qiagen Buffer EB
- Bio-Rad 10% Tween 20

Thaw at 65°C:

- Thaw Buffer for Sample Cleanup 1 for 10 min at 65°C at max speed on a thermomixer. Let cool to room temperature. Verify that the Buffer for Sample Cleanup 1 is completely dissolved with no visible crystals.

Prepare 80% Ethanol (15 ml for 8 samples)

Post GEM-RT Cleanup & cDNA Amplification

3.1. Post GEM-RT Cleanup – Silane DynaBeads

a) Remove the foil seal and add 125 µl Recovery Agent to each well containing post incubation GEMs. Wait 60 sec and then transfer the entire volume to an 8-tube strip.

NOTE

To maximize recovery of the aquous phase, the plate can be lightly sealed with a Microseal® 'B' Adhesive Seal and spun down in a plate centrifuge at 1200 rpm for 30 sec.

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.

NOTE

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

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

DynaBeads Cleanup Mix	1X (µl)	8.8X (µl)
Buffer for Sample Cleanup 1	182	1602
DynaBeads MyOne Silane	14	123
Additive A	4	35
Total	**200**	**1760**

e) Immediately add 200 µl DynaBeads Cleanup Mix to each sample. Pipette mix 5 times and incubate at room temperature for 10 min. After the first 5 min pipette mix again 5 times.

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

Elution Solution I	1 reaction (µl)	10 reactions (µl)
Buffer EB	98	980
10% Tween20	1	10
Additive A	1	10
Total	**100**	**1000**

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

n) Remove and discard any remaining ethanol and allow the samples to air dry for 2 min.

o) Remove the tube strip from the magnet and add 50.5 µl Elution Solution I. Pipette mix at least 15 times and incubate at room temperature for 1 min.

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

q) Transfer 50 µl the eluted sample to a new tube strip.

3.2. Post GEM-RT Cleanup – SPRIselect

a) Prepare Elution Solution II by adding appropriate volume of reagents in the order shown below. Vortex mix thoroughly and centrifuge briefly.

Elution Solution II	1 reaction (µl)	10 reactions (µl)
Buffer EB	99	990
Additive A	1	10
Total	**100**	**1000**

b) Vortex the SPRIselect Reagent until fully resuspended. Add 30 µl SPRIselect Reagent to each sample in the tube strip and pipette mix 15 times.

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

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

e) Carefully remove and discard the supernatant.

f) Add 125 µl 80% ethanol to the pellet and stand for 30 sec.

g) Carefully remove and discard the ethanol wash.

Repeat

h) Repeat steps f and g 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 1 min. **Do not exceed 1 min** as this will lead to decreased elution efficiency.

k) Remove the tube strip from the 10x Magnetic Separator and add 35.5 µl Elution Solution II.

l) Pipette mix 15 times and incubate 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 35 µl of sample to a new tube strip and cap the sample wells.

3.3. cDNA Amplification Reaction

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

cDNA Amplification Reaction Mix	1X (µl)	8.8X (µl)
Nuclease-Free Water	8	70
Amplification Master Mix	50	440
cDNA Additive	5	44
cDNA Primer Mix	2	18
Total	**65**	**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 80 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. The run will take ~45 min.

Lid Temperature	Reaction Volume	
105°C	100 µl	
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	10

STOP

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

3.4. Post cDNA Amplification Reaction Cleanup – SPRIselect

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

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 55.5 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.

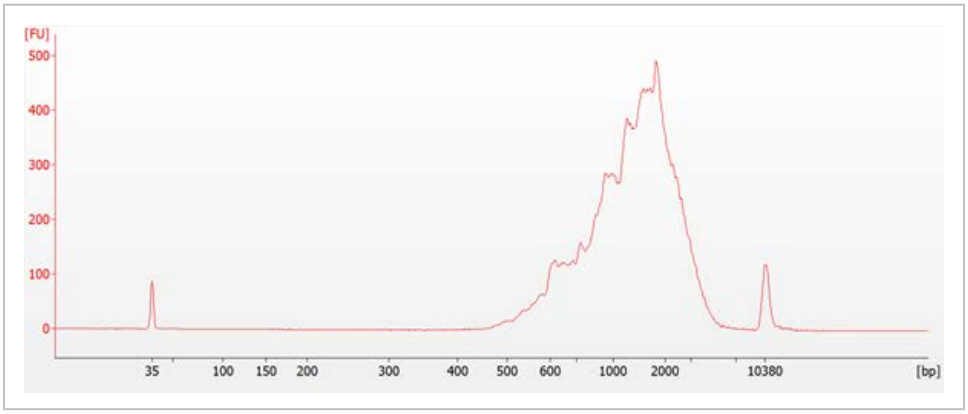
m) Transfer 55 µl of sample to a new tube strip and cap the sample wells.

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.

3.5. Post cDNA Amplification Reaction QC

a) Run 1 µl of sample at 1:5 dilution in Nuclease-Free Water on the Agilent Bioanalyzer High Sensitivity chip for qualitative analysis. Traces should resemble the overall shape of the sample electropherogram 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.

Protocol Step 4

Library Construction

Insert P5, Read 1, and Sample Index to prepare for sequencing

4. Library Construction

Tips

General

The final Single Cell 3' Libraries contain the P5 and P7 primers used in Illumina bridge amplification PCR. The Single Cell 3' Barcode is added to the molecules during the GEM-RT incubation. P7 primer and Read 2 (primer site for sequencing read 2) are added to the cDNA molecules during the GEM-RT incubation. The P5 primer, Read1 (primer site for sequencing read 1), and Sample Index 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 0.1 ng/µl to 70 ng/µl 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 – Shearing

Use Covaris's recommended settings for the available instrument to achieve a target peak size of 200 bp for a standard DNA sample.

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:

- End Repair and A-tailing Buffer (verify no precipitate)
- SI-PCR Primer
- Ligation Buffer (verify no precipitate)
- R1 Adaptor Mix
- Single Cell 3' Sample Index Plate
- Beckman Coulter SPRIselect Reagent
- Agilent Bioanalyzer High Sensitivity Kit (if used for QC)
- Agilent TapeStation ScreenTape and Reagents (if used for QC)
- Kapa DNA Quantification Kit for Illumina Platforms

Obtain:

- Qiagen Buffer EB

Place on ice:

- End Repair and A-tailing Enzyme
- DNA Ligase
- Amplification Master Mix

Prepare 80% Ethanol (20 ml for 8 samples)

Library Construction

4.1. Shearing

a) Shear 51 µl of the sample obtained from Post cDNA Amplification Reaction Cleanup according to Covaris's recommended settings to achieve target peak size of 200 bp for a standard DNA sample.

b) Centrifuge tubes briefly and transfer 50 µl of sheared sample to a new tube strip

NOTE

If the shearing instrument used requires a larger input volume, the Post cDNA Amplification Reaction Cleanup product may be further diluted in Nuclease-Free Water. Recover the full volume post shearing and scale the volumes of SPRIselect Reagent and trasnferred supernatants in step 4.2 accordingly.

4.2. Post Shearing Size Selection – SPRIselect

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

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 to each sample in the tube strip and pipette mix 15 times.

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 the supernatant.

NOTE

Due to the extremely low volume of the SPRIselect Reagent used in this step it is crucial to not discard any of the beads with the supernatant. Verify the supernatant is free of magnetic beads before continuing. If there are beads present in the pipette tips, transfer the supernatant back into the tube strip and reduce the volume of supernatant removed by 5 µl.

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 extremely low volume of beads used during this SPRI clean-up 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 Magnetic Separator in the High position until the solution is clear.

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

4.3. Library Construction: End Repair & A-tailing

a) Vortex the End Repair and A-tailing Buffer. Verify there is no precipitate before proceeding.

b) Prepare End Repair and A-tailing Mix by adding the reagents in the order shown below. Mix thoroughly and centrifuge briefly.

End Repair and A-tailing Mix	**1X (µl)**	**8.8X (µl)**
End Repair and A-tailing Buffer	7	62
End Repair and A-tailing Enzyme	3	26
Total	**10**	**88**

c) Add 10 µl End Repair and A-tailing Mix to each tube containing 50 µl sample from the Post Shearing Size Selection. Pipette mix 15 times and centrifuge briefly.

d) Incubate in a thermal cycler with the following protocol. The run will take ~1 h 5 min.

Lid Temperature	**Reaction Volume**	
85°C	60 µl	
Step	**Temperature**	**Time**
End Repair	20°C	30:00
A-tailing	65°C	30:00
Hold	4°C	Hold

e) Proceed immediately to the next step.

4.4. Library Construction: Adaptor Ligation

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

Adaptor Ligation Mix	1X (µl)	8.8X (µl)
Nuclease-Free Water	7.5	66
Ligation Buffer	30	264
DNA Ligase	10	88
R1 Adaptor Mix	2.5	22
Total	**50**	**440**

b) Add 50 µl Adaptor Ligation Mix to each tube containing 60 µl sample from the End Repair and A-tailing step. Pipette mix 15 times and centrifuge briefly.

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

Lid Temperature	Reaction Volume	
30°C	110 µl	
Step	**Temperature**	**Time**
1	20°C	15:00

d) Proceed immediately to the next step.

4.5. Post Ligation Cleanup 1 – SPRIselect

a) Vortex the SPRIselect Reagent until fully resuspended. Add 88 µl SPRIselect Reagent to each sample in the tube strip and pipette mix 15 times.

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 50.5 µl Buffer EB. Pipette mix 15 times.

k) Incubate the tube strip 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 50 µl of sample to a new tube strip.

4.6. Post Ligation Cleanup 2 – SPRIselect

a) Vortex the SPRIselect Reagent until fully resuspended. Add 50 µl SPRIselect Reagent to each sample in the tube strip and pipette mix 15 times.

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 125 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 30.5 µl Buffer EB. Pipette mix 15 times.

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

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

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

4.7. Sample Index PCR

NOTE

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

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

Sample Index PCR Mix	1X (µl)	8.8X (µl)
Nuclease-Free Water	8	70
Amplification Master Mix	50	440
SI-PCR Primer	2	18
Total	**60**	**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 Single Cell 3' Sample Index to each well and record their assignment. Pipette mix 15 times and centrifuge briefly.

d) Index the library DNA in a thermal cycler for a total of 10 cycles. The run will take ~25 min.

Lid Temperature	Reaction Volume	
105°C	100 µl	
Step	**Temperature**	**Time**
1	98°C	0:45
2	98°C	0:20
3	60°C	0:30
4	72°C	0:20
5	Go to step 2, 9X (for 10 cycles in total)	
6	72°C	1:00
7	4°C	Hold

STOP

e) Store the tube strip at 4 °C for up to 72 h or proceed directly to Post Sample Index PCR Cleanup.

4.8. Post Sample Index PCR Cleanup 1 – SPRIselect

a) Vortex the SPRIselect Reagent until fully resuspended. Add 100 µl SPRIselect Reagent to each sample in the tube strip and pipette mix 15 times.

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 50.5 µl Buffer EB. Pipette mix 15 times.

k) Incubate the tube strip 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 50 µl of sample to a new tube strip.

4.9. Post Sample Index PCR Cleanup 2 – SPRIselect

a) Vortex the SPRIselect Reagent until fully resuspended. Add 50 µl SPRIselect Reagent to each sample in the tube strip and pipette mix 15 times.

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 125 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 35.5 µl Buffer EB. Pipette mix 15 times.

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

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

m) Transfer 35 µl of sample to a new tube strip.

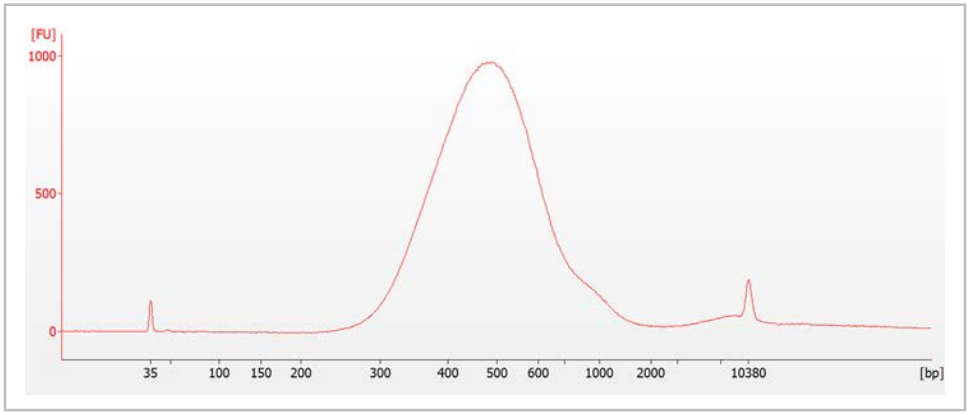
STOP

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

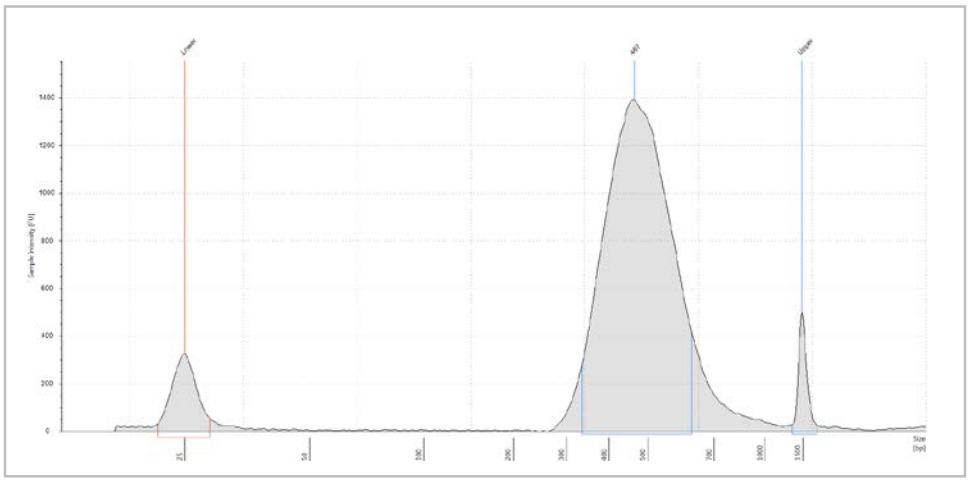
4.10. Post Library Construction QC

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.



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

d) Dispense 16 µl of Quantification Master Mix for sample dilutions and DNA Standards to 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 trace is used as the insert size for accurate library quantification in qPCR.

Protocol Step 5

Sequencing

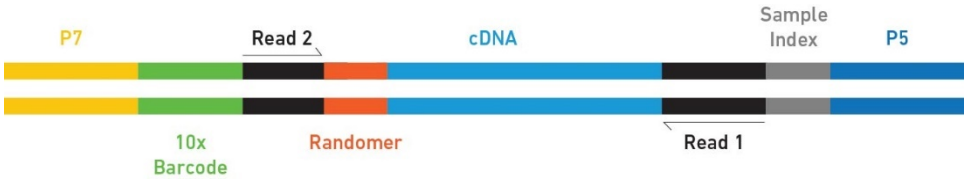
Sequencing prepared libraries

5

5. Sequencing Libraries

The Single Cell 3' Solution produces Illumina-ready sequencing libraries. This section of the protocol describes the steps required to ensure the successful sequencing of Single Cell 3' libraries to deliver the full value of the Single Cell 3' Solution.

A Single Cell 3' Library comprises standard Illumina paired-end constructs which begin and end with P5 and P7. The Single Cell 3' 14 bp 10x™ Barcodes are encoded at the start of the i7 index read, while sample index sequences are incorporated as the i5 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 cDNA fragment, while Read 2 is used to sequence the 10 bp randomer attached during the reverse transcription step.



Each sample index provided in the Single Cell 3' Sample Index Kit combines 4 different sequences in order to balance across all 4 nucleotides.

5.1. Sequencing Run Parameters

a) Single Cell libraries have been validated for sequencing on the Illumina NextSeq 500 (V2 chemistry, 150 cycle kit) and the HiSeq 2500 in Rapid Run mode (V1 and V2 chemistry, 200 cycle kits).

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

c) Single Cell libraries must be run using paired-end sequencing with dual indexing. The following read lengths are recommended:

   i. Read 1: 98 nt (shorter reads may result in reduced alignment rates)

   ii. i7 Index: 14 nt (any other length will result in a failed run)

   iii. i5 Index: 8 nt (any other length will result in a failed run)

   iv. Read 2: 10 nt (any other length will result in a failed run)

5.2. Loading Single Cell 3' Libraries

a) Once quantified and normalized, Single Cell 3' Libraries should be denatured and diluted as recommended for the Illumina HiSeq 2500 sequencer or the Illumina NextSeq 500. Refer to Illumina documentation for Denaturing and Diluting Libraries for the HiSeq and NextSeq sequencers. Suggested loading concentrations are 10 pM for HiSeq 2500 Rapid Run mode and 2.1 pM for NextSeq 500. Sequencing runs should be set up per Illumina recommendations for the number of cycles specified in section 5.1.

Practical Tips & Troubleshooting

Processing Fewer than 8 Reactions

Assembling a Chip, 10x™ Chip Holder & 10x™ Gasket

Pipetting Gel Beads

Pipetting GEMs

Surrogate Fluid

Reagent Clogs during GEM Generation

Wetting Failures

Chromium™ Controller Errors

Glossary of Terms

6

6. Practical Tips & Troubleshooting

6.1. 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 4 freeze-thaw cycles.

c) Never store Gel Beads at –20°C.

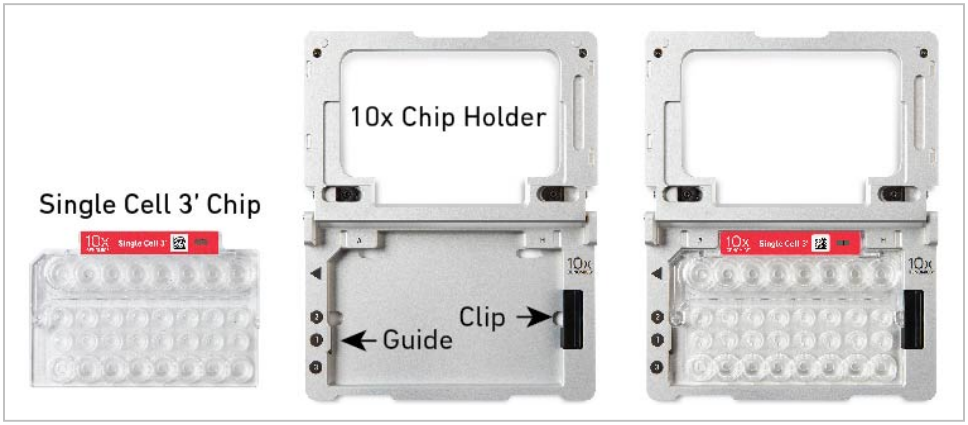
d) Reagent volumes should be calculated with a 10% excess of 1X values quoted in the protocol. e.g. For 3 samples, multiply the 1X volume quoted in the protocol by 3.3 to determine the suitable volume to prepare. Using larger reagent excesses may reduce the total number of reactions that can be run using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

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

6.2. Assembling a Chip, 10x™ Chip Holder & 10x™ Gasket

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.

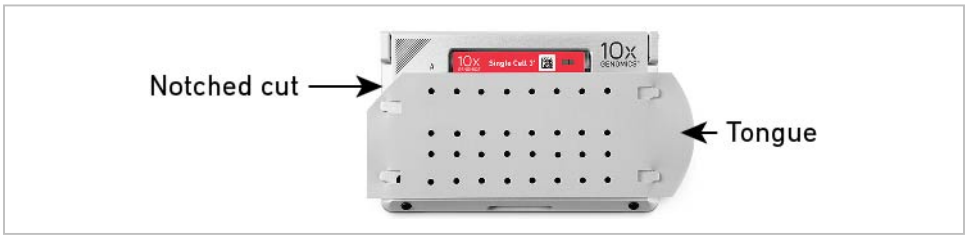
b) Depress the right-hand side of the Chromium Chip until the spring-loaded clip engages the Chromium Chip.



c) 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.

d) 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.

e) 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.



Click back to Loading the Single Cell 3' Chip

6.3. 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.

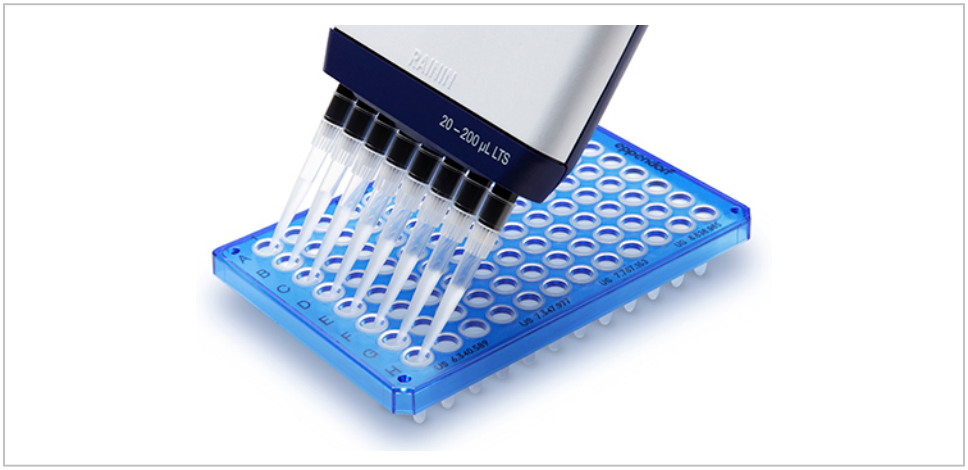
   iv. With the pipette tips still in the Gel Bead Strip, very slowly aspirate the required volume of Gel Beads. After aspiration stops, leave the pipette tips in the wells for an additional 5 sec to allow pressure to equilibrate.

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.

6.4. 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 Single Cell 3' Chip after the Chromium Controller run into the Eppendorf® twin-tec 96-well PCR plate (on a chilled metal block resting on ice), the pipette tips should be positioned against the side walls of the wells as shown below.



Click back to Loading the Single Cell 3' Chip

6.5. Surrogate Fluid

Surrogate Fluid is glycerol in a ~50% volume/volume aqueous solution. It is critical all unused wells in Rows labeled 1, 2 and 3 of the Single Cell 3' Chip contain Surrogate Fluid prior to running the Chromium™ Controller. If the amount of Surrogate Fluid provided in the Library Kit is insufficient, the following commercially available glycerol solution can be used as a substitute:

Ricca Chemical Company, Glycerin (glycerol), 50% (v/v) Aqueous Solution, P/N 3290-32

Alternatively, additional Surrogate Fluid 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) Surrogate Fluid should be equilibrated to room temperature before use.

6.6. Reagent Clogs during GEM Generation

GEM reagents are manufactured in a cleanroom environment to minimize the level of fibers and debris in GEM reagents that could cause a clog during GEM generation.

It is also important for users to minimize exposure of reagents, chips, and gaskets to sources of fibers such as reagent reservoirs, KimWipes, repeat-usage of flip-cap tubes, and the general laboratory environment. When care is taken to minimize the introduction of additional debris, the clog rate is typically very low. In the unlikely event that a clog occurs during GEM generation, **it is recommended that the sample be remade.**

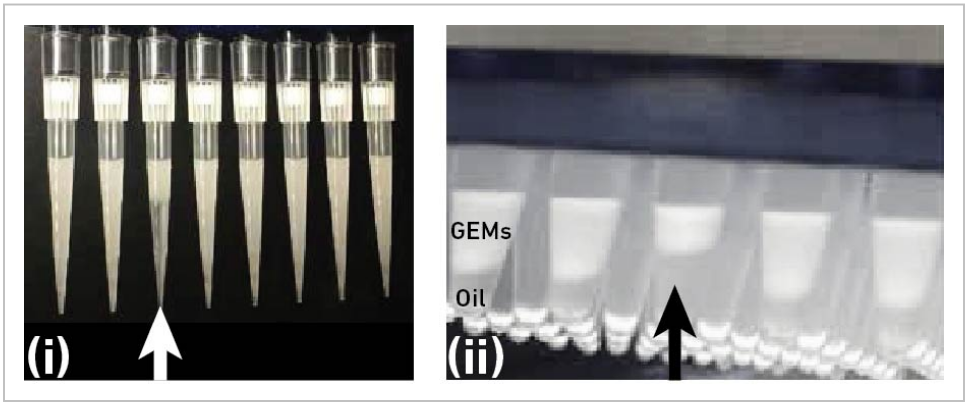
There are several ways to identify if a clog has occurred. If any of the following occur, take a picture and send it to techsupport@10xgenomics.com for further assistance. Continue processing the remaining samples:

a) After removing a Chromium™ Chip from the Chromium Controller, one or more wells contain abnormally high volume.

b) During GEM recovery:

   i. When transferring GEMs from the Chromium Chip to a PCR plate, there is excess Partitioning Oil in a pipette tip after aspiration. Note the excess Partitioning Oil present in the third tip from the left (below left, arrow).

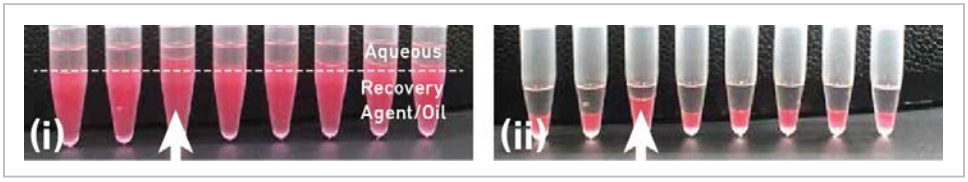
   ii. Excess Partitioning Oil can also be observed by comparing the volume uniformity in the PCR plate. After the GEMs are transferred to a PCR plate, a clog occurred if the Partitioning Oil volume (clear, bottom of well) in one or more wells is increased compared to other wells (below right, arrow).



c) After transfer of the GEMs + Recovery Agent to a tube strip:

   i. Wells with decreased aqueous sample indicate a clog during GEM generation when compared to wells with other normal samples (below left, arrow).

   ii. After aspirating the designated volume of Recovery Agent/Partitioning Oil, a greater volume of Partitioning Oil (pink) remaining in the PCR tubes (below right, arrow) also indicates a clog occurred.



6.7. 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. If a wetting failure occurs during GEM generation, it is recommended that the sample be remade.

The occurrence of a wetting failure can be recognized during GEM recovery by the absence of uniform GEM emulsion in an outlet well or pipette tip (below, arrow). If this occurs, take a picture and send it to techsupport@10xgenomics.com for further assistance. Continue processing the remaining samples.

6.8. Chromium™ Controller Errors

If the Chromium 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 techsupport@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 techsupport@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 techsupport@10xgenomics.com for further assistance.

ii. If this message is received after a few minutes into the run, it is likely one or more of the reagents was not loaded into the Chromium Chip. In this case, the Chromium Chip must be discarded. **Do not try running this Chromium Chip again as this will 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 techsupport@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 techsupport@10xgenomics.com for further assistance.

Click back to Running the Chromium Controller

6.9. 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™ Single Cell 3' Chip

The Chromium Single Cell 3' Chip is a microfluidic chip specifically designed to run the Single Cell 3' Protocol in the Chromium Controller. The Single Cell 3' Chip 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 Single Cell 3' Chip, 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.