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Multiplexed Fixed Single Cell RNA Sequencing of frozen human post mortem brain

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We are still developing and optimizing this protocol

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Disclaimer

In development
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Abstract

This protocol describes the processing of post-mortem frozen brain tissue for fixed single-cell sequencing using 10x Genomics for the library preparation. This protocol can be applied and generalized for infectious tissues that need to be fixed before processing.

Materials

10x Genomics Kits:
Chromium Nuclei Isolation Kit with RNase Inhibitor, 16 rxns (Ref: 1000494)
Chromium Fixed RNA Kit, Human Transcriptome, 4rxns x 4 BC (Ref: 1000475)
Chromium Next GEM Chip Q Single Cell Kit, 16 rxns (Ref: 1000422)
Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit, 16 rxns (Ref: 1000414)
Dual Index Kit TS Set A, 96 rxn (Ref: 1000251)

Additional Reagents and Plastic Wear not included:
DNA LoBind Tubes 1,5ml (Eppendorf; Ref: 022431021)
PCR Tubes 0.2ml 8-tube strips (Eppendorf; Ref: 951010022)
15ml Centrifuge Tube (Sarstedt; Ref: 62.554.016)
Pre-Separation Filters (30µm) (Milentyi Biotec; Ref: 130-041-407)
Nuclease-free Water
Low TE Buffer (10mM Tris-HCL pH 8.0 0.1mM EDTA) (Thermo Fisher; Ref: 12090-105)
Tween 20 Surfact-Amps Detergent Solution (10%) (Thermo Fisher; Ref: 28320)
SPRIselect Reagent Kit (Beckman Coulter; Ref: B23318)
Ethanol (Millipore Sigma; Ref: E7023-500ml)
Gylercol (Millipore Sigma; Ref: G5516)
Buffer EB (Qiagen; Ref: 19086)
37% Formaldehyde (Thermo Fisher; Ref: BP531-500)
Bovine Serum Albumin In DPBS (10%) (Millipore Sigma; Ref: A1595)
DAPI 10mg (Thermo Fisher; Ref: D3571)
High Sensitivity D5000: ScreenTape (Agilent; Ref: 5067-5592)
High Sensitivity D500: Reagents (Agilent; Ref: 5067-5593)

Equipment:
10x Magnetic Seperator
10x Chromium iX/X
FACS Sorter
Brightfield-Microscope
Agilent TapeStation
Mini Centrifuge
Thermocycler (100µl Volume Capacity)
Eppendorf 1.5ml ThermoMixer C
One of the Following Illumina Sequencers: iSeq. MiSeq, NextSeq 500/550, NextSeq 1000/2000, NovaSeq

Before start

For this protocol, frozen brain tissue from liquid nitrogen is recommended. The samples may be highly variable in cell counts and quality due to various dependencies, such as disease status, the time it took to transfer the samples into liquid nitrogen after dissection, the time elapsed between the patient's death and the dissection, and the brain region being observed. This protocol was performed using striatal tissue.
Using ~30mg of tissue led to 100.000-400.000 cells counted in FACS, which resulted in 2000-13,000 unique cells sequenced.
To get an estimate on the quality of the sample, a percentage was sorted to single cell fraction with FACS.
5-20% or greater single cells is optimal, and it is safe to continue. A higher yield of 6,000-13,000 cells in the end may be expected.
2-5% Single Cells are of average quality to continue, if the result of 3,000-6,000 Cells are enough for analysis.
Less than 2% indicates a poor cell quality, and a low yield of reads with poor quality is expected **even** if a high number of Cells (e.g. 200.000) have been collected in the Single Cell Fraction. Continue **at own risk.**

Nuclei Isolation

2h 15m 40s

1 **Note:** Nuclei Isolation is following the standard 10x Genomics "Nuclei Isolation" and "Fixation Protocol" with some tweaks for maximizing Nuclei yield:
https://www.10xgenomics.com/support/flex-gene-expression/documentation/steps/sample-prep/chromium-nuclei-isolation-kit-sample-prep-user-guide
https://www.10xgenomics.com/support/flex-gene-expression/documentation/steps/sample-prep/fixation-of-cells-and-nuclei-for-chromium-single-cell-gene-expression-flex

Preparation of reagents and centrifuge

1.1 Obtain the 10x Nuclei Isolation Kit, Nuclease-Free Water, PBS, and 10x BSA.

1.2 Pre-chill the **centrifuge** to 4 °C .

1.3 Obtain materials and reagents as listed:

10m

Action	Item	10x PN	Preparation & Handling	Storage
Place on Ice	Lysis Reagent	2000558	Vortex, verify no precipitate, and centrifuge briefly.	4°C
	Surfactant A	2000559	Vortex, verify no precipitate, and centrifuge briefly.	4°C
	Debris Removal Reagent	2000560	Vortex, verify no precipitate or layering, and centrifuge briefly.	4°C
	Reducing Agent B	2000087	Thaw to room temperature, vortex, verify no precipitate, and centrifuge briefly.	-20°C
	RNase Inhibitor	2000565	Centrifuge briefly.	-20°C
	Nuclei Isolation Consumables: • *Nuclei Isolation Column* • *Collection Tube*	 2000562 2000563	Pre-chill assembled Nuclei Isolation Column(s) and Collection Tube(s) on ice.	Ambient
	Nuclease-free Water	—	See Buffer Preparation.	Ambient
	1X PBS	—	See Buffer Preparation.	Ambient
	10% BSA	—	See Buffer Preparation.	4°C
Place on Dry Ice	Frozen Tissue Sample	—	See Tips & Best Practices.	Liquid Nitrogen (long-term) or -80°C (short-term)
	Sample Dissociation Tube	2000564	Pre-chill on dry ice.	Ambient
Obtain	Pestles	2000561	Keep on lab bench.	Ambient
	Nucleic Acid Staining Fluorescent Dye	—	See Tips & Best Practices.	4°C
	Vortex	—	See Nuclei Isolation Protocol.	—

2 Prepare Lysis Buffer and place On ice . 5m

Lysis Buffer (500 µl/rxn) *Add reagents in the order listed*	PN	1X+10% (µl)	4X + 10% (µl)	8X + 10% (µl)
Lysis Reagent	2000558	**550**	2,200	4,400
Reducing Agent B	2000087	**0.55**	2.2	4.4
Surfactant A	2000559	**5.5**	22	44
Total	–	**556.05**	2,224.2	4,448.4

2.1 Prepare Debris Removal Buffer and place On ice . 5m

Debris Removal Buffer (500 µl/rxn) *Add reagents in the order listed*	PN	1X+10% (µl)	4X + 10% (µl)	8X + 10% (µl)
Debris Removal Reagent	2000560	**550**	2,200	4,400
Reducing Agent B	2000087	**0.55**	2.2	4.4
Total	–	**550.55**	2,202.2	4,404.4

2.2 Prepare the Wash and Resuspension Buffer and place On ice . 5m

Wash and Resuspension Buffer (3 ml/rxn) *Add reagents in the order listed*	PN	1X+10% (µl)	4X + 10% (µl)	8X + 10% (µl)
1X PBS (not provided)	-	**2,887.5**	11,550	23,100
10% BSA (not provided)	-	**330**	1,320	2,640
RNase Inhibitor	2000565	**82.5**	330	660
Total	–	**3,300**	13,200	26,400

3 Nuclei Isolation

1h

3.1 Cut 30 mg of the frozen tissue on dry ice and put it in the sample dissociation tube. 10m

3.2 Add 200µl of Lysis Buffer to the tissue On ice . 1m

3.3 Homogenize the tissue until no clumps remain, then add an additional 300 µL of Lysis Buffer and mix 10 times with a pipette. 2m

3.4 Incubate for 00:10:00 On ice . 10m

3.5 Transfer the lysate to the Nuclei Isolation Column. 1m

3.6 Centrifuge 16.000 rcf for 00:00:20 . 20s

3.7 Keep the Collection Tube and discard the column.
Resuspend the nuclei by vortexing the Collection Tube for 00:00:10 at 3.200 rpm . 1m

3.8 Centrifuge the Collection Tube 00:03:00 at 500 rcf . 3m

3.9 Discard the supernatant.
Strongly recommended: a small amount of 200 µL may be left behind if a low yield is expected. 2m

3.10 Add 500 µL Debris Removal Reagent and resuspend by pipetting. 1m

3.11 Centrifuge at 700 rcf for 00:10:00 . 10m

3.12 Discard the supernatant. 2m

Note: The nuclei will be sitting at the bottom-side of the tube, and there will be a lot of debris/fat on the upper side of the tube. Gently take off the supernatant from the side of the wall, without going too low to take up the nuclei.
Strongly recommended: an amount of 200 µL may be left behind if a low yield is expected.

3.13 Resuspend in 1 mL Wash and Resuspension Buffer. 1m

3.14 Centrifuge at 500 rcf for 00:05:00 . 5m

3.15 Discard the supernatant. 1m
Strongly recommended: a small amount of 100 µL may be left behind if a low yield is expected.

3.16 Add 1 mL of Fixation Buffer, resuspend by pipetting and leave at 4 °C for 16:00:00 - 24:00:00 . 20s

Buffers for Fixation - Prepare fresh			
Fixation Buffer Maintain at room temperature	Stock	Final	Per Sample (µl)
Nuclease-free Water	-	-	791.9
Conc. Fix & Perm Buffer* (10x Genomics PN-2000517)	10X	1X	100
Formaldehyde	37%	4%	108.1

FACS sorting 2h 27m

4 **Cell sorting is mandatory, as ~95% of the pellet will be debris or clumps**

If the cells have been fixed overnight, continue with the cell sorting.

Note: Ensure that all samples have been fixed for the same duration.

5 **Prepare the buffers and reagents and place** On ice : 5m

-Quenching Buffer:

Quenching Buffer Maintain at 4°C	Stock	Final	Per Sample (µl)
Nuclease-free Water	-	-	875.0
Conc. Quench Buffer* (10x Genomics PN-2000516)	8X	1X	125.0

-PBS+2% BSA:

4 mL PBS

1 mL 10% BSA

-300µM DAPI:

Dilute 300 µL 1mM DAPI stock solution with 700 µL of PBS.

6 **FACS Sorting**

6.1 Centrifuge fixed cells at 900 rcf 00:05:00 at Room temperature . 5m

6.2 Take off the supernatant. 2m

Note: the pellet should be more compact now, and the full removal of supernatant is possible.

6.3 Resuspend the pellet in 500 µL cold Quenching Buffer and incubate 00:05:00 On ice . 5m

6.4 Centrifuge cells at 900 rcf 00:05:00 at Room temperature . 5m

6.5 Remove supernatant and resuspend in 200 µL - 500 µL PBS+2% BSA. 2m

Note: if the cell suspension is too dense for FACS sorting, adjust with PBS+2%BSA to achieve a sort rate of ~10000 events/second during sorting to avoid clotting of the machine.

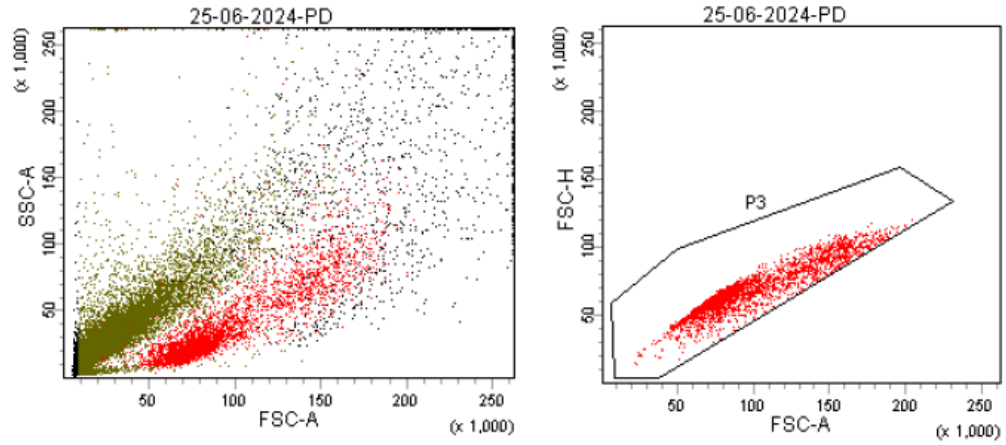
6.6 Pass the sample through a 30µm strainer directly into the FACS sorting tubes. 2m

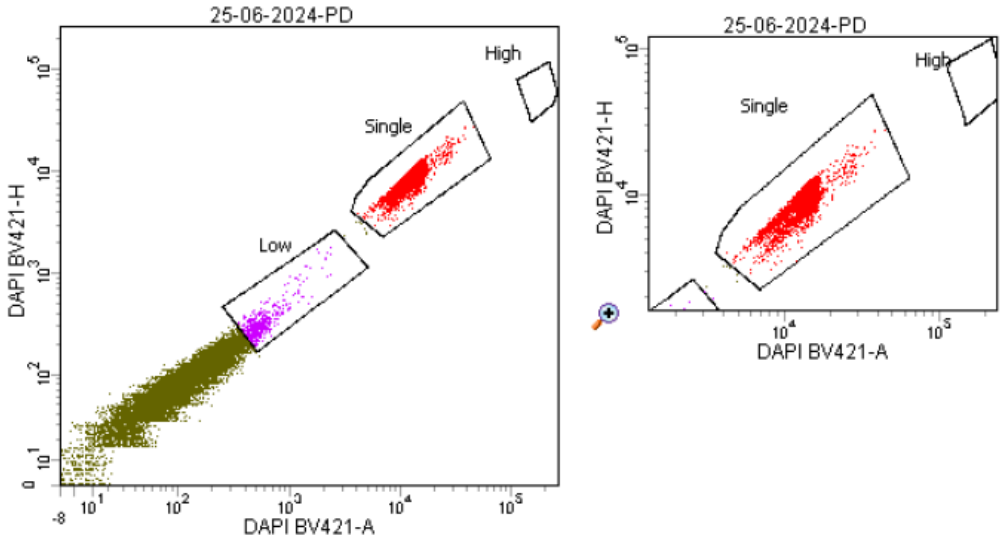
6.7 Add 5 µL 300µM DAPI to 1000 µL of cell suspension. 1m

6.8 Sort the single DAPI positive events to a fresh 1.5 ml LoBind Tube and place On ice . 2h

Note: Each sample will take about 30 minutes to sort
Depending on tissue quality, expect 50000-300000 single nuclei
If it is less than 100000 nuclei, it is not recommended to continue as there is more nuclei loss expected during the preparation of the samples

6.9





Tube: PD

Population	#Events	%Parent	%Total
All Events	30,000	####	100.0
P3	22,536	75.1	75.1
Low	498	2.2	1.7
Single	4,071	18.1	13.6
High	0	0.0	0.0

Example of a good sort with a high fraction of 13.6% single DAPI positive events.

Probe Hybridization

16h 35m

7 **Note:** Library preparation is following the standard 10x Genomics "Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples" protocol with some tweaks for maximizing nuclei yield: https://www.10xgenomics.com/support/flex-gene-expression/documentation/steps/library-prep/chromium-single-cell-gene-expression-

5m

flex-reagent-kits-for-multiplexed-samples

Prepare the following reagents as listed:

Action	Item	10x PN	Preparation & Handling	Storage
Thaw & Keep Warm				
☐	**Hyb Buffer B**	2000485/ 2000483	Thaw at 42°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. DO NOT keep the thawed buffer on ice, or the solution will precipitate. Thawed Hyb Buffer B can be kept at 42°C for up to 1 h.	−20°C
☐	**Enhancer**	2000482	Thaw for 10 min at 65°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min.	−20°C
Place on Ice				
☐	**Fixed Cell Suspension**	—	Consult Chromium Fixed RNA Profiling - Protocol Planner (CG000528) for details on applicable Demonstrated Protocols.	—
☐	**Human WTA Probes BC001-BC016** OR **Mouse WTA Probes BC001-BC016**	2000495-2000510 2000703-2000718	Thaw on ice. Vortex and centrifuge briefly.	−20°C

8 Prepare the Hyb Mix at Room temperature : 2m

Hyb Mix *Add reagents in the order listed*	PN	1X* (µl)	1X* + 20% (µl)	4X* + 20% (µl)	16X* + 20% (µl)
Hyb Buffer B *Thaw at 42°C. Add warm to the mix and if appears milky keep it back on 42°C.*	2000485/ 2000483	70.0	84.0	336.0	1344.0
Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.*	2000482	10.0	12.0	48.0	192.0
Total	-	**80.0**	**96.0**	**384.0**	**1536.0**

8.1 Incubate Hyb Mix at 42 °C for 00:05:00 . 5m

8.2 Centrifuge fixed nuclei at 900 x g for 00:05:00 and take off the Supernatant. 5m

Very Important: When removing the supernatant, leave about 20 µL behind. This drastically increases the yield and does not affect the hybridization performance.

8.3 Resuspend the pellet in 80 µL Hyb Mix and transfer to a 8-strip tube. **Keep the samples at** Room temperature 2m

8.4 Add 20 µL of Unique Human WTA-Probe Barcode per sample and gently mix by pipetting. 1m

8.5 Incubate samples for **16-24h** at 42 °C in a thermocycler with heated lid. 16h
Continue with counting and GEM generation the next day.

8.6 Count the nuclei using 5 µL . 15m

Note: A high nuclei loss can occur here. For example, starting with 150,000-400,000 nuclei after FACS can result in 35,000-140,000 after probe hybridization and then a subsequent final read count of 3,500-13,000 per sample.

GEM Generation and Barcoding

3h 34m 30s

9 Prepare the following reagents and materials as listed: 5m

Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐	**Single Cell TL v1 Gel Beads**	2000538	Equilibrate to room temperature 30 min before loading the chip.	−80°C
☐ ○	**Reducing Agent B**	2000087	Vortex, verify no precipitate, centrifuge briefly.	−20°C
Thaw & Keep Warm				
☐	**Enhancer**	2000482	Thaw for 10 min at 65°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min.	−20°C
Place on Ice				
☐	**Conc. Post-Hyb Buffer**	2000533	Thaw at room temperature and keep on ice.	−20°C
☐	**GEM Enzyme Mix**	2000490	Centrifuge briefly before adding to the mix.	−20°C
☐	**GEM Reagent Mix**	2000491	Thaw at room temperature. Vortex, verify no precipitate, centrifuge briefly. Keep on ice.	−20°C
Obtain				
☐	**Partitioning Oil**	2000190	—	Ambient
☐	**Next GEM Chip Q**	2000518	See Tips & Best Practices.	Ambient
☐	**Chromium Next GEM Secondary Chip Holder**	3000332	See Tips & Best Practices.	Ambient
☐	**10x Gasket**	370017/	See Tips & Best Practices.	Ambient

Action	Item	10x PN	Preparation & Handling	Storage
		3000072		
☐	**Sample Filters Sysmex Sterile Single-pack CellTrics Filters/Miltenyi Biotec Pre-Separation Filters (30 µm)**	—	Manufacturer's recommendations.	Ambient
☐	**10x Vortex Adapter**	330002	See Tips & Best Practices.	Ambient
☐	**50% glycerol solution** for adding to unused wells	—	See Tips & Best Practices.	
☐	**Glycerol for molecular biology, ≥99%** Prepare fresh 50% glycerol solution for sample storage	—	See Tips & Best Practices.	—

10 Prepare the following buffer: 5m

Post-Hyb Wash Buffer *Add reagents in the order listed*	PN	Pooling 4 samples (ml)*	Pooling 16 samples (ml)*
Nuclease-free Water	-	4.95	13.86
Conc. Post-Hyb Buffer	2000533	0.275	0.77
Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.*	2000482	0.275	0.77
Total	-	**5.5**	**15.40**

Volumes for 4 wells/GEM reactions (includes 10% overage)

Post-Hyb Wash Buffer *Add reagents in the order listed*	PN	Pooling 4 samples (ml)*	Pooling 16 samples (ml)*
Nuclease-free Water	-	19.80	55.44
Conc. Post-Hyb Buffer	2000533	1.10	3.08
Enhancer *Heat at 65°C for 10 min. Vortex and verify no precipitate. Add warm to the mix.*	2000482	1.10	3.08
Total	-	**22.0**	**61.6**

Use the upper table, if multiplexing 4 samples, and the lower table for up to 16 samples

11 Pooling and Washing of the samples

11.1 Pool the samples, using the cell count you got after hybrdization in a 15ml Falcon. 5m

NOTE: Usually, take all nuclei of the sample with the lowest concentration, and an equal amount of nuclei from the other samples to create one pool, where there are similar amount of cells from each sample.
If one sample has way lower concentration than the others, it also worked out to Pool the full amount of all samples. But expect less reads from the sample with the low concentration.

11.2 Add 3 mL Post-Hyb Wash Buffer and mix by inverting 5 times. 1m

11.3 Centrifuge the pool at 900 x g for 00:05:00 and take off the supernatant. 5m

11.4 Resuspend cell pellet in 1 mL Post-Hyb Wash Buffer and transfer to a 1.5ml Tube. 1m

11.5 Incubate at 42 °C for 00:10:00 . 10m

11.6 Centrifuge at 900 x g for 00:05:00 and take off the supernatant. 5m

11.7 Resuspend cell pellet in 0.5 mL Post-Hyb Wash Buffer. 1m

11.8 Incubate at 42 °C for 00:10:00 . 10m

11.9 **Critical step**: Filter the nuclei through a 30µm Strainer into a new 1.5ml Tube, to avoid clumps going into the GEM generation. 1m

11.10 Centrifuge at 900 x g for 00:05:00 and take off the supernatant. 5m

11.11 Prepare Post-Hyb Resuspension Buffer and maintain at 4 °C : 2m

Post-Hyb Resuspension Buffer *Add reagents in the order listed*	PN	1 Pool + 10% (µl)	4 Pools + 10% (µl)
Nuclease-free Water	-	1567.5	6270.0
Conc. Post-Hyb Buffer	2000533	82.5	330.0
Total	**-**	**1650.0**	**6600.0**

11.12 Resuspend the pellet in an appropriate amount of Post-Hyb Resuspension Buffer and place On ice : 2m

Starting Total Cell Number in Pool	Post-Hyb Resuspension Buffer (µl)
<1 x 10^6	500
1 x 10^6-4 x 10^6	750
5 x 10^6-8 x 10^6	1,000
9 x 10^6-12 x 10^6	1,250
13 x 10^6-16 x 10^6	1,500

11.13 Count the nuclei and calculate the total amount. 5m

Note: The lowest concentration should be 2000 nuclei/µl so 1.000.000 in total. If there are less nuclei, centrifuge at 900 x g for 00:05:00 and remove as much supernatant as needed, to achieve a concentration of 2000 nuclei/µl.
For example if you have 100.000 Nuclei in 500µl, remove 450µl of supernatant after centrifugation to achieve a concentration of 2000 Nuclei/µl in 50µl.

12 **Prepare GEM Master Mix + Sample Dilution**

12.1 Prepare Master Mix On ice. Pipette mix 15x and centrifuge briefly. 2m

GEM Master Mix *Add reagents in the order listed*	PN	1X* (µl)	4X* + 10% (µl)
● GEM Reagent Mix	2000491	20.9	92.1
○ Reducing Agent B	2000087	1.7	7.3
● GEM Enzyme Mix	2000490	12.4	54.6
Total	-	**35.0**	**154.0**

*1X = 1 well/GEM reaction, 4X = 4 wells/GEM reactions

12.2 In a PCR 8-tube strip, prepare a dilution depending on the cell stock concentration and target cell recovery. 2m

Volume of Cell Suspension Stock per reaction (µl) | Volume of Post-Hyb Resuspension Buffer per reaction (µl)

Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery										
	2000	4000	8000	12000	16000	20000	24000	28000	32000	36000	40000
500	6.6 33.4	13.2 26.8	26.4 13.6	39.6 0.4	n/a	n/a	n/a	n/a	n/a	n/a	n/a
750	4.4 35.6	8.8 31.2	17.6 22.4	26.4 13.6	35.2 4.8	n/a	n/a	n/a	n/a	n/a	n/a
1000	3.3 36.7	6.6 33.4	13.2 26.8	19.8 20.2	26.4 13.6	33.0 7.0	39.6 0.4	n/a	n/a	n/a	n/a
1250	2.6 37.4	5.3 34.7	10.6 29.4	15.8 24.2	21.1 18.9	26.4 13.6	31.7 8.3	37.0 3.0	n/a	n/a	n/a
1500	2.2 37.8	4.4 35.6	8.8 31.2	13.2 26.8	17.6 22.4	22.0 18.0	26.4 13.6	30.8 9.2	35.2 4.8	39.6 0.4	n/a
1750	1.9 38.1	3.8 36.2	7.5 32.5	11.3 28.7	15.1 24.9	18.9 21.1	22.6 17.4	26.4 13.6	30.2 9.8	33.9 6.1	37.7 2.3
2000	1.7 38.4	3.3 36.7	6.6 33.4	9.9 30.1	13.2 26.8	16.5 23.5	19.8 20.2	23.1 16.9	26.4 13.6	29.7 10.3	33.0 7.0
2250	1.5 38.5	2.9 37.1	5.9 34.1	8.8 31.2	11.7 28.3	14.7 25.3	17.6 22.4	20.5 19.5	23.5 16.5	26.4 13.6	29.3 10.7
2500	1.3 38.7	2.6 37.4	5.3 34.7	7.9 32.1	10.6 29.4	13.2 26.8	15.8 24.2	18.5 21.5	21.1 18.9	23.8 16.2	26.4 13.6
2750	1.2 38.8	2.4 37.6	4.8 35.2	7.2 32.8	9.6 30.4	12.0 28.0	14.4 25.6	16.8 23.2	19.2 20.8	21.6 18.4	24.0 16.0
3000	1.1 38.9	2.2 37.8	4.4 35.6	6.6 33.4	8.8 31.2	11.0 29.0	13.2 26.8	15.4 24.6	17.6 22.4	19.8 20.2	22.0 18.0
3250	1.0 39.0	2.0 38.0	4.1 35.9	6.1 33.9	8.1 31.9	10.2 29.8	12.2 27.8	14.2 25.8	16.2 23.8	18.3 21.7	20.3 19.7
3500	0.9 39.1	1.9 38.1	3.8 36.2	5.7 34.3	7.5 32.5	9.4 30.6	11.3 28.7	13.2 26.8	15.1 24.9	17.0 23.0	18.9 21.1
3750	0.9 39.1	1.8 38.2	3.5 36.5	5.3 34.7	7.0 33.0	8.8 31.2	10.6 29.4	12.3 27.7	14.1 25.9	15.8 24.2	17.6 22.4
4000	0.8 39.2	1.7 38.4	3.3 36.7	5.0 35.1	6.6 33.4	8.3 31.8	9.9 30.1	11.6 28.5	13.2 26.8	14.9 25.2	16.5 23.5
4250	0.8 39.2	1.6 38.4	3.1 36.9	4.7 35.3	6.2 33.8	7.8 32.2	9.3 30.7	10.9 29.1	12.4 27.6	14.0 26.0	15.5 24.5
4500	0.7 39.3	1.5 38.5	2.9 37.1	4.4 35.6	5.9 34.1	7.3 32.7	8.8 31.2	10.3 29.7	11.7 28.3	13.2 26.8	14.7 25.3
4750	0.7 39.3	1.4 38.6	2.8 37.2	4.2 35.8	5.6 34.4	6.9 33.1	8.3 31.7	9.7 30.3	11.1 28.9	12.5 27.5	13.9 26.1
5000	0.7 39.3	1.3 38.7	2.6 37.4	4.0 36.0	5.3 34.7	6.6 33.4	7.9 32.1	9.2 30.8	10.6 29.4	11.9 28.1	13.2 26.8
5250	0.6 39.4	1.3 38.7	2.5 37.5	3.8 36.2	5.0 35.0	6.3 33.7	7.5 32.5	8.8 31.2	10.1 29.9	11.3 28.7	12.6 27.4
5500	0.6 39.4	1.2 38.8	2.4 37.6	3.6 36.4	4.8 35.2	6.0 34.0	7.2 32.8	8.4 31.6	9.6 30.4	10.8 29.2	12.0 28.0
5750	0.6 39.4	1.1 38.9	2.3 37.7	3.4 36.6	4.6 35.4	5.7 34.3	6.9 33.1	8.0 32.0	9.2 30.8	10.3 29.7	11.5 28.5
6000	0.6 39.5	1.1 38.9	2.2 37.8	3.3 36.7	4.4 35.6	5.5 34.5	6.6 33.4	7.7 32.3	8.8 31.2	9.9 30.1	11.0 29.0
Yellow boxes	Indicate a low transfer volume that may result in higher cell load variability										

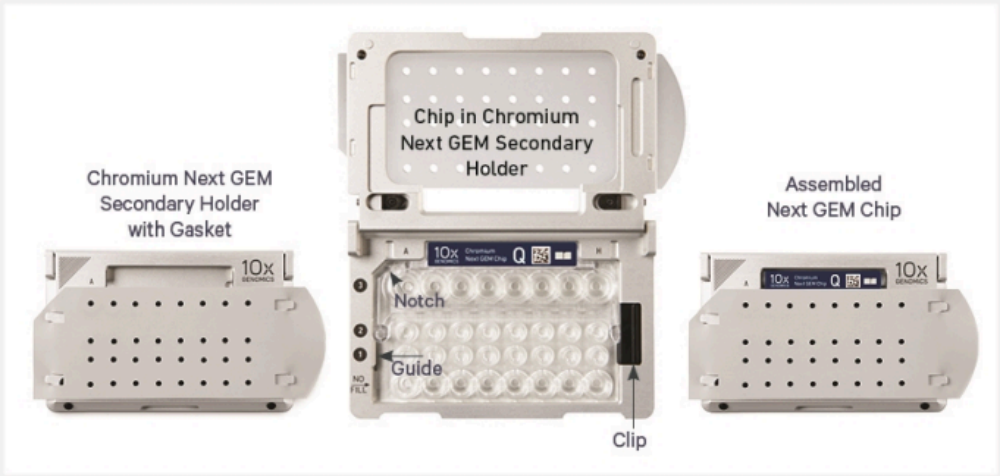
Note: If you have a concentration of 2000 Cells/µl and aim for a Cell Recovery of 40000 cells, dilute 33 µL of Cell Stock with 7 µL Post-Hyb Resupension Buffer.

12.3 Add 35 µL of prepared GEM Master Mix into each tube containing the cell dilution and **immediately** proceed to the next step. 1m

Note: Don´t mix the sample at this point.

13 **Assembly and Loading of the Next GEM Chip Q**

13.1 2m



- Attach the gasket, with the smooth side facing the chip holder
- Open the chip holder
- Remove the chip from the bag, align the chip with the notch on the left side of the Holder and secure the chip with the clip on the right side
- The chip is now ready for loading
- **Do not** touch the smooth side of the gasket

13.2 When loading the chip, ensure the chip lays flat and raise the pipette tip as you dispense. This should take 00:00:05 .

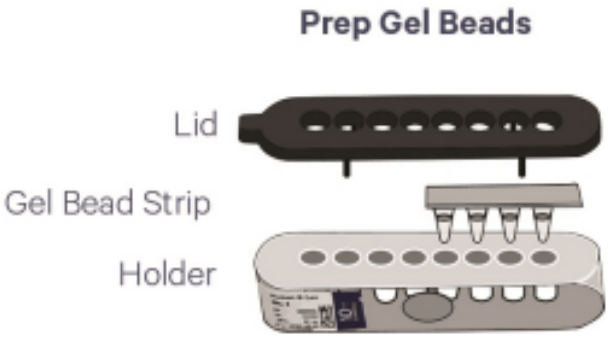
13.3 **Add 50% glycerol solution to each unused well:** 2m

- 70 µl in each unused well in row labeled 1
- 50 µl in each unused well in row labeled 2
- 150 µl in each unused well in row labeled 3

13.4 5m

Prepare Gel Beads:

- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex 00:00:30 .
- Centrifuge the Gel Bead strip for 00:00:05 . Confirm there are no bubbles at the bottom of the tubes & the liquid levels are even.



- Place the Gel Bead strip back in the holder. Secure the holder lid.

13.5 2m

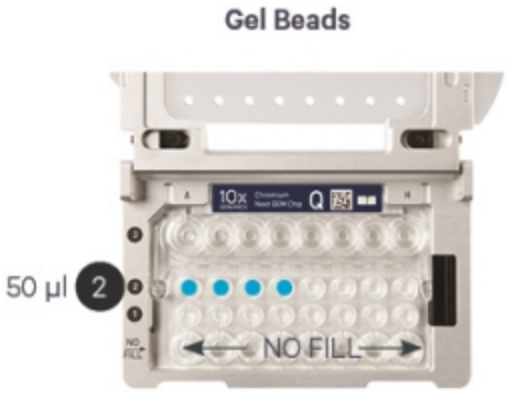
Load Row 1:

- With pipette set to 70 µl, gently pipette mix the GEM Master Mix + Sample 15x.
- Using the same pipette tips, dispense 70 µL GEM Master Mix + Sample into the bottom center of wells in row labeled 1 without

introducing bubbles.

13.6 2m

Load Row Labeled 2:



- Puncture the foil seal of the Gel Bead tubes. Slowly aspirate 50 µL Gel Beads.
- Dispense into the wells in row labeled 2 without introducing bubbles.
- Wait 00:01:00 .

13.7 2m

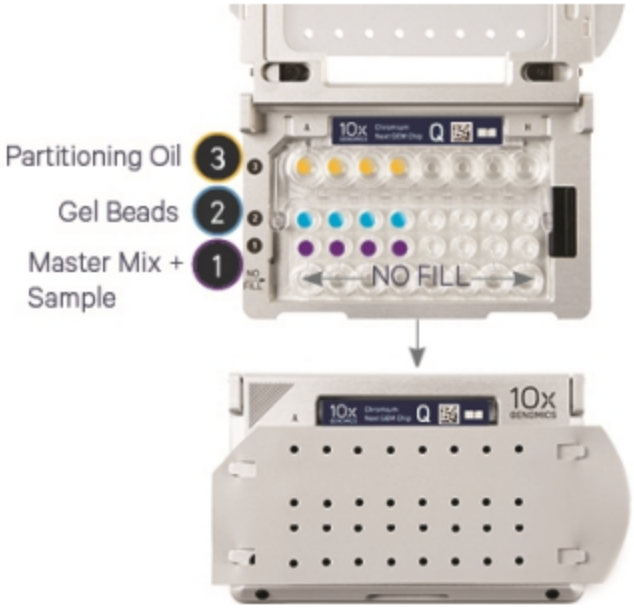
Load Row Labeled 3:

- Dispense 45 µL Partitioning Oil into the wells in row labeled 3 from a reagent reservoir.

13.8 1m

Prepare for Run:

- Close the lid (gasket already attached). **DO NOT** touch the smooth side of the gasket. **DO NOT** press down on the top of the gasket.



14 **Run the Chromium X/iX**

14.1 5m 30s

- Press the eject button on the Chromium X to eject the tray. If the eject button is not touched within 1 min, tray will close automatically. System requires a few seconds before the tray can be ejected again.
- Place the assembled chip with the gasket in the tray, ensuring that the chip stays horizontal. Press the button to retract the tray.

- Press the play button.
- At completion of the run (00:05:30), Chromium X/iX will chime.

  Immediately proceed to the next step.

15 **Transfer GEMs**

15.1 5m

- Place a tube strip on ice.
- Press the eject button of the Chromium X/iX 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.
- Check the volume in rows labeled 1-2. Abnormally high volume in any well indicates a clog.
- Slowly aspirate 100 µL GEMs from the lowest points of the recovery wells in the top row labeled 3, without creating a seal between the tips and the bottom of the wells.
- Withdraw pipette tips from the wells. GEMs should appear opaque and uniform across all channels.
- Over the course of 00:00:20 , dispense GEMs into the tube strip on ice with the pipette tips against the sidewalls of the tubes.

16 **GEM Incubation**

16.1 2h

Incubate in a Thermal Cycler using the following protocol:

Lid Temperature	Reaction Volume	Run Time
80°C	100 µl	~125 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	25°C	00:60:00
2	60°C	00:45:00
3	80°C	00:20:00
Hold	4°C	Hold

Note: Store at 4 °C for up to a week, or proceed immediately to the next step.

GEM Recovery and Pre-Amplification

1h 18m 30s

17 Prepare the following reagents and materials as listed: 5m

Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐ ○	**Reducing Agent B**	2000087	Thaw, vortex, verify no precipitate, centrifuge briefly.	−20°C
☐ ●	**Pre-Amp Primers B** Verify name & PN	2000529	Thaw, vortex, centrifuge briefly.	−20°C
☐	**Beckman Coulter SPRIselect Reagent**	—	Manufacturer's recommendations.	Ambient
Place on Ice				
☐ ○	**Amp Mix**	2000103	Vortex and centrifuge briefly.	−20°C
Obtain				
☐ ○	**Recovery Agent**	220016	—	Ambient
☐	**Qiagen Buffer EB**	—	Manufacturer's recommendations.	Ambient
☐	**10% Tween 20**	—	Manufacturer's recommendations.	Ambient
☐	**10x Magnetic Separator/10x Magnetic Separator B**	230003/ 2001212	—	Ambient
☐	**Prepare 80% Ethanol** Prepare 2.5 ml for 4 GEM reactions.	—	Prepare fresh.	—

18 **Post-Gem Incubation-Recovery**

18.1 Add 125 µL Recovery Agent to each sample at Room temperature. **DO NOT** pipette mix or vortex the biphasic mixture. 2m

18.2 Mix by inverting 5x and incubate for 00:02:00. 2m

18.3 Centrifuge briefly and slowly remove 125 µL Recovery Agent (pink phase). 2m

Note: A small amount of 20 µL Recovery Agent is normal and does not affect the performance.

19 **Pre-Amplification PCR**

19.1 Prepare Pre-Amplification Mix on ice. Vortex and centrifuge briefly. 2m

Pre-Amplification Mix *Add reagents in the order listed*	PN	1X (µl)	4X + 10% (µl)
○ **Amp Mix**	2000103	25.0	110.0
● **Pre-Amp Primers B**	2000529	10.0	44.0
Total		**35.0**	**154.0**

19.2 Add 35 µL Pre-Amplification Mix to the aqueous sample. 1m

19.3 Mix by inverting 8x and centrifuge briefly. 1m

19.4 Incubate in a Thermocycler using the following protocol: 40m

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~30-45 min
Step	**Temperature**	**Time** *hh:mm:ss*
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, 7x (total 8 cycles)	
6	72°C	00:01:00
7	4°C	Hold

19.5 Store at 4 °C for up to 72:00:00 or -20 °C for 1 week, or proceed to the next

step.

20 **DNA Cleanup- SPRISelect**

20.1 Prepare the Elution Solution. Vortex briefly. 2m

Elution Solution *Add reagents in the order listed*	PN	1000 µl
Buffer EB		980
10% Tween 20	-	10
○ Reducing Agent B	2000087	10
Total		1000

20.2 Centrifuge the sample for 00:00:30 in a microcentrifuge and transfer 70 µL of the upper layer to a fresh 8-tube strip. 2m

Note: If a low yield is expected, transfer all of the upper layer and adjust the Volume of SPRIselect accordingly.

20.3 Vortex to resuspend the SPRIselect reagent. Add 126 µL SPRIselect reagent (1.8X) to each sample and pipette mix 15x. 5m
Incubate for 00:05:00 at Room temperature .

20.4 Place on the magnet **"high"** until the solution clears. 2m

20.5 Remove the supernatant and wash the beads with 200 µL 80% Ethanol. 30s
Wait for 00:00:30 .

20.6 Remove the ethanol and repeat the previous step for a total of 2 washes. 5m

20.7 Centrifuge briefly and place on the magnet **"low"**.
Remove any remaining ethanol, but **do not** let the beads dry out. 1m

20.8 Remove from the magnet. Add 101 µL Elution Solution. Wait 00:01:00 before resuspending. Pipette mix 15x. 2m

20.9 Incubate 00:02:00 at Room temperature . 2m

20.10 Place the tube strip on the magnet **"High"** until the solution clears. 1m

20.11 Transfer 100 µL supernatant to a new 8-tube strip. 1m

20.12 Store at 4 °C for up to 72:00:00 or -20 °C for 1 week, or proceed to the next
step.

Library Construction 1h 30m 30s

21 Prepare the following reagents and materials as listed: 5m

Action	Item	10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐ ⚠	**Dual Index Plate TS Set A** Verify name & PN. Use indicated plate only	3000511	Vortex and centrifuge briefly.	−20°C
☐	**Beckman Coulter SPRIselect Reagent**	—	Manufacturer's recommendations.	—
☐	**Agilent Bioanalyzer High Sensitivity Kit** If used for QC	—	Manufacturer's recommendations.	—
☐	**Agilent TapeStation ScreenTape & Reagents** If used for QC	—	Manufacturer's recommendations.	—
Place on Ice				
☐ ◯	**Amp Mix**	2000103	Vortex and centrifuge briefly.	−20°C
☐	**KAPA Library Quantification Kit for Illumina Platforms**	—	Manufacturer's recommendations.	—
Obtain				
☐	**Qiagen Buffer EB**	—	Manufacturer's recommendations.	Ambient
☐	**10x Magnetic Separator/ 10x Magnetic Separator B**	230003/ 2001212	See Tips & Best Practices.	Ambient
☐	**Prepare 80% Ethanol** Prepare 2.5 ml for 4 GEM reactions.	—	Prepare fresh.	Ambient

21.1 Choose an appropriate index so there are no overlaps during one sequencing run.

21.2 Prepare the Sample Index PCR Mix On ice : 5m

Sample Index PCR Mix *Add reagents in the order listed*		PN	1X (µl)	1X + 10% (µl)	4X + 10% (µl)
◯	**Amp Mix**	2000103	50.0	55.0	220.0
	Nuclease-free Water	—	10.0	11.0	44.0
	Total		**60.0**	**66.0**	**264.0**

22 Transfer **only** 20 µL of the cleaned up sample to a new strip and add 60 µL of the prepared Sample Index PCR Mix. 2m

22.1 Add 20 µL of an individual Dual Index TS Set A to each sample. Pipette mix 5x. 1m

22.2 Incubate in a Thermal Cycler using the following protocol: 30m

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25-40 min
Step	**Temperature**	**Time** *hh:mm:ss*
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 total # of cycles	
6	72°C	00:01:00
7	4°C	Hold

Depending on the targeted cell recovery, choose the number of cycles from the Table below.
Note: For analyzing 10.000 cells/barcode (40.000 in total), choose 10 cycles for the PCR program.

Targeted Cell Recovery	Total Cycles* for Cell Lines	for PBMCs & Nuclei	for Cells from Fixed & Dissociated Tissues**	for Cells from FFPE Tissue Sections
500-2,000	11	15	14-15	16
2,000-4,000	10	14	13-14	15
4,000-7,000	9	13	12-13	14
7,000-12,000	8	12	11-12	13
12,000-25,000	7	11	10-11	12
25,000-50,000	6	10	9-10	11
50,000-128,000	5	9	8-9	10

*Optimization of cycle number may be needed based on the total RNA content of the sample. The ideal target library concentration is 50 - 200 nM. However, if the concentration is between 10-50 nM or between 200-500 nM and if the libraries do not contain low or high molecular weight peaks, sequencing can still be performed. If optimization is needed, additional Amp Mix can be obtained using the Fixed RNA Feature Barcode Kit (PN-1000419). For dissociated tumor cells, cycle numbers for cell lines can be used as a starting point. For dissociated primary cells, cycle numbers for PBMCs can be used as a starting point.

**For cells derived from the fixed and dissociated tissue samples, the cycle number will depend on the RNA expression level of the tissue and on overall quality of the tissue prior to fixation. Additional optimization may be required.

22.3 Store at 4 °C for ≤ 72:00:00, or proceed to the next step.

23 Post Sample Index PCR Size Selection – SPRIselect

23.1 Vortex to resuspend the SPRIselect reagent. Add 100 µL SPRIselect Reagent (1.0X) to each sample. Pipette mix 15x and incubate for 00:05:00 at Room temperature . 5m

23.2 Place on the magnet **"high"** until the solution clears. 2m

23.3 Remove the supernatant and wash the beads with 200 µL 80% ethanol. Wait for 00:00:30 . 30s

23.4 Remove the ethanol and repeat the previous step for a total of 2 washes. 5m

23.5 Centrifuge briefly and place on the magnet **"low"**.
Remove any remaining Ethanol, but **do not** let the beads dry out. 2m

23.6 Remove from the magnet. Add 41 µL Buffer EB. Pipette mix 15x. 1m

23.7 Place the tube strip on the magnet **"low"** until the solution clears. 1m

23.8 Transfer 40 µL to a new tube strip. 1m
The Library is now finished and can be prepared for Illumina Sequencing as needed.

23.9 Store at 4 °C for up to 72:00:00 or at -20 °C for long-term storage.

24 Post Library Construction QC

24.1 Run 1 µL sample at 1:80 dilution on an **Agilent Bioanalyzer** High Sensitivity chip or on a **Tapestation** D5000 Screen Tape Assays. 30m
Select the region between 150-300 bp to determine average size of the library.