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

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We use this protocol and it's working

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Abstract

This protocol has been adapted from the 10X User Guide CG000420, Rev D.

GEM Generation and Barcoding

1 Prepare RT Mastermix

1.1 Prepare RT Master Mix on ice. Pipette mix 15x and centrifuge briefly

	Matermix reagent	Product number	16X +10% (µl)
	RT Reagent B	2000435/ 2000165	660.0
	Template Switch Oligo	3000228	82.7
	Reducing Agent B	2000087	68.6
	RT Enzyme C	2000085/ 2000436	308.0

1.2 Add 63.6 µl Master Mix into each tube of a PCR 8-tube strip on ice.

2 Load Chromium Next GEM Chip M

2.1 Load 130 µl 50% glycerol to row 1, 140 µl to rows 2A, 2B, 3A, and 3B.

2.2 Vortex gel beads

2.3 Mix master mix and cell suspension, and add nuclease free water to get a final volume of 105 µl.

2.4 Add 130 µl beads to each well in row 1.

2.5 Add 140 µl Master Mix + Cell Suspension to Row 2A and 2B.

2.6 Load 140 µl Partitioning Oil into rows labeled 3A and 3B.

2.7 Run on 10X Chromium Controller.

Post GEM–RT Cleanup & cDNA Amplification

3 **Transfer GEMs and perform RT**

3.1 Label four tube strips and place on ice.

3.2 Press the eject button of Chromium X and remove the chip.

3.3 Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees.

3.4 Visually check the volume in rows labeled 1, 2A, and 2B. Abnormally high volume relative to other wells indicates a clog. Significant volume of non-sample fluid is expected in rows 2A and 2B after a successful run and does not indicate a sample clog.

3.5 Retrieve GEMs from row labeled 3A: Slowly aspirate 90 µl GEMs from the lowest points of the recovery wells in the top row labeled 3A without creating a seal between the tips and the bottom of the wells.

3.6 Withdraw pipette tips from the wells. GEMs should appear opaque and uniform across all channels. Excess Partitioning Oil (clear) in the pipette tips indicates a potential clog.

3.7 Over the course of ~20 sec, dispense GEMs into first tube strip on ice with the pipette tips against the sidewalls of the tubes.

3.8 Using the same pipette tips, slowly aspirate remaining 90 µl GEMs from the wells in the top row labeled 3A and dispense in second tube strip as described above.

3.9 Retrieve from row labeled 3B: Slowly aspirate 90 µl GEMs from the lowest points of the recovery wells in the bottom row labeled 3B without creating a seal between the tips and the bottom of the wells.

3.10 10. Repeat steps 8 and 9, dispensing GEMs into third tube strip on ice with the pipette tips against the sidewalls of the tubes.

3.11 11. Using the same pipette tips, slowly aspirate remaining 90 µl GEMs from the wells in the bottom row labeled 3B and dispense in fourth tube strip as described above.

3.12 12. Transfer tube strips to a Bio-Rad T1000 thermacycler (Bio-Rad) for cDNA synthesis, performed in two steps; cDNA generation at 53°C for 45 minutes followed and reverse transcriptase deactivation at 85°C for 5 minutes.

4 **Post GEM-RT Cleanup – Dynabeads**

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

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

4.3 Prepare Dynabeads Cleanup Mix accoding to table below:

	Dynabeads Cleanup reagents	Product number	32X +10% (µl)
	Cleanup Buffer	2000088/ 2000438	6406.4
	Dynabeads MyOne SILANE	2000048	281.6
	Reducing Agent B	2000087	176
	Nuclease-free Water	-	176

4.4 Vortex and add 200 µl Dynabeads Cleanup Mix to each tube. Pipette mix 10x.

4.5 Incubate 10 min at room temperature. Pipette mix again at ~5 min after start of incubation to resuspend settled beads.

4.6 Prepare Elution Solution I according to table below:

Elution solution reagents	Product number	40X (µl)
Buffer EB	-	3920
10% Tween 20	-	40
Reducing Agent B	2000087	40

4.7 At the end of 10 min incubation, place the tube strips in slots 1-4 of a 10x Magnetic Separator HT (magnet High) until the solution clears.

4.8 Remove the supernatant.

4.9 Add 300 µl 80% ethanol to the pellet while on the magnet. Wait 30 sec.

4.10 Remove the ethanol.

4.11 Add 200 µl 80% ethanol to pellet. Wait 30 sec.

4.12 Remove the ethanol.

4.13 Centrifuge briefly. Place on the magnet (at Low).

4.14 Remove remaining ethanol. Air dry for 1 min.

4.15 To avoid over drying the pellet, immediately add 36 µl Elution Solution I to the tube strips, cap the tubes, remove all tube strips from the magnet, and centrifuge briefly.

4.16 10. Pipette mix (pipette set to 30 µl) without introducing bubbles.

4.17 11. Incubate 2 min at room temperature.

4.18 12. Place on the magnet (at Low) until the solution clears.

4.19 13. Transfer 35 µl sample to a new tube strip.

5 cDNA Amplification

5.1 Prepare cDNA Amplification Mix on ice according to table below:

	Amplification mixture reagents	Product number	32X + 10%
	Amp Mix	2000047/ 2000440	1760
	Feature cDNA Primers 3	2000289	528

5.2 Add 65 µl cDNA Amplification Reaction Mix to 35 µl sample.

5.3 Pipette mix 15x (pipette set to 90 µl). Centrifuge briefly.

5.4 Incubate in a Bio-Rad T1000 thermacycler for amplification to be performed in six steps; the enzyme was first activated at 98°C for three minutes, followed by 11 cycles where DNA was linearized at 98°C for 15 seconds, the primer annealing was performed at 63°C for 20 seconds and DNA elongation at 72°C for one minute. This was followed by a final elongation at 72°C for one minute.

6 **cDNA Cleanup –SPRIselect**

6.1 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).

6.2 Incubate 5 min at room temperature.

6.3 Place on the magnet (at High) until the solution clears.

6.4 Transfer and save 75 µl supernatant each into two new tube strips (one for Cell Surface Protein and one for Cell Multiplexing) without disturbing the pellet. Maintain at room temperature. DO NOT discard the transferred supernatant (cleanup for Cell Surface Protein and Cell Multiplexing-step 2.3B).

6.5 Remove the remaining supernatant from the pellet without disturbing the pellet. DO NOT discard the pellet (cleanup for 3' Gene Expression library construction). Immediately proceed to Pellet Cleanup.

7 **Pellet Cleanup for 3' Gene Expression**

7.1 Add 200 µl 80% ethanol to the pellet. Wait 30 sec.

7.2 Remove the ethanol.

7.3 Repeat steps i and ii for a total of 2 washes.

7.4 Centrifuge briefly and place on the magnet (at Low).

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

7.6 Remove from the magnet. Add 41 µl Buffer EB. Pipette mix 15x (pipette set to 35 µl).

7.7 Incubate 2 min at room temperature.

7.8 Place the tube strip on the magnet (at High) until the solution clears.

7.9 Transfer 40 µl sample to a new tube strip.

8 **Transferred Supernatant Cleanup for Multiplexing**

8.1 Vortex to resuspend the SPRIselect reagent. Add 70 µl SPRIselect reagent (2.0X) to 75 µl of the transferred supernatant and pipette mix 15x (pipette set to 130 µl).

8.2 Incubate for 5 min at room temperature.

8.3 Place on the magnet (at High) until the solution clears.

8.4 Remove supernatant.

8.5 Add 300 µl 80% ethanol to the pellet. Wait 30 sec.

8.6 Remove the ethanol.

8.7 Repeat steps 5 and 6 for a total of 2 washes.

8.8 Centrifuge briefly and place on the magnet (at Low).

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

8.10 10. Remove from the magnet. Add 41 µl Buffer EB. Pipette mix 15x (pipette set to 35 µl).

8.11 11. Incubate 2 min at room temperature.

8.12 12. Place the tube strip on the magnet (at High) until the solution clears.

8.13 13. Transfer 40 µl sample to a new tube strip.

9 **Post cDNA Amplification QC & Quantification**

9.1 Validate fragment size by capillary electrophoresis using the Tapestation 4200 (Agilent).

3' Gene Expression Library Construction

10 **Fragmentation, End Repair & A-tailing**

10.1 Transfer ONLY 20 µl purified cDNA sample from Pellet Cleanup to a tube strip.

10.2 Add 15 µl Buffer EB to each sample.

10.3 Add 15 µl Fragmentation Mix to each sample.

10.4 Pipette mix 15x (pipette set to 35 µl) on ice. Centrifuge briefly.

10.5 Transfer to Bio-Rad T1000 thermacycler and incubate for 5 minutes at 32°C for cDNA fragmentation and thereafter at 65°C for 30 minutes for end repair and A-tailing to occur.

11 **Post Fragmentation, End Repair & A-tailing Double Sided – SPRIselect**

11.1 Vortex to resuspend SPRIselect reagent. Add 30 µl SPRIselect (0.6X) reagent to each sample. Pipette mix 15x (pipette set to 75 µl).

11.2 Incubate 5 min at room temperature.

11.3 Place on the magnet (at High) until the solution clears. DO NOT discard supernatant.

11.4 Transfer 75 µl supernatant to a new tube strip.

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

11.6 Incubate 5 min at room temperature.

11.7 Place on the magnet (at High) until the solution clears.

11.8 Remove 80 µl supernatant. DO NOT discard any beads.

11.9 Add 125 µl 80% ethanol to the pellet. Wait 30 sec.

11.10 10. Remove the ethanol.

11.11 11. Repeat steps 9 and 10 for a total of 2 washes.

11.12 12. Centrifuge briefly. Place on the magnet (at Low) until the solution clears. Remove remaining ethanol pipetting slowly. DO NOT over dry to ensure maximum elution efficiency.

11.13 13. Remove from the magnet. Add 51 µl Buffer EB to each sample. Pipette mix 15x.

11.14 14. Incubate 2 min at room temperature.

11.15 15. Place on the magnet (at High) until the solution clears.

11.16 16. Transfer 50 µl sample to a new tube strip pipetting slowly.

12 Adaptor Ligation

12.1 Prepare adaptor ligation master mix according to table below:

	Adaptor ligation reagents	Product number	16X + 10%
	Ligation Buffer	2000092	352
	DNA Ligase	220110	176
	Adaptor Oligos	2000094	352

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

12.3 Incubate in a Bio-Rad T1000 thermacycler for 20 minutes at 20°C.

13 Post Ligation Cleanup – SPRIselect

13.1 Vortex to resuspend SPRIselect Reagent. Add 80 µl SPRIselect Reagent (0.8X) to each sample. Pipette mix 15x.

13.2 Incubate 5 min at room temperature.

13.3 Place on the magnet (at High) until the solution clears.

13.4 Remove the supernatant.

13.5 Add 200 µl 80% ethanol to the pellet. Wait 30 sec.

13.6 Remove the ethanol.

13.7 Repeat steps 5 and 6 for a total of 2 washes.

13.8 Centrifuge briefly. Place on the magnet (at Low).

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

13.10 10. Remove from the magnet. Add 31 µl Buffer EB. Pipette mix 15x.

13.11 11. Incubate 2 min at room temperature.

13.12 12. Place on the magnet (at Low) until the solution clears.

13.13 13. Transfer 30 µl sample to a new tube strip.

14 **Sample Index PCR**

14.1 Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x sample index name (PN-3000431 Dual Index Plate TT Set A well ID) used.

14.2 Add 50 µl Amp Mix (PN-2000047) to 30 µl sample.

14.3 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.

14.4 Transfer to a Bio-Rad T1000 thermacycler and run an initial DNA denaturation and enzyme activation for 45 seconds at 98°C, followed by 5-16 cycles (depending in cDNA input),of 98°C for 15 seconds, 54°C for 30 seconds, and 72°C for 20 seconds. A final elongation at 72°C was run for one minute.

15 **Post Sample Index PCR Double Sided Size Selection – SPRIselect**

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

15.2 Incubate 5 min at room temperature.

15.3 Place the magnet (at High) until the solution clears. DO NOT discard supernatant.

15.4 Transfer 150 µl supernatant to a new tube strip.

15.5 Vortex to resuspend the SPRIselect reagent. Add 20 µl SPRIselect Reagent (0.8X) to each transferred supernatant. Pipette mix 15x (pipette set to 150 µl).

15.6 Incubate 5 min at room temperature.

15.7 Place the magnet (at High) until the solution clears.

15.8 Remove 165 µl supernatant. DO NOT discard any beads.

15.9 With the tube still in the magnet, add 200 µl 80% ethanol to the pellet. Wait 30 sec.

15.10 10. Remove the ethanol.

15.11 11. Repeat steps 9 and 10 for a total of 2 washes.

15.12 12. Centrifuge briefly. Place on the magnet (at Low). Remove remaining ethanol.

15.13 13. Remove from the magnet. Add 36 µl Buffer EB. Pipette mix 15x.

15.14 14. Incubate 2 min at room temperature.

15.15 15. Place on the magnet (at Low) until the solution clears.

15.16 16. Transfer 35 µl to a new tube strip.

15.17 17. Validate size by capillary electrophoresis using the Tapestation 4200 (Agilent).

Cell Multiplexing Library Construction

16 **Sample Index PCR**

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

16.2 Prepare sample index PCR mixture according to table below:

	Amplification mixture reagents	Product number	16X + 10%
	Amp Mix	2000047	880

Buffer EB		352

16.3 Transfer ONLY 10 µl DNA sample from the Transferred Supernatant Cleanup to a new tube strip.

16.4 Add 70 µl Sample Index PCR Mix to each sample.

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

16.6 Incubate in a Bio-Rad T1000 thermacycler for amplification to be performed in six steps; the enzyme was first activated at 98°C for 45 seconds, followed by 6 cycles where DNA was linearized at 98°C for 20 seconds, the primer annealing was performed at 54°C for 30 seconds and DNA elongation at 72°C for 20 seconds. This was followed by a final elongation at 72°C for one minute.

17 **Post Sample Index PCR Size Selection – SPRIselect**

17.1 Vortex to resuspend the SPRIselect reagent. Add 120 µl SPRIselect Reagent (1.2X) to each sample. Pipette mix 15x (pipette set to 150 µl).

17.2 Incubate 5 min at room temperature.

17.3 Place the magnet (at High) until the solution clears.

17.4 Remove the supernatant.

17.5 Add 300 µl 80% ethanol to the pellet. Wait 30 sec.

17.6 Remove the ethanol.

17.7 Add 200 µl 80% ethanol to the pellet. Wait 30 sec.

17.8 Remove the ethanol.

17.9 Centrifuge briefly. Place on the magnet (at Low). Remove remaining ethanol. Air dry for 2 min.

17.10 10. Remove from the magnet. Add 41 µl Buffer EB. Pipette mix 15x.

17.11 11. Incubate 2 min at room temperature.

17.12 12. Place on the magnet (at Low) until the solution clears.

17.13 13. Transfer 40 µl to a new tube strip.

17.14 14. Validate size by capillary electrophoresis using the Tapestation 4200 (Agilent).

Sequencing

18 Sequencing was performed on a NovaSeq 6000 S4 v1.5 Flowcell (Illumina) to a depth of > 20,000 reads/cell for gene expression and > 5,000 reads/cell for hashtag antibody libraries.

19 Library Loading

	Instrument	Loading concentration	PhiX (%)
	NovaSeq	150*/300	1

20 **Pooling for Gene expression**

	A	B	C	D	E	F	G	H	I	J	K
	Samp	**# of**	**# of Read**	**Total # of**	**Perc**	**nM**	**Targ**	**Indi**	**uL FOR**	**uL H2**	**Fin**

	A	B	C	D	E	F	G	H	I	J	K
	le ID	**Cells**	**s/Cell**	**Reads**	**ent of Pool**		**et nM**	**vVol**	**5nM**	**O**	**al Vol.**
	HC_NR_CELSR2 Neg	60,000	20,000	1,200,000,000	25	87.4	5	100	5.72	94.28	100
	HC_NR_CELSR2 Pos	60,000	20,000	1,200,000,000	25	88.3	5	100	5.66	94.34	100
	PD_R_CELSR2 Neg	60,000	20,000	1,200,000,000	25	93.6	5	100	5.34	94.66	100
	PD_R_CELSR2 Pos	60,000	20,000	1,200,000,000	25	84.9	5	100	5.89	94.11	100

21 **Pooling for cell multiplexing**

	A	B	C	D	E	F	G	H	I	J	K
	Sample ID	**# of Cells**	**# of Reads/Cell**	**Total Reads**	**Percent of Pool**	**nM**	**Target nM**	**IndivVol**	**uL FOR 5nM**	**uL H2O**	**Final Vol.**
	HC_NR_CELSR2 Neg	60,000	5,000	300,000,000	25	144	5	100	3.47	96.53	100
	HC_NR_CELSR2 Pos	60,000	5,000	300,000,000	25	55.8	5	100	8.96	91.04	100

	A	B	C	D	E	F	G	H	I	J	K
	PD_R_CELSR2 Neg	60,000	5,000	300,000,000	25	106	5	100	4.72	95.28	100
	PD_R_CELSR2 Pos	60,000	5,000	300,000,000	25	45	5	100	11.11	88.89	100

22 **NovaSeq sequencing**

22.1 Inspect the underside of each cartridge to make sure that the reservoirs are free of ice, which indicates that the reagents are thawed.

22.2 Invert each cartridge 10 times to mix reagents.

22.3 Gently tap the bottom of each cartridge on the bench to reduce air bubbles.

22.4 Without disturbing the library at the bottom, insert the uncapped library tube containing the denatured and diluted library pool into the Library Tube position (#8) of the cluster cartridge.

22.5 Insert the library tube into position #8 of the cluster cartridge.

22.6 Normalize and pool libraries and add PhiX control according to the table below:

	POOL ID	**# OF SAMPLES**	**# OF READS/SAMPLE**	**TOTAL # OF READS**	**PERCENT OF LANE**	**nM**	**DILUTION CONC (nM)**	**FINAL VOLUME**	**uL FOR 2nM**	**uL RSB**	**FINAL VOL.**
	Gene expression	240,000	20,000	4.80E+09	50.36	6.5	2	156.12	48	10	156.12

										8.11	
	Cell multiplexing	240,000	5,000	1.20E+09	12.59	5.75	3	39.03	20.37	18.66	39.03
	Other sample	108,582	5,000	5.43E+08	5.7	4.54	2	17.66	7.78	9.88	17.66
	Other sample	108,582	20,000	2.17E+09	22.78	6.73	2	70.63	21	49.63	70.63
	Other sample	108,582	5,000	5.43E+08	5.7	6.12	3	17.66	8.66	9	17.66
	Other sample	5,932	5,000	2.97E+07	0.31	0.9	2	0.96	2.14	-1.18	0.96
	Other sample	5,932	20,000	1.19E+08	1.24	6.98	2	3.86	1.11	2.75	3.86
	Other sample	5,932	5,000	2.97E+07	0.31	1.06	2	0.96	1.81	-0.85	0.96
	Phix	1	95,000,000	9.50E+07	1	2.1	2	3.09	2.94	0.15	3.09
	Other sample	1	1,000,000	1.00E+06	0.01	0.03	2	0.03	2.51	-2.48	0.03
	TOTAL # OF READS:	9.53E+09				**TOTAL VOLUME uL:**	193.68	310			

22.7 Thaw the ExAmp reagents.

22.8 Prepare a fresh dilution of NaOH according to the NovaSeq 6000 Denature and Dilute Guide (document # 1000000106351).

22.9 Denature and neutralize library pool according to the NovaSeq 6000 Denature and Dilute Guide (document # 1000000106351).

22.10 Prepare the flow cell and dock

22.11 Prepare the ExAmp master mix according to table below:

	Addition order	Reagents	Volume for four-lane flow cell
	1	DPX1/JPX1	315
	2	DPX2/JPX2	45
	3	DPX3	165

22.12 Add 105 µl ExAmp master mix to 45 µl library pool

22.13 Load ExAmp/library mix onto the flow cell.

22.14 Load an empty library tube into position #8 of the cluster cartridge

22.15 Set Up a Sequencing Run according to table below

3' Gene Expression Library Sequencing Parameters

	A	B
	Parameters	**Description**
	Sequencing Depth	>20,000 read pairs per cell

	A	B
	Sequencing Type	Paired-end, dual indexing
	Sequencing Read	Recommended Number of Cycles
	Read 1	28 cycles
	i7 Index	10 cycles
	i5 Index	10 cycles
	Read 2	90 cycles

Cell Multiplexing Library Sequencing Parameters

	A	B
	Parameters	**Description**
	Sequencing Depth	>5,000 read pairs per cell
	Sequencing Type	Paired-end, dual indexing
	Sequencing Read	Recommended Number of Cycles
	Read 1	28 cycles
	i7 Index	10 cycles
	i5 Index	10 cycles
	Read 2	90 cycles