May 03, 2024

Multiple Myeloma Immune Atlas Consortium: Gene Expression Profiling of the Bone Marrow Microenvironment

DOI
dx.doi.org/10.17504/protocols.io.q26g718z3gwz/v1

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.q26g718z3gwz/v1

Protocol Citation: Manoj Bhasin 2024. Multiple Myeloma Immune Atlas Consortium: Gene Expression Profiling of the Bone Marrow Microenvironment . **protocols.io** https://dx.doi.org/10.17504/protocols.io.q26g718z3gwz/v1

Protocol status: Working
We use this protocol and it's working

Created: April 19, 2024

Last Modified: May 03, 2024

Protocol Integer ID: 98507

Keywords: Multiple Myeloma, scRNA-seq, Single-cell RNA-seq

Abstract

A comprehensive gene expression (GEX) profiling protocol that assists in generating the most granular map of single cell landscape from clinical trials supported by Multiple Myeloma Research Foundation and identify key markers of response along with opening avenues for new therapies.

Materials

S.No.	Company	Reagent	Cat. No.
1	10X genomics	Dynabeads MyOne TM SILANE	2000048 (store at 4°C)
2	10X genomics	Chromium Next GEM Single Cell 3’ Gel Bead Kit v3.1, 16 rxns	1000122 (store at -20°C and -80°C according to manufacturer’s instructions)
3	10X genomics	Chromium Next Gem Chip G Single Cell Kit, 48 rxns	1000120 (store at RT)
4	10X genomics	Dual Index Plate TT Set A	3000431 (store at −20°C)
5	Ambion	Nuclease free Water	AM9937
6	Thermo Fisher Scientific	Low TE Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA)	12090-015
7	Millipore Sigma	Ethanol, Pure (200 Proof, anhydrous)	E7023-500ML
8	Beckman Coulter	SPRIselect Reagent Kit	B23318
9	Bio-Rad	10% Tween 20	1662404
10	Ricca Chemical Company	Glycerin (glycerol), 50% (v/v) Aqueous Solution	3290-32
11	Qiagen	Qiagen Buffer EB	19086

Materials for GEM Generation and Barcoding - 1

Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature	Chromium Single Cell 3’ v3 Gel Beads	2000164	Equilibrate to RT 30 min before loading the chip.	−80°C
RT Reagent B	2000165	Vortex, verify no precipitate, centrifuge briefly.	−20°C	
Template Switch Oligo	3000228	Centrifuge briefly, resuspend in 80 µl Low TE Buffer. Vortex 15 sec at maximum speed, centrifuge briefly,	−80°C	

		leave at room temperature for ≥ 30 min. After resuspension, store at −80°C.		
Reducing Agent B	2000087	Vortex, verify no precipitate, centrifuge briefly.	−20°C	
Place on Ice	RT Enzyme C	2000085/ 2000102	Centrifuge briefly before adding to the mix.	−20°C
Cell Suspension				
Obtain	Partitioning Oil	2000190	-	Ambient
Chromium Next GEM chip G	2000177	-	Ambient	
10x Gasket	370017/ 3000072		Ambient	
10x Chip Holder	330019		Ambient	
10x Vortex Adapter	330002		Ambient	
50% glycerol solution If using <8 reactions	-		-Ambient	

Materials for GEM Generation and Barcoding -2

Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature	Reducing Agent B	2000087	Thaw, vortex, verify no precipitate, centrifuge.	−20°C
cDNA primers	2000089	Vortex, centrifuge briefly.	−20°C	
Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendations.	-	

	Agilent Bioanalyzer High Sensitivity Kit If used for QC and quantification	-	Manufacturer's recommendations.	-	
	Agilent TapeStation ScreenTape and Reagents If used for QC and quantification	-	Manufacturer's recommendations.	-	
	Qubit dsDNA HS Assay Kit If used for QC and quantification	-	Manufacturer's recommendations.	-	
	Dynabeads MyOne SILANE	2000048	Vortex thoroughly (≥30 sec) immediately before adding to the mix.	4°C	
	Place on ice	Amp Mix	2000047/ 2000103	Vortex, centrifuge briefly.	−20°C
	Thaw at 65°C	Cleanup Buffer	2000088	Thaw for 10 min at 65°C at max speed on a thermomixer. Verify no visible crystals. Cool to room temperature.	−20°C
	Obtain	Recovery Agent	220016	-	Ambient
	Qiagen Buffer EB	-	Manufacturer's recommendations.	-	
	Bio-Rad 10% Tween 20	-	Manufacturer's recommendations.	-	
	10x Magnetic Separator	230003	-	Ambient	
	Prepare 80% Ethanol - Prepare 15 ml for 8 reactions.	-	-		

Materials for Post GEM-RT Cleanup & cDNA Amplification

	Obtain the following 10X kit and other components				
	Action	Item	10x PN	Preparation & Handling	Storage
	Equilibrate to Room Temperature	Fragmentation Buffer	2000091	Vortex, verify no precipitate, centrifuge briefly.	−20° C
	Adaptor Oligos	2000094	Vortex, centrifuge briefly.	−20°C	
	Ligation Buffer	2000092	Vortex, verify no precipitate, centrifuge briefly.	−20°C	
	Dual Index Plate TT Set A	3000431	-	−20°C	
	Beckman Coulter SPRIselect Reagent	B23318		Ambient	
	Agilent Bioanalyzer High Sensitivity kit If used for QC	-			
	Place on Ice	Fragmentation Enzyme DNA Ligase	2000090/ 2000104	Centrifuge briefly.	−20° C
	DNA Ligase	220110	Centrifuge briefly.	−20°C	
	Amp Mix	220131	Centrifuge briefly.	−20°C	

Materials for 3’ Gene Expression Dual Index Library Construction

Thawing & Washing bone marrow (BM) derived cells

1 **Preparation**

1.1 Warm water bath to 37 °C prior to commencing the thawing Protocol.

1.2 Prepare ~ 42 mL warm complete growth medium (e.g. 10% FBS in RPMI-1640) per sample by incubating in a 37 °C water bath prior to use.

1.3 Prepare 1X PBS with 0.04% BSA solution (keep on ice).

1.4 Thaw a vail of NIH3T3 viably as detailed in **10X genomics protocol**:

CG00039_Demonstrated_Protocol_FreshFrozenHumanPBMCs_RevD.pdf

Keep the thawed cells on ice till spiking into viably thawed patients' sample (step 2.19)

1.5 Add 1 mL cold 1X PBS with 0.04% BSA (40 mg/100ml) and gently pipette mix 5 times with a wide bore tip. Take an 10 µL aliquot for counting.

1.6 Centrifuge cells at 370 g for 00:05:00 at 4 °C. 5m

1.7 Remove supernatant without disrupting the cell pellet. Save the removed until the Protocol is complete.

1.8 Using a regular-bore pipette tip, add 1 mL 1X PBS with 0.04% BSA or an appropriate volume to achieve a cell concentration of ~1 x 106 cells/ml. Gently pipette mix to completely suspended the cells (keep resuspended cells on ice).

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

1.9 If needed, use a cell strainer (a 40 µm Flowmi™ Tip Strainer) to remove cell debris and large clumps.

1.10 Determine the cell concentration, viability using a Bio-Rad T20 Cell Counter.

2 **Thawing, Washing & Counting Cells** (do at Room temperature)

2.1 Remove cryovial(s) from liquid or vapor-phase nitrogen storage and immediately thaw in the water bath at 37 °C for 00:02:00 to 00:03:00 . Do not submerge the entire vial in the water bath. Remove from the water bath when a tiny ice crystal remains. 2m

2.2 After thawing is complete, clean the vial with 70% alcohol and Kim Wipes.

2.3 In a biosafety hood, gently transfer thawed cells to a 50 mL conical tube using a wide-bore pipette tip.

2.4 Using a wide-bore pipette tip, rinse the cryovial with 1 mL warm complete growth medium.

2.5 Using a wide-bore pipette tip, add the rinse medium dropwise (1 drop per 5 sec*) to the 50 ml conical tube while intermittently gently shaking the tube.

*very important- add medium slowly as described.

2.6 Serially dilute cells with complete growth medium a total of 5 times by 1:1 volume additions with ~ 00:01:00 wait between additions (for e.g. after the first addition of 1ml, wait 00:01:00 , then add 2 mL , wait 00:01:00 , then add 4 ml and so on). Add complete growth medium at a speed of 3-5 ml/sec to a total of 32 mL . 3m

2.7 Centrifuge cells at 370 g for 00:05:00 at Room temperature . 5m

2.8 Remove most of the supernatant*, resuspend cell pellet in the remaining media using a regular-bore pipette tip (or tap gently against hand palm). *Save the removed supernatant in another tube until the protocol is complete.

2.9 Add an additional 9 mL complete growth medium (at a speed of 3-5 ml/sec) to achieve a total volume of ~ 10 mL .

2.10 Determine the cell concentration using a Bio-Rad T20 Cell Counter. Calculate the total cell number (N) based on the total volume (V) and concentration (C) where N = C x V. Use the form below to note cell counts.

#	Sample ID	Cells per ul	Cells to capture	Vol of suspension	Vol. of Water	
1						
2						
3						
4						
5						
6						

Appendix A

2.11 If total cell number is ≤2 x 106 cells, use the entire sample for washing. If total cell number is >2 x 106 cells, transfer ~2 million cells into a new tube for further processing. 1h

(Note: Excess cells will be pelleted, frozen and stored for future purposes).

Determine cell viability. All bone marrow mononuclear cell (BMMC) samples should be cleaned-up using the dead-cell removal kit prior to analysis (e.g. Miltenyi Dead Cell Removal Kit, Cat. No. 130-090-101).

1. Centrifuge cells at 370 g for 00:05:00 at Room temperature .
2. Remove most of the supernatant by decanting gently. Remove as much supernatant as possible. Add 100 µL dead cell removal microbeads per 107 total cells. Gently resuspend cells and microbead solution using a wide-orfice pipette. Incubate for 00:15:00 at Room temperature .
3. During the 00:15:00 incubation, prepare 1X binding buffer from 20X binding buffer stock solution, e.g. dilute 500 µL of 20X binding buffer stock solution with 9.5 mL of sterile, double-distilled water. Choose appropriate MACS column (LS column or autoMACS column) and MACS separator (Magnet ot autoMACS Pro Separator) for magnetic separation.

If using LS column:

1. Place LS column in the magnetic field of a MACS separator and place a 15 mL column underneath.
2. Prepare column by rinsing with 3 mL of 1X binding buffer.
3. After initial 3 mL has run through column, remove 15 mL comical and replace with a new 15 mL conical tube.
4. After cell suspension has incubated for 00:15:00 with microbeads, apply cell suspension to column.
5. Wash the tube holding cells with microbeads with 3 mL of 1X binding buffer and apply to the column.
6. Wash column with 3 mL of 1X binding buffer three more times (add subsequent wash each time the column is empty from previous wash).
7. Take resulting suspension in 15 mL conical and spin down at 370 g for 00:05:00 at 4 °C .

If using autoMACS column:

1. Dilute 0.25 mL of 20X binding buffer with 4.75 mL of distilled water.
2. Add 500 µL l of 1X binding buffer to each tube
3. Run each through DepleteS selection on the autoMACS.
4. Toss the positive fractions. Negative fractions containing the live cells should be spun down at 370 g for 00:05:00 at 4 °C .

2.12 Remove supernatant without disrupting the cell pellet. Save the removed supernatant in another tube until the protocol is complete.

2.13 Add 1 mL cold 1X PBS with 0.04% BSA (40 mg/100ml) and gently pipette mix 5 times with a wide bore tip. Take an 10 µL aliquot for counting

2.14 Centrifuge cells at 370 g for 00:05:00 at 4 °C 5m

2.15 Remove supernatant without disrupting the cell pellet. Save the removed until the Protocol is complete.

2.16 Using a regular-bore pipette tip, add 1 mL 1X PBS with 0.04% BSA or an appropriate volume to achieve a cell concentration of ~1 x 106 cells/ml. Gently pipette mix to completely suspended the cells (keep resuspended cells on ice).

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

2.17 If needed, use a cell strainer (a 40 µm Flowmi™ Tip Strainer) to remove cell debris and large clumps.

2.18 Determine the cell concentration, viability using a Bio-Rad T20 Cell Counter.

2.19 Spiking in NIH3T3 cells: Adjust volume of cells to get about 2 million cells per ml. Spike in NIH3T3 cells into the patient sample at a ratio of 50:1 patient sample: murine sarcoma cells. For e.g., mix 1:1 of 100 µL of 200,000 multiple myeloma sample cells and 100 µL of 4000 NIH3T3 cells.

2.20 Proceed with the 10X Genomics ® Single Cell Protocol.

GEM Generation and Barcoding

3 Prepare Master Mix on ice according to the standard 10X protocol and dispense 31.8 µL per sample into a 8 strip tube kept on ice.

Master Mix	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
RT Reagent B	2000165	18.8	82.2	165.0
Template Switch Oligo	3000228	2.4	10.4	20.8
Reducing Agent B	2000087	2.0	8.6	17.3
RT Enzyme C	2000085/ 2000102	8.7	38.4	76.8
Total	-	31.8	139.9	279.8

4 Assemble Chromium Chip G in a Chromium Next GEM Secondary Holder according to the manufacturer's instructions.

5 Add 50% glycerol solution in wells that will not be used for single cell prep (70 µL in row 1, 50 µL in row 2, 45 µL in row 3).

6 Add appropriate volume of nuclease free water based on the cell concentration measured in step 2.19. according to appendix B (for targeting 5000 cells) to the master mix. Mix 4-5 times. Mix the cells and add 5000 cells (volume calculated according to the table – appendix B) to the diluted master mix. Gently mix 5X. Gently dispense 70 µL Master Mix + Cell Suspension into the bottom center of each well in row labeled 1 without introducing bubbles.

	Sample ID	Total counts	Live counts	Viability	Dilution	Total counts	Live counts	Viability
1								
2								
3								
4								

Appendix B

7 Vortex beads for 00:00:30. Centrifuge the Gel Bead strip for ~ 00:00:05. Confirm there are no bubbles at the bottom of the tubes and the liquid levels are even. Place the Gel Bead strip back in the holder. Secure the holder lid. Slowly aspirate 50 µL Gel Beads. Dispense into the bottom of wells in row labeled 2 without introducing bubbles. 35s

8 Wait 00:00:30 and then dispense 45 µL Partitioning Oil into the wells in row labeled 3. Attach 10x Gasket and load the chip on the 10X chromium controller and run the GEM generation program (Firmware V4 or higher required). Proceed to next step as soon as the run is over (00:17:00). Note any errors that occur during run. 17m 30s

9 Place a tube strip on ice. Press the eject button of the Controller and remove the chip. Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees. Ensure that the partitioning oil from the wells does not spill when exposing the wells.

10 Slowly aspirate (over ~ 00:00:20) 100 µL GEMs from the lowest points of the Recovery Wells in the top row without creating a seal between the tips and the bottom of the wells. Withdraw pipette tips from the wells. Check GEMs. GEMs should appear opaque and 40s

uniform across all channels. Over the course of ~ 00:00:20 , dispense GEMs into the tube strip on ice with the pipette tips positioned at an angle against the sidewalls of the tubes.

11 Load the GEM samples from step 7 into a PCR machine and run the standard 10X recommended program for cDNA generation. 3d 0h 55m

Lid Temperature 53 °C , Reaction Volume 125 µL , Run Time ~ 00:55:00

Step	Temperature	Time
1	53°C	00:45:00
2	85°C	00:05:00
3	4°C	Hold

Store at 4 °C for up to 72:00:00 or at -20 °C for up to a week, or proceed to the next step.

Post GEM-RT Cleanup & cDNA Amplification

12 **Post GEM cleanup**

12.1 Add 125 µL Recovery Agent to each sample at room temperature. DO NOT pipette mix or vortex the biphasic mixture. Wait 00:02:00 . The resulting biphasic mixture contains Recovery Agent/Partitioning Oil (pink) and aqueous phase (clear), with no persisting emulsion (opaque). 2m

12.2 Slowly remove 125 µL Recovery Agent/ Partitioning Oil (pink) from the bottom of the tube. DO NOT aspirate any of the top aqueous sample.

12.3 Prepare Dynabeads cleanup mix (DCM) according to number of reactions. Vortex well.

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

	Dynabeads MyOne SILANE Vortex thoroughly (≥30 sec) immediately before adding to the mix. If still clumpy, pipette mix to resuspend completely. DO NOT centrifuge before use.	2000048	8	35	70
	Reducing Agent B	2000087	5	22	44
	Nuclease-free Water		5	22	44

12.4 Add 200 µL of Dynabeads Mix to each sample. Pipette mix and Incubate for 00:10:00 * at Room temperature . 25m

*At 5 minutes, pipette mix 5X. At the end of 00:10:00 incubation, gently place on a 10x Magnetic Separator (high end) until the solution clears (~ 00:05:00).

12.5 Prepare 80% ethanol. Also prepare Elution solution 1 (ES1)

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

12.6 Remove the supernatant (from step 12.4). Add 300 µL 80% ethanol to the pellet while on the magnet. Wait 00:00:30 and remove the ethanol. 30s

12.7 Add 200 µL 80% ethanol to pellet. Wait 00:00:30 . Remove the ethanol. 30s

12.8 Centrifuge briefly. Place on the magnet (low end). Remove remaining ethanol. Air dry for 00:01:00 . 1m

12.9 Remove from the magnet. Immediately add 35.5 µL Elution Solution I. Pipette mix without introducing bubbles.

12.10 Incubate 00:02:00 at room temperature. 2m

12.11 Place on the magnet until the solution clears (~ 00:05:00). 5m

12.12 Transfer 35 µL sample to a new tube strip. Keep on ice

13 cDNA Amplification

13.1 Prepare cDNA Amplification Mix on ice according to 10X recommendation. Vortex and centrifuge briefly.

DNA Amplification Reaction Mix Add reagents in the order listed	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Amp Mix	2000047/ 2000103	50	220	440
cDNA Primers	2000089	15	66	172
total		65	286	572

13.2 Add 65 µL cDNA Amplification Reaction Mix to 35 µL sample from step 12.12. Pipette mix and centrifuge briefly. Incubate in a thermal cycler and run the 10X recommended cDNA amplification program.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~30-45 min

Step	Temperature	Time
1	98°C	00:03:00
2	98°C	00:00:15
3	63°C	00:00:20
4	72°C	00:01:00

5	Go to Step 2, see table below for total # of cycles	
6	72°C	00:01:00
7	4°C	Hold

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

13.3 Store at 4 °C for up to 72:00:00 or proceed to the next step. 3d

13.4 Vortex to resuspend the SPRIselect reagent. Add 60 µL SPRIselect reagent (0.6X) to each sample and pipette mix 15x (pipette set to 150 µL). Incubate 00:05:00 at room temperature and place on the magnet (high end) until the solution clears (~ 00:05:00). 10m

13.5 Discard the transferred supernatant without disturbing the pellet. DO NOT discard the pellet (cleanup for 3′ Gene Expression library construction).

14 cDNA cleanup - SPRIselect

3d 0h 4m 30s

1. Add 200 µL 80% ethanol to the pellet. Wait 00:00:30.
2. Remove the ethanol.
3. Repeat steps a and b for a total of 2 washes.
4. Centrifuge briefly and place on the magnet (low end).
5. Remove any remaining ethanol. Air dry for 2min. DO NOT exceed 00:02:00 as this will decrease elution efficiency.
6. Remove from the magnet. Add 40.5 µL Buffer EB. Pipette mix 15x.
7. Incubate 00:02:00 at Room temperature.
8. Place the tube strip on the magnet (High end) until the solution clears.
9. Transfer 40 µL sample to a new tube strip.
10. Store at 4 °C for upto 72:00:00 or at -20 °C for up to 4 weeks or proceed to 3′ Gene Expression Dual Index Library Construction.
11. Quantify cDNA concentration with Qubit dsDNA HS Assay Kit on the Qubit Flurometer 3. Run 1 µL of sample from Pellet Cleanup from above (dilute if need to 1ng/ul) on an Agilent Bioanalyzer High Sensitivity chip to check for quality.

3′ Gene Expression Dual Index Library Construction

15 Fragmentation, end-repair and labeling

15.1 Prepare a thermal cycler with the 10X protocol for fragmentation.

Lid Temperature	Reaction Volume	Run Time
65°C	50 µl	~35 min

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

15.2 Prepare Fragmentation Mix on ice according to instructions.

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

Pipette mix on ice and centrifuge briefly.

15.3 Transfer ONLY 10 µL purified cDNA sample from from step 14.9 to a tube strip. Add 25 µl Buffer EB to each sample followed by adding 15 µl Fragmentation Mix to each sample.

15.4 Pipette mix on ice and centrifuge briefly. Transfer into the pre-cooled thermal cycler (4 °C) and press “SKIP” to initiate the protocol. After completion, proceed to next step.

16 Post Fragmentation, End Repair & A-tailing: Double Sided Size Selection – SPRIselect

16.1 Vortex to resuspend SPRIselect reagent. Add 30 µL SPRIselect (0.6X) reagent to each sample from above step. Pipette mix and incubate 00:05:00 at Room temperature *. 5m
* Prepare 80% ethanol

16.2 Place on the magnet (high end) until the solution clears and then transfer 75 µL supernatant to a new tube strip.

16.3 Vortex to resuspend SPRIselect reagent. Add 10 µL SPRIselect reagent (0.8X) to each sample. Pipette mix and incubate 00:05:00 at Room temperature . 5m

16.4 Place on the magnet (high end) until the solution clears.

16.5 Remove 80 µL supernatant. DO NOT discard any beads.

16.6 Wash beads by adding 125 µL 80% ethanol to the pellet. Wait 00:00:30 . 30s

16.7 Remove the ethanol.

16.8 Repeat steps 16.6 and 16.7 for a total of 2 washes.

16.9 Centrifuge briefly. Place on the magnet (Low end) until the solution clears. Remove remaining ethanol.

16.10 Remove from the magnet. Add 50.5 µL Buffer EB to each sample. Pipette mix and incubate 00:02:00 at Room temperature . 2m

16.11 Place on the magnet (high end) until the solution clears and transfer 50 µL sample to a new tube strip.

17 Adaptor Ligation

17.1 Prepare Adaptor Ligation Mix according to 10X protocol

	Adaptor Ligation Mix Add reagents in the order listed	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
	Ligation Buffer	2000092	20	88	176
	DNA Ligase	220110/ 220131	10	44	88
	Adaptor Oligos	2000094	20	88	176
	Total		50	220	440

Pipette mix and centrifuge briefly. Add 50 µL Adaptor Ligation Mix to 50 µL sample from step 6.4.2.k. Pipette mix and centrifuge briefly.

17.2 Incubate in a thermal cycler with the 10X adaptor ligation protocol.

	Lid Temperature	Reaction Volume	Run Time
	30°C	100 µl	15 min

	Step	Temperature	Time
	1	20°C	00:15:00
	2	4°C	Hold

18 **Post Ligation Cleanup with SPRIselect**

18.1 Vortex to resuspend SPRIselect Reagent. Add 80 µL SPRIselect Reagent (0.8X) to each sample. Pipette mix and incubate 00:05:00 at Room temperature . 5m

18.2 Place on the magnet (high end) until the solution clears. Remove the supernatant.

18.3 To wash, add 200 µL 80% ethanol to the pellet. Wait 00:00:30 . 30s

18.4 Remove the ethanol.

18.5 Repeat steps 18.3 and 18.4 for a total of 2 washes.

18.6 Centrifuge briefly and place on the magnet (low end).

18.7 Remove any remaining ethanol. Air dry for 00:02:00 . DO NOT exceed 00:02:00 as this will decrease elution efficiency. Remove from the magnet. Add 30.5 µL Buffer EB. Pipette mix and incubate 00:02:00 at Room temperature . 6m

18.8 Place on the magnet (Low end) until the solution clears.

18.9 Transfer 30 µL sample to a new tube strip.

Sample Index PCR

19 Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x Sample Index name (PN-1000215 Dual Index Plate TT Set A well ID) used.

20 Add 50 µL Amp Mix (PN-2000047 or 2000103) to 30 µL sample.

21 Add 20 µL of an individual Dual Index TT Set A to each sample and record the well ID used. Pipette mix 5x (pipette set to 90 µL). Centrifuge briefly.

22 Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25-40 min

Step	Temperature	Time
1	98°C	00:00:45
2	98°C	00:00:20
3	54°C	00:00:30
4	72°C	00:00:20
5	Go to step 2, see below for # of cycles	
6	72°C	00:01:00
	4°C	Hold

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

23 Store at 4 °C up to 72:00:00 or proceed. 3d

Post Sample Index PCR Double Sided Size Selection with SPRIselect

24 Vortex to resuspend the SPRIselect reagent. Add 60 µL SPRIselect Reagent (0.6X) to each sample. Pipette mix and incubate 00:05:00 at room temperature. 5m

25 Place on the magnet until the solution clears. DO NOT discard supernatant. Transfer 150 µL supernatant to a new tube strip.

26 Vortex to resuspend the SPRIselect reagent. Add 20 µL SPRIselect Reagent (0.8X) to each sample. Pipette mix and incubate 00:05:00 at Room temperature. 5m

27 Place on the magnet until the solution clears. Then remove 165 µL supernatant. DO NOT discard any beads.

28 With the tube still in the magnet, add 200 µL 80% ethanol to the pellet to wash the beads. Wait 00:00:30. 30s

29 Remove the ethanol.

30 Repeat steps 28 and 29 for a total of 2 washes.

31 Centrifuge briefly. Place on the magnet. Remove remaining ethanol.

32 Remove from the magnet. Add 35.5 µL Buffer EB. Pipette mix and incubate 00:02:00 at Room temperature and then place on the magnet until the solution clears. 2m

33 Transfer 35 µL to a new tube strip. Store at 4 °C for up to 72:00:00 or at -20 °C for long-term storage. 3d

Post Library Construction QC

34 Determine concentration using Qubit fluorimeter. Run 1 µL sample (dilute to 1-5ng/ul) on an Agilent Bioanalyzer High Sensitivity chip.
Determine the average fragment size from the Bioanalyzer trace. This will be used as the insert size for library quantification.

Sequencing the libraries

35 3’ Gene Expression Library Sequencing Depth & Run Parameters

Sequencing Depth: Minimum 50,000 read pairs per cell
Sequencing Type: Paired-end, dual indexing

Sequencing Read	Recommended Number of Cycles
Read 1 i7 Index i5 Index Read 2	28 cycles 10 cycles 10 cycles 90 cycles

Protocol references

CG00039_Demonstrated_Protocol_FreshFrozenHumanPBMCs_RevD (Fresh Frozen Human PBMCs for Single Cell RNA Sequencing Protocols)

https://assets.ctfassets.net/an68im79xiti/1eX2FPdpeCgnCJtw4fj9Hx/7cb84edaa9eca04b607f9193162994de/CG000204_ChromiumNextGEMSingleCell3_v3.1_Rev_D.pdf

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