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Single Cell Sequencing 10x Chromium 5' VDJ

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ABSTRACT

Single cell RNA sequencing technique (scRNA-Seq) obtains gene expression profiles of individual cells for analysis, as opposed to comparing averaged gene expression signals between bulk samples of cells. The ability to examine transcriptional changes between individual cells at a high resolution uniquely define rare cell populations, identify heterogeneity within cell populations, investigate cell population dynamics in depth over time, and even interrogate cell signaling pathways.

ScRNA sequencing enables the exploration of the cellular heterogeneity from the various biospecimen tissues; tumor, biopsy samples and organoid cells. To profile the immune repertoire of cells, full-length (5' UTR to constant region), paired T-cell receptor (TCR) and/or B-cell receptor (BCR) transcripts from 500-10,000 individual cells per sample can be assessed. A pool of approximately 750,000 barcodes are sampled separately to index each cell's transcriptome and antigen specificity. It is done by partitioning thousands of cells into nanoliter-scale Gel Beads-in-emulsion (GEMs), where all generated cDNA share a common 10x Barcode. Libraries are generated and sequenced and 10x Barcodes are used to associate individual reads back to the individual partitions.

PROTOCOL integer ID: 94914

MATERIALS

- Chromium Next GEM Single Cell 5' Library and Gel Bead Kit v1.1 16 rxns (10x Genomics #1000165)
- Chromium Single Cell 5’ Library Construction Kit 16 rxns (10x Genomics #1000020)
- Chromium Single Cell V(D)J Enrichment Kit, Human B Cell 96 rxns (10x Genomics #1000016)
- Chromium Single Cell V(D)J Enrichment Kit, Human T Cell 96 rxns (10x Genomics #1000005)
- Single Index Kit T Set A 96 rxns (10x Genomics #1000213)
- Chromium Next GEM Chip G Single Cell Kit, 48 rxns (10x Genomics #1000202)
- Chromium Controller & Next GEM Accessory Kit (#1000202)
- Thermocycler - Veriti 96 well (Thermo Fisher Scientific #4375786)
- Thermomixer 1.5 mL (Eppendorf #EP5382000023)
- Quant Studio 3 (Thermo Fisher Scientific #A28567)
- 2100 Bioanalyzer (Agilent #G2939BA)
- 4200 Tape Station (Agilent #G2991A)
- Nuclease Free Water (Ambion #AM9937)
- Ethanol (Acros Organics #AC615095000)
- Elution Buffer (Qiagen #19086)
- Tween 20 (Thermo Fisher Scientific #BP337-100)
- SPRISelect (Beckman Coulter #B23319)
- 50% GLYCEROL (RICCA CHEMICAL, CAT# 329032)
- DNA LOBIND TUBE 1.5 ML (EPPENDORF #022431021)
- PCR TUBE STRIPS 0.2 ML (EPPENDORF #951010022)

SAFETY WARNINGS

- All personnel must have completed the necessary training, including annual refresher training, on the safe handling of potentially infectious material.
- Personal protective equipment (PPE), which includes gowns, gloves, and protective goggles.

GEM Generation and RT Reaction

1 Prepare master mix on ice. Pipette mix 15x and centrifuge briefly.

Master Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
RT Reagent B	2000165	18.8	82.7	165.4
Poly-dT RT Primer	2000007	6.4	28.2	56.3
Additive A	220074	2.0	8.8	17.6
RT Enzyme Mix B	2000010/ 2000021	10.0	44.0	88.0
Total	-	37.2	163.7	327.3

2 Add 37.2 µl Master Mix into each tube of a PCR 8-tube strip on ice.

3 Assemble Chromium Next GEM Chip G:

Assemble Chromium Next GEM Chip G

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

TIPS

See Tips & Best Practices for chip handling instructions.

- Align notch on the chip (upper left corner) and the holder.
- Insert the left-hand side of the chip under the guide. Depress the right-hand side of the chip until the spring-loaded clip engages.
- Close the lid before dispensing reagents into the wells.
- The assembled chip is ready for loading the indicated reagents. Refer to step 1.2 for reagent volumes and loading order.

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

4 Load 50% Glycerol into Unused Chip Wells (if <8 samples per chip): i. 70 ul to unused wells in row labeled 1. ii. 50 ul to unused wells in row labeled 2. iii. 45 ul to unused wells in row labeled 3.

5 Prepare Master Mix + Cell Suspension. Refer to the Cell Suspension Volume Calculator Table. Add the appropriate volume of nuclease-free water and corresponding volume of single cell suspension to Master Mix for a total of 75 ul in each tube. Gently pipette mix the cells suspension before adding to the Master Mix.

6 Gently pipette mix the Master Mix + Cell Suspension and using the same pipette tip, dispense 70 ul Master Mix + Cell Suspension into the bottom center of each well in row labeled 1 without introducing bubbles.

7 Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex 30 sec. Centrifuge the Gel Bead strip for ~5 seconds. 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.

8 Puncture the foil seal of the Gel Bead tubes. Slowly aspirate 50 ul Gel Beads. Dispense into the wells in row labeled 2 without introducing bubbles. Wait 30 seconds.

9 Dispense 45 ul Partitioning Oil into the wells in row labeled 3 from a reagent reservoir. Failure to add Partitioning Oil to the top row labeled 3 will prevent GEM generation and can damage the Chromium Controller.

10 Attach 10x gasket (notch on top left)

11 Place the assembled chip with the gasket into the tray of the Chromium Controller, ensuring the chip stays horizontal. Close the tray and press the play button to begin run (18 minutes). Complete next steps during run.

12 Place a PCR 8-tube strip on ice.

13 When chip run is complete, press the eject button of the Controller to remove the chip. Discard the gasket, open chip holder, and fold the lid back until it clicks to expose the wells at a 45-degree angle.

14 Transfer 100 µl of GEMs into PCR strip tube. Pipette slowly. It should take ~20 seconds to pipette GEMs.

15 If multiple chips are run back-to-back, cover the GEM-containing strip tube and place on ice for no more than 1 hour.

16 Run the following thermocycler program:

Lid Temperature	Reaction Volume	Run Time
53°C	125 µl	~55 min
Step	**Temperature**	**Time**
1	53°C	00:45:00
2	85°C	00:05:00
3	4°C	Hold

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

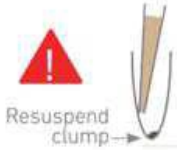
Post GEM Incubation Cleanup

17 Add 125 ul Recovery Agent to each sample at room temperature. DO NOT pipette mix or vortex the biphasic mixture. Gently invert tube 10x to mix. Centrifuge briefly.

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

19 Prepare Dynabeads Cleanup Mix:

Dynabeads Cleanup Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water		5	22	44
Buffer Sample Clean Up 1	220020	182	801	1602
Dynabeads MyOne SILANE Vortex thoroughly (≥30 sec) **immediately** before adding to the mix. Aspirate the full liquid volume with a pipette tip to verify that the beads have not settled in the bottom of the tube. If clumps are present, pipette mix to resuspend completely. DO NOT centrifuge before use.	2000048	8	35	70
Additive A	220074	5	22	44
Total	-	200	880	1760



20 Vortex and add 200 µl of Dynabeads Cleanup Mix to each sample. Pipette mix 5x (pipette set to 200 ul).

21 Incubate 10 min at room temperature.

22 Prepare Elution Solution I. Vortex and centrifuge briefly:

Elution Solution I *Add reagents in the order listed*	PN	1X (µl)	10X (µl)
Buffer EB	-	98	980
10% Tween 20	-	1	10
Additive A	220074	1	10
Total	-	100	1000

23 At the end of 10 min incubation, place on the 10x Magnetic Separator, high position (Magnet – High) until the solution clears.

24 Remove the supernatant.

25 Add 300 ul freshly prepared 80% ethanol to the pellet while on the magnet – High. Wait 30 seconds.

26 Remove the ethanol.

27 Add 200 ul 80% ethanol to the pellet. Wait 30 seconds.

28 Remove the ethanol.

29 Centrifuge briefly. Place on the magnet – Low.

30 Remove the remaining ethanol.

31 Remove from the magnet. Immediately add 35.5 ul Elution Solution I to avoid clumping.

32 Pipette mix 15x (pipette set to 30 ul) without introducing bubbles.

33 Incubate 2 minutes at room temperature

34 Centrifuge briefly. Place on the magnet – Low until the solution clears.

35 Transfer 35 ul sample to a new tube strip.

cDNA Amplification

36 Prepare cDNA Amplification Mix on ice. Vortex and centrifuge briefly.

cDNA Amplification Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	8	35	70
Amplification Master Mix	220125	50	220	440
cDNA Additive	220067	5	22	44
cDNA Primer Mix	220106	2	9	18
Total	-	**65**	**286**	**572**

37 Add 65 ul cDNA Amplification Reaction Mix to 35 ul sample. Pipette mix 15x (pipette set to 90 ul). Centrifuge briefly.

38 Incubate in a thermal cycler with the following protocol. NOTE: Samples can be stored at 4°C up to 72 hours or at -20°C for up to 1 week or proceed to the next step.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25-50 min
Step	**Temperature**	**Time**
1	98°C	00:00:45
2	98°C	00:00:20
3	67°C	00:00:30
4	72°C	00:01:00
5	Go to Step 2, see table below for total # of cycles	
6	72°C	00:01:00
7	4°C	Hold

The optimal number of cycles is a trade-off between generating sufficient final mass for library construction and minimizing PCR amplification artifacts. Cycle numbers were optimized assuming that sample includes >80% T or B cells. If testing cells types with a known fraction of T and/or B cells, adjust cycle number based on that fraction to generate sufficient product. See Tips and Best Practices for examples.

Recommended starting point for cycle number optimization.

Targeted Cell Recovery	Primary Cells Total Cycles	Cell Lines Total Cycles
100 -500	18	16
501-2,000	16	14
2,001-6,000	14	12
6,001-10,000	13	11

39 Ensure sample volume is 100 µl (bring up to 100 µl with Buffer EB if needed). Vortex to resuspend the SPRIselect reagent. Add 60 ul SPRIselect reagent (0.6X) to each sample and pipette mix 15x (pipette set to 150 ul).

40 Incubate 5 min at room temperature.

41 Place on the magnet – High until the solution clears (~5 minutes).

42 Remove the supernatant.

43 Add 200 ul of 80% ethanol to the pellet. Wait 30 sec.

44 Remove the ethanol.

45 Repeat steps 43 and 44 for a total of 2 washes.

46 Centrifuge briefly and place on the magnet – High.

47 Remove any remaining ethanol. Air dry for 2 minutes. DO NOT exceed 2 minutes as this will decrease elution efficiency.

48 Remove from magnet. Add 45.5 ul Buffer EB. Pipette mix 15x.

49 Incubate 2 min at room temperature.

50 Place the tube strip on the magnet – High until the solution clears (~2 minutes).

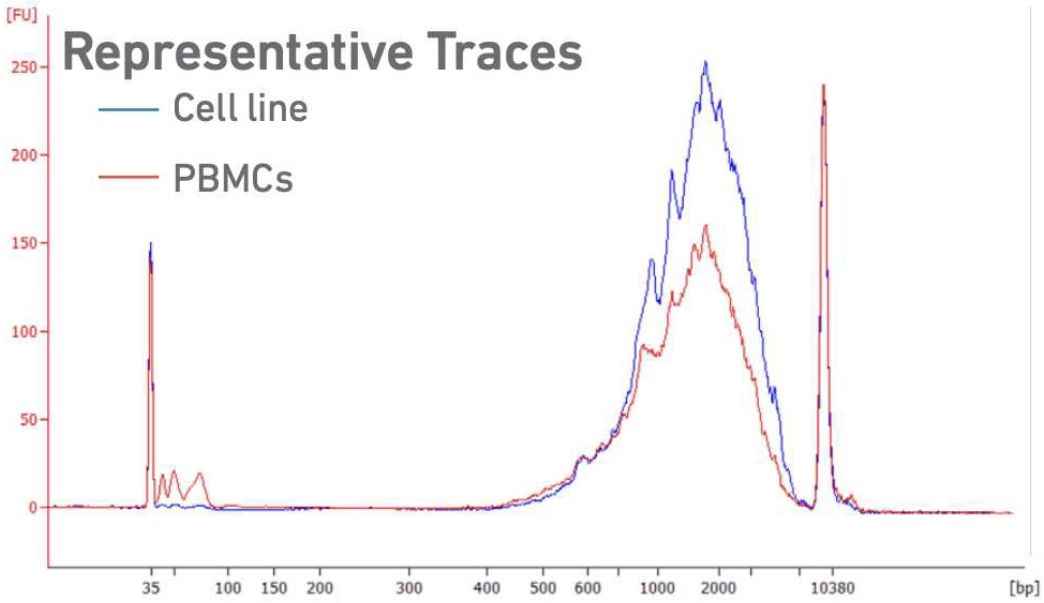
51 Transfer 45 ul sample to a new tube strip and *Label the top of tubes with "cDNA". NOTE: Store at 4°C for up to 72 hours or at −20°C for up to 4 weeks or proceed to the next step.

cDNA QC & Quantification

52 Run 1 ul of sample on Qubit dsDNA HS Assay.

53 Either run 1 ul of 1 ng/ul diluted sample on an Agilent Bioanalyzer High Sensitivity chip to determine fragment size.

54 OR run 2 ul of 1 ng/ul diluted sample on the Agilent Tapestation High Sensitivity D1000 ScreenTape to determine fragment size.



Gene Expression Library Construction

55 Initiate the following thermocycler protocol to pre-cool the block.

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

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

Fragmentation Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	15	66	132
Fragmentation Buffer	220108	5	22	44
Fragmentation Enzyme Blend	220107/ 220130	10	44	88
Total	-	30	132	264

57 Determine the volume for 50 ng mass of sample. Dispense the sample volume in a tube strip on ice. If the volume required for 50 ng is less than 20 µl, adjust the total volume of each sample to 20 µl with nuclease-free water. If the volume for 50 ng exceeds 20 µl, carry ONLY 20 µl sample into library construction.

58 Aliquot 30 µl of Fragmentation Mix to each well, mix 15x and spin down before returning to ice, let cool for 1 minute.

59 Transfer to pre-cooled thermal cycler (4°C) and press “SKIP” to initiate the protocol.

60 After the cycle is complete, vortex to resuspend SPRIselect reagent. Add 30 ul SPRIselect (0.6X) reagent to each sample. Pipette mix 15x (pipette set to 75 ul).

61 Incubate 5 min at room temperature.

62 Place on the magnet – High until the solution clears (~5 minutes). DO NOT discard the supernatant.

63 Transfer 75 ul supernatant to a new tube strip.

64 Vortex to resuspend SPRIselect reagent. Add 10 ul SPRIselect reagent (0.8X) to each sample. Pipette mix 15x (pipette set to 80 ul).

65 Incubate 5 min at room temperature.

66 Place on the magnet – High until the solution clears.

67 Remove 80 ul supernatant, making sure not to discard any beads.

68 Add 125 ul 80% ethanol to the pellet. Wait 30 seconds.

69 Remove the ethanol.

70 Repeat steps 68 and 69 for a total of 2 washes.

71 Centrifuge briefly and place on the magnet – Low until the solution clears. Remove remaining ethanol. DO NOT over dry to ensure maximum elution efficiency.

72 Remove from the magnet. Add 50.5 ul Buffer EB to each sample. Pipette mix 15x.

73 Incubate 2 min at room temperature.

74 Place on the magnet – High until the solution clears.

75 Transfer 50 ul sample to a new tube strip.

76 Prepare Adaptor Ligation Mix on ice. Pipette mix and centrifuge briefly.

Adaptor Ligation Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	17.5	77	154
Ligation Buffer	220109	20	88	176
DNA Ligase	220110/ 220131	10	44	88
Adaptor Mix	220026	2.5	11	22
Total	-	50	220	440

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

78 Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
30°C	100 µl	15 min
Step	**Temperature**	**Time**
1	20°C	00:15:00
2	4°C	Hold

79 Vortex to resuspend SPRIselect Reagent. Add 80 ul SPRIselect Reagent (0.8X) to each sample. Pipette mix 15x (pipette set to 150 ul).

80 Incubate 5 min at room temperature.

81 Place on the magnet – High until the solution clears.

82 Remove the supernatant.

83 Add 200 ul 80% ethanol to the pellet. Wait 30 sec.

84 Remove the ethanol.

85 Repeat steps 83 and 84 for a total of 2 washes.

86 Centrifuge briefly. Place on the magnet – Low.

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

88 Remove from the magnet. Add 30.5 ul Buffer EB. Pipette mix 15x.

89 Incubate 2 min at room temperature.

90 Place on the magnet – Low until the solution clears.

91 Transfer 30 ul sample to a new tube strip.

92 Choose the appropriate sample index set to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x sample index name (PN-2000240 Single Index Plate T Set A well ID) used.

93 Prepare Sample Index PCR Mix:

Sample Index PCR Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	8	35	70
Amplification Master Mix	220125	50	220	440
SI-PCR Primer Verify name & PN	220111	2	9	18
Total	-	**60**	**264**	**528**

94 Add 60 ul Sample Index PCR Mix to 30 ul sample.

95 Add 10 ul of an individual Single Index to each well and record the well ID used. Pipette mix 5x (pipette set to 90 ul). Centrifuge briefly.

96 Incubate in a thermal cycler with the following protocol. NOTE: Store at 4°C for up to 72 hours or at −20°C for up to 4 weeks or proceed to the next step.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~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 table below for # cycles	
6	72°C	00:01:00
7	4°C	Hold

The table recommends starting point for optimization. If less than 50 ng was carried into 5' Gene Expression Library Construction, refer to the product yield calculation example in step 3.3 to determine the mass input into Library Construction.

Input into Library Construction	Total Sample Index Cycles
1-25 ng	16
26-50 ng	14

97 Vortex to resuspend the SPRIselect reagent. Add 60 ul SPRIselect Reagent (0.6X) to each sample. Pipette mix 15x (pipette set to 150 ul).

98 Incubate 5 minutes at room temperature.

99 Place on the magnet – High until the solution clears (~2 minutes).

100 Discard the supernatant

101 Add 200 ul 80% ethanol to the pellet. Wait 30 seconds.

102 Remove the ethanol.

103 Repeat steps 101 and 102 for a total of 2 washes.

104 Centrifuge briefly. Place on the magnet – Low.

105 Remove remaining ethanol.

106 Remove from the magnet. Immediately add 35.5 ul Buffer EB. Pipette mix 15x

107 Incubate 2 minutes at room temperature.

108 Centrifuge briefly. Place on the magnet – Low.

109 Transfer 35 ul sample to a new tube strip. NOTE: Samples can be stored at 4°C up to 72 hours or -20°C for long-term storage.

V(D)J Target Enrichment from cDNA

110 Add 33ul of Nuclease-Free water to PCR tube on ice.

111 Add 2ul of cDNA Amp product to water.

112 Prepare Target Enrichment 1 master mix on ice.

Target Enrichment 1 Reaction Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	5	22	44
Amplification Master Mix	220125	50	220	440
cDNA Additive	220067	5	22	44
T Cell Mix 1 or B Cell Mix 1	Human 2000008/ Mouse 2000075 or Human 2000035/ Mouse 2000080	5	22	44
Total	-	65	286	572

113 Add 65ul of master mix to each sample, mix 5x on ice.

114 Run the following thermocycler conditions. NOTE: Samples can be stored at 4°C up to 72 hours before the next step.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~20-30 min
Step	**Temperature**	**Time**
1	98°C	00:00:45
2	98°C	00:00:20
3	67°C	00:00:30
4	72°C	00:01:00
5 Different cycle numbers for T & B cells	T Cell: Go to Step 2, 9x (total 10 cycles) B Cell: Go to Step 2, 5x (total 6 cycles)	
6	72°C	00:01:00
7	4°C	Hold

115 Vortex to resuspend SPRIselect Reagent. Add 80 ul SPRIselect Reagent (0.8X) to each sample. Pipette mix 15x (pipette set to 150 ul).

116 Incubate 5 min at room temperature.

117 Place on the magnet – High until the solution clears.

118 Remove the supernatant.

119 Add 200 ul 80% ethanol to the pellet. Wait 30 sec.

120 Remove the ethanol.

121 Repeat steps 10 and 11 for a total of 2 washes.

122 Centrifuge briefly. Place on the magnet – Low.

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

124 Remove from the magnet. Add 30.5 ul Buffer EB. Pipette mix 15x.

125 Incubate 2 min at room temperature.

126 Place on the magnet – Low until the solution clears.

127 Transfer 30 ul sample to a new tube strip. NOTE: Samples can be stored at 4°C up to 72 hours or -20°C up to 1 week before the next step.

128 Prepare Target Enrichment 2 master mix on ice.

Target Enrichment 2 Reaction Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	5	22	44
Amplification Master Mix	220125	50	220	440
cDNA Additive	220067	5	22	44
T Cell Mix 2 or B Cell Mix 2	Human 2000009/ Mouse 2000079 or Human 2000036/ Mouse 2000081	5	22	44
Total	-	65	286	572

129 Add 65ul of master mix to each sample, mix 5x on ice.

130 Run the following thermocycler conditions. NOTE: Samples can be stored at 4°C up to 72 hours before the next step.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25-30 min
Step	**Temperature**	**Time**
1	98°C	00:00:45
2	98°C	00:00:20
3	67°C	00:00:30
4	72°C	00:01:00
5 Different cycle numbers for T & B cells	T Cell: Go to Step 2, 9x (total 10 cycles) B Cell: Go to Step 2, 7x (total 8 cycles)	
6	72°C	00:01:00
7	4°C	Hold

131 Vortex to resuspend SPRIselect Reagent. Add 50 ul SPRIselect Reagent (0.8X) to each sample and pipette mix 15x (pipette set to 145 ul).

132 Incubate 5 min at room temperature.

133 Place on the magnet – High until the solution clears. DO NOT discard the supernatant.

134 Transfer 145 µl supernatant to a new tube strip.

135 Add 30 µl of SPRIselect Reagent, mix 15x, incubate 5 min.

136 Place on the magnet – High for 5 min., then remove 170 µl of supernatant.

137 Add 200 ul 80% ethanol to the pellet. Wait 30 sec.

138 Remove the ethanol.

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

140 Centrifuge briefly. Place on the magnet – Low.

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

142 Remove from the magnet. Add 45.5 ul Buffer EB. Pipette mix 15x.

143 Incubate 2 min at room temperature.

144 Place on the magnet – Low until the solution clears.

145 Transfer 45 ul sample to a new tube strip. NOTE: Samples can be stored at 4°C up to 72 hrs. or -20°C up to 1 week before the next step.

V(D)J Target Enrichment Library Construction

146 Initiate following thermocycler protocol to pre-cool block:

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

147 Prepare Fragmentation Mix on ice

Fragmentation Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	15	66	132
○ Fragmentation Buffer	220108	5	22	44
● Fragmentation Enzyme Blend	220107/ 220130	10	44	88
Total	-	**30**	**132**	**264**

148 Determine the volume for 50 ng mass of sample (see example calculation at step 3.3). Dispense the sample volume in a tube strip on ice. If the volume required for 50 ng is less than 20 µl, adjust the total volume of each sample to 20 µl with nuclease-free water. If the volume for 50 ng exceeds 20 µl, carry ONLY 20 µl sample into library construction.

149 Aliquot 30 µl of Fragmentation Mix to each well, mix 15x and spin down before returning to ice, let cool for 1 minute.

150 Transfer to pre-cooled thermal cycler (4°C) and press “SKIP” to initiate the protocol.

151 Prepare Adaptor Ligation Mix on ice.

Adaptor Ligation Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	17.5	77	154
Ligation Buffer	220109	20	88	176
DNA Ligase	220110/ 220131	10	44	88
Adaptor Mix	220026	2.5	11	22
Total	-	50	220	440

152 Remove tube strip containing Fragmentation Rxn from the thermocycler.

153 Add 50 µl of Adaptor Ligation Mix to each sample, mix 15x, and spin briefly.

154 Run the following thermocycler program:

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

155 Remove tube from thermocycler. Add 80 µl of SPRIselect Reagent, mix 15x, incubate 5 minutes, complete next step during incubation.

156 Prepare Sample Index PCR Mix on ice.

Sample Index PCR Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	8	35	70
Amplification Master Mix	220125	50	220	440
SI-PCR Primer	220111	2	9	18
Total	-	60	264	528

157 Place on the magnet – High until the solution clears.

158 Remove the supernatant...

159 Add 200 ul 80% ethanol to the pellet. Wait 30 sec.

160 Remove the ethanol.

161 Repeat steps 159 and 160 for a total of 2 washes.

162 Centrifuge briefly. Place on the magnet – Low.

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

164 Remove from the magnet. Add 30.5 ul Buffer EB. Pipette mix 15x.

165 Incubate 2 min at room temperature.

166 Place on the magnet – Low until the solution clears.

167 Transfer 30 ul sample to a new tube strip.

168 Add 60 µl of Sample Index PCR Mix to each sample

169 Add 10 µl of an individual sample index (Single Index Plate T Set A) to each well and record their assignment.

170 Mix by pipetting 15x and spin briefly. Run the following thermocycler program:NOTE: Samples can be stored at 4°C up to 72 hours before the next step

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~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 table below for # cycles	
6	72°C	00:01:00
7	4°C	Hold

171 Vortex to resuspend SPRIselect reagent. Add 80 ul SPRIselect reagent to each sample. Pipette to mix 15x.

172 Incubate 5 minutes at room temperature.

173 Place on the magnet – High until the solution clears (~2 minutes).

174 Discard the supernatant

175 Add 200 ul 80% ethanol to the pellet. Wait 30 seconds.

176 Remove the ethanol.

177 Repeat steps 175 and 176 for a total of 2 washes.

178 Centrifuge briefly. Place on the magnet – Low.

179 Remove remaining ethanol.

180 Remove from the magnet. Immediately add 35.5 ul Buffer EB. Pipette mix 15x

181 Incubate 2 minutes at room temperature.

182 Centrifuge briefly. Place on the magnet – Low.

183 Transfer 35 ul sample to a new tube strip. NOTE: Samples can be stored at 4°C up to 72 hours or -20°C for long-term storage.

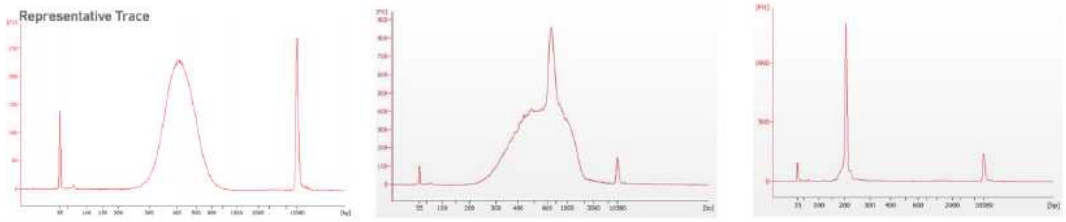
LIBRARY QUANTIFICATION

184 Qubit: Run 1 ul sample at 1:5 dilution on the Qubit dsDNA HS Assay Kit

185 Bioanalyzer/Tapestation:

1. EITHER Run 1 ul sample diluted to 3 ng/ul on the Agilent Bioanalyzer High Sensitivity DNA chip to determine fragment size. Lower molecular weight product (<150 bp) may be present. This does not affect sequencing.
2. OR Run 2 ul sample diluted to 1 ng/ul on the Agilent Tapestation High Sensitivity D1000 ScreenTape to determine fragment size.

Gene Expression TCR/BCR Feature Barcode



186 qPCR

186.1 Thaw KAPA Library Quantification Kit for Illumina Platforms

186.2 Dilute 1 ul sample with deionized water to appropriate dilutions that fall within the linear detection range of the KAPA Library Quantification Kit for Illumina Platforms. (For more accurate quantification, make the dilution(s) in duplicate).

186.3 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

186.4 Dispense 16 ul Quantification Master Mix for sample dilutions and DNA Standards into a 96 well PCR plate.

186.5 Incubate in a thermal cycler with the following protocol.

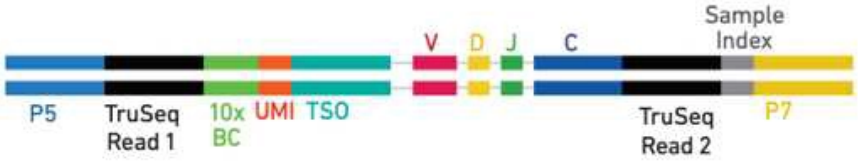
Step	Temperature	Run Time
1	95°C	00:03:00
2	95°C	00:00:05
3	67°C	00:00:30
4	Go to Step 2, 29X (Total 30 cycles)	

186.6 Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration using the average size in the region of 175 – 1,000 bp for gene expression libraries.

SEQUENCING

187 Sequencing Libraries: Chromium Single Cell V(D)J Enriched libraries, 5' Gene Expression libraries, and Cell Surface Protein libraries comprise standard Illumina paired-end constructs which begin with P5 and end with P7. These libraries include 16 bp 10x Barcodes encoded at the start of TruSeq Read 1. Sample index sequences are incorporated as the i7 index read for V(D)J Enriched and 5' Gene Expression libraries; as i7 index read N for Cell Surface Protein library. TruSeq Read 1, TruSeq Read 2, and Nextera Read 2 (Read 2N) are all standard Illumina sequencing primer sites. TruSeq Read 1 and TruSeq Read 2 are used in paired-end sequencing of V(D)J Enriched and 5' Gene Expression libraries. TruSeq Read 1 and Nextera Read 2 (Read 2N) are used for paired-end sequencing of Cell Surface Protein library. Sequencing these libraries produce a standard Illumina BCL data output folder.

Chromium Single Cell V(D)J Enriched Library



Chromium Single Cell 5' Gene Expression Library

Chromium Single Cell 5' Cell Surface Protein Library

188 Illumina Sequencer Compatibility

The compatibility of the listed sequencers has been verified by 10x Genomics. Some variation in assay performance is expected based on sequencer choice.

- MiSeq
- NextSeq 500/550 (High Output)

- HiSeq 2500 (Rapid Run)
- HiSeq 3000.4000
- NovaSeq

189 Sample Indices

Each sample index in the Chromium i7 Sample Index Kit (PN-120262) and Chromium i7 Sample Index Kit Plate N, Set A (PN-1000084) is a mix of 4 different sequences to balance across all 4 nucleotides. If multiple samples are pooled in a sequence lane, the sample index name (i.e. the Chromium i7 Sample Index plate well ID) is needed in the sample sheet used for generating FASTQs with “cellranger mkfastq”. If multiple libraries are pooled in a sequence lane, a separate sample index is needed with each library.

190 Sequencing Depth & Run Parameters

Sequencing Depth	Minimum 5,000 read pairs per cell for V(D)J Enriched library Minimum 20,000 read pairs per cell for 5' Gene Expression library Minimum 5,000 read pairs per cell for Cell Surface Protein library
Sequencing Type	Paired-end, single indexing
Sequencing Read	Recommended cycles for all library types – 26 x 91 bp Recommended cycles for all library type combinations – 26 x 91 bp

V(D)J Enriched libraries (alone or in combination with 5' Gene Expression and/or Cell Surface Protein libraries) may be sequenced at 150 x 150 bp.

191 Library Pooling

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

Library Pooling Examples:

Libraries	Sequencing Depth (read pairs per cell)	Library Pooling Ratio
Example 1		
V(D)J Enriched library	5,000	1
5' Gene Expression library	20,000	4
Cell Surface Protein library	5,000	1
Example 2		
V(D)J Enriched library	5,000	1
5' Gene Expression library	50,000	10
Cell Surface Protein library	5,000	1