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### OPEN ACCESS



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### **MANUSCRIPT CITATION:**

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**Protocol status:** In development
We are still developing and optimizing this protocol

Created: Dec 14, 2023

### Fixed RNA - FFPE Resection Tissue (gentleMACS dissociation)

### Ksenija Sabic<sup>1</sup>

<sup>1</sup>Icahn School of Medicine at Mount Sinai



Ksenija Sabic

### **ABSTRACT**

This protocol utilizes the gentleMACS OctoDissociator, for pestle dissociation please refer to the original 10x protocol. This protocol assumes multiplexing exactly 4 or 16\* samples with 8,000 cells per sample, if fewer samples are being multiplexed or sub-pools are required, refer to the 10x protocol for pooling recommendations.

\*If 16 samples are being pooled, it is recommended to divide the samples into two batches due to the 8 sample limitation of the gentleMACS OctoDissociator.

For video instructions: https://www.10xgenomics.com/support/single-cell-gene-expression-flex

### **IMAGE ATTRIBUTION**

10x Genomics

### **GUIDELINES**

Please review and consult the full 10x Genomics protocols prior to starting and at any point during the procedure if needed.

### **MATERIALS**

### From 10x Genomics:

Conc. Quench Buffer 10x
Genomics Catalog #2000516

### Note

\*\*Included in the 10x Genomics Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit, 16 rxns (PN-1000414); also includes Enhancer (PN-2000482) if storing fixed cells.

Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 4 BC 10x Genomics Catalog #1000475

OR

Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 16 BC 10x Genomics Catalog #1000476

Last Modified: Dec 20, 2023

### **PROTOCOL integer ID:** 92347

Chromium Next GEM Chip Q Single Cell Kit, 48 rxns10x

Genomics Catalog #1000418

OR

- Chromium Next GEM Chip Q Single Cell Kit, 16 rxns10x
  Genomics Catalog #1000422
- Dual Index Kit TS Set A, 96 rxns 10x
  Genomics Catalog #1000251

### Miscellaneous:

Xylene Merck MilliporeSigma (Sigma-Aldrich) Catalog #214736

### Safety information

Xylene is a highly flammable liquid and vapor causes irritation to eyes, skin, and respiratory tract. Only use in chemical hood and dispose in designated Xylene waste container.

- Liberase TH Merck MilliporeSigma (Sigma-Aldrich) Catalog #5401135001
- GentleMACS C tube Miltenyi
  Biotec Catalog #130-093-237
- VitaStain AOPI Staining
  Solution Nexcelom Catalog #CS2-0106-5ml
- Cell Counting Plates
- Pre-Separation Filters (30 μm) Miltenyi
  Biotec Catalog #130-041-407
- PBS 1x without calcium & magnesium VWR International Catalog #Cat# 21-040-CVR

or similar

- RPMI 1640 with Lglutamine Corning Catalog #10040CV

  or similar
- Ethyl alcohol, 200 proof, anhydrous, ≥99.5% Merck MilliporeSigma (Sigma-Aldrich) Catalog #459836

or similar

Nuclease-Free Water (not DEPC-Treated) Thermo Fisher Scientific Catalog #AM9937

or similar

## Equipment gentleMACS™ Dissociator NAME tissue dissociator TYPE Miltenyi Biotec BRAND 130-093-235 SKU https://www.miltenyibiotec.com/US-en/products/gentlemacs-dissociator.html LINK 8 tubes, etc... SPECIFICATIONS

Equipment	
Cellaca MX High-throughput Automated Cell Counter	NAME
cell counter	TYPE
Nexcelom	BRAND
MX0112-0127	SKU

### SAFETY WARNINGS

Perform Xylene steps in chemical hood. During the first Ethanol incubation, samples may be moved to the bench.

### **BEFORE START INSTRUCTIONS**

Set water bath to  $\$ 65 \, ^{\circ}\text{C}$ . Set heat block to  $\$ 42 \, ^{\circ}\text{C}$ . Chill PBS in  $\$ 4 \, ^{\circ}\text{C}$  if not already.

## Isolation of Cells from FFPE Tissue Sections for Chromium Fixed RNA...

20m

Buffer Preparation (~ 🕙 00:20:00 min):

Note

All buffers should be prepared fresh.

Prepare Dissociation Enzyme Mix; incubate at \$\scrtex\$ 37 °C for 00:10:00 min before proceeding with dissociation:

10m

A	В	С	D	E	F
Dissociation Enzyme Mix	Stock	Final	1 rxn (µl)	4 rxn + 10% (µl)	8 rxn + 10% (µl)
Liberase TH (mg/ml)	5	1	420	1848	3696
RPMI	-	-	1680	7392	14,784
Total Volume (µl)			2100	9240	18,480

Reconstitute Liberase TH using 1mL Nuclease-free water.

1.2 Prepare Quenching Buffer, maintain at 4 °C

A	В	С	D	E	F
Quenching Buffer	Stock	Final	1 rxn (μl)	4 rxn + 10% (µl)	8 rxn + 10% (µl)
Nuclease-free water	-	-	437.5	1925	3850
Conc. Quench Buffer (10x Genomics)	8X	1X	62.5	275	550
Total Volume (µl)			500	2200	4400

Thaw Quench Buffer at room temperature, keep on ice.

**1.3** Prepare fresh 70% and 50% Ethanol (1 ml each/sample).

A

2

Note: Scrolls need to be intact and remain intact during the subsequent steps until the gentleMACS run. If scrolls appear as shards, request new scrolls from the biorepository. If scrolls disintegrate after adding xylene, take extra care when aspirating solutions.

Transfer either one  $\rightarrow$  60  $\mu$ m or two  $\rightarrow$  25  $\mu$ m FFPE scrolls to a gentleMACS C tube keeping the scrolls intact.

Add A 3 mL Xylene; incubate for 00:10:00 min.

- **3.1** Remove without breaking the scrolls.
- **3.2** Repeat Step 3 and 3.1.
- 4 Add Add Add 100% Ethanol; incubate for 00:00:30 sec.

200

- **4.1** Remove without breaking the scrolls.
- Repeat sequentially ( 00:00:30 sec each) with 1 mL 100% Ethanol, 1 mL 70% Ethanol, 1 mL 30s Ethanol.
- 6 Add A 1 mL Nuclease-free water; incubate for 30 sec.
- **6.1** Remove without breaking the scrolls.
- 7 Add A 1 mL chilled PBS; maintain on ice.

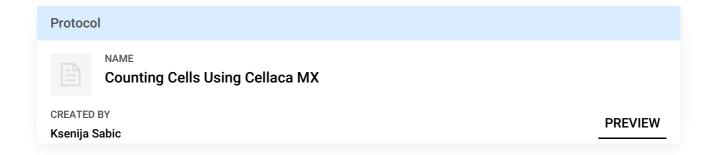
- 7.1 When ready to begin gentleMACS Octo Dissociator, remove PBS.
- Add <u>A 2 mL</u> Dissociation Enzyme Mix; secure cap, attach to gentleMACS with heater attached and run the following program (duration: ~ 00:48:00 min):

A	В
temp ON	
spin - 20 rpm	5' 0"
loop 3X	
spin 20 rpm	14' 0"
spin 1700 rpm	7"
spin 1700 rpm	1"
spin -1700 rpm	2"
spin 1700 rpm	1"
spin 1700 rpm	4"
end loop	
end	

saved as 'fixed\_ffpe' program

- 9 Centrifuge 300 rcf, 00:01:00 min.
- 10 Resuspend pellet in supernatant; filter through a 30µm strainer.
- 10.1 Wash strainer with 🗓 2 mL chilled PBS (~ 🗓 4 mL total volume).
- 11 Centrifuge ( 850 rcf, 00:05:00 min.

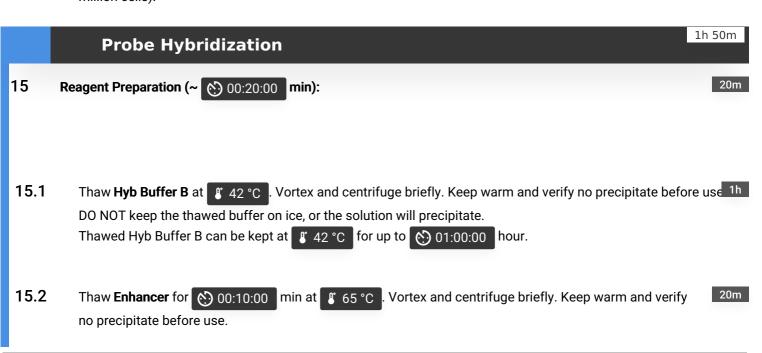
- **11.1** Remove supernatant.
- 12 Add 🛕 500 µL Tissue Resuspension Buffer; resuspend pellet.
- Count cells using below protocol and keep in mind that concentrations provided by the counter assume 1mL volume, but the actual volume is half.



14 Confirm cells numbers are in the correct range to move forward with hybridization:



- If <100,000 total cells, do not move forward to hybridization. Consider having thicker scrolls cut and repeat the procedure.
- If >2,000,000 total cells, divide resuspension before centrifuging in step 16 (ideally, you would proceed with 1 2 million cells).



DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at \$\mathbb{\epsilon}\$ 42 °C for up to \$\infty\$ 00:10:00 min.

15.3 Thaw Human WTA Probes on ice. Vortex and centrifuge briefly.

15.4 Prepare Hyb Mix at B Room temperature . Pipette mix 10x.

A	В	С	D	E	F
Hyb Mix (add reagents in order listed)	PN	1X (μl)	1X + 20% (μl)	4X + 20% (μl)	16X + 20% (µl)
Hyb Buffer B	2000485/ 2000483	70	84	336	1344
Enhancer	2000482	10	12	48	192
Total (µl)		80	96	384	1536

Ensure Enhancer has been incubated at 65C for 10 mins prior to use.

15.5 Incubate Hyb Mix at \$\mathbb{I}\$ 42 °C for \( \mathbb{O} \) 00:05:00 5m

15.6 Set thermal cycler to the following program:

A	В	С
Step	Temperature	Time
Pre-equilibriate	42C	Hold
Probe hybridization	42C	24 h*

Saved as 'hybridization'

Lid Temperature: 🕴 42 °C

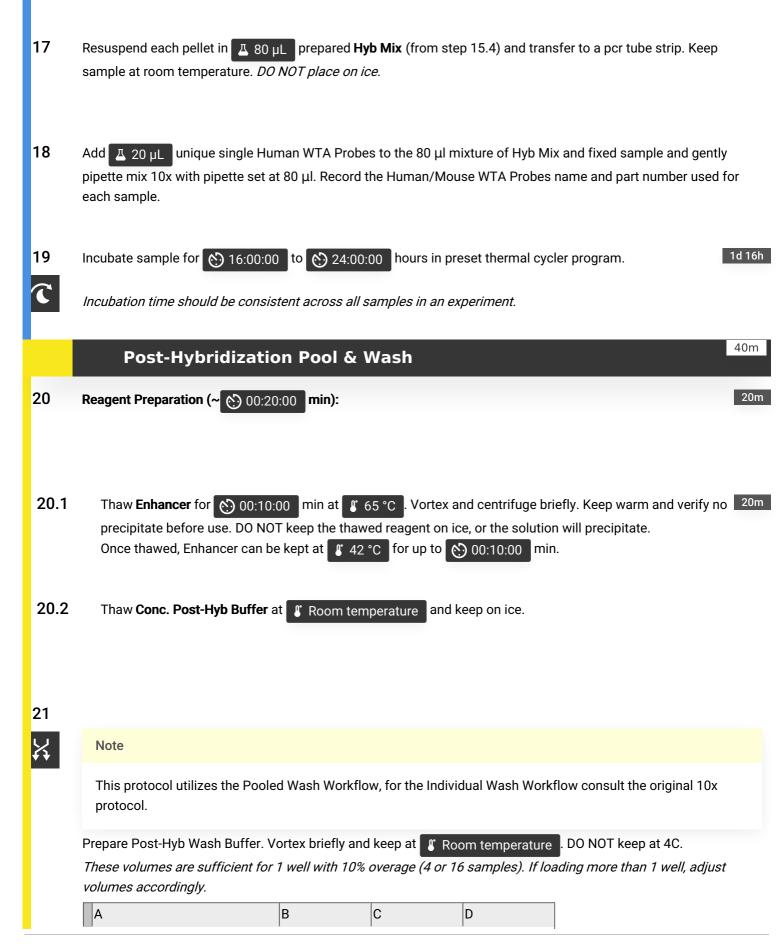
Reaction Volume: A 100 µL

Runtime: Overnight

\*24 h is the maximum incubation, be mindful of this time depending on experiment.

16 Centrifuge fixed cells/nuclei resuspended in Quenching Buffer at 850 rcf, 00:05:00 min at 4 °C

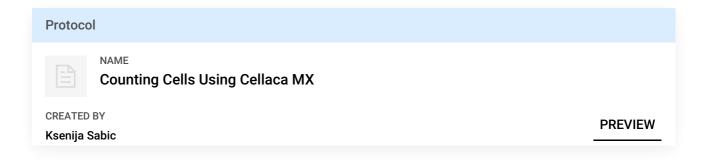
16.1 Remove the supernatant.



A	В	С	D
Post-Hyb Wash Buffer (add reagents in order listed)	PN	Pooling 4 samples (mL)	Pooling 16 samples (mL)
Nuclease-free water	-	4.95	13.86
Hyb Buffer B	2000533	0.275	0.77
Enhancer	2000482	0.275	0.77
Total (µl)		5.5	15.40

Note: volumes are in ml not μl.

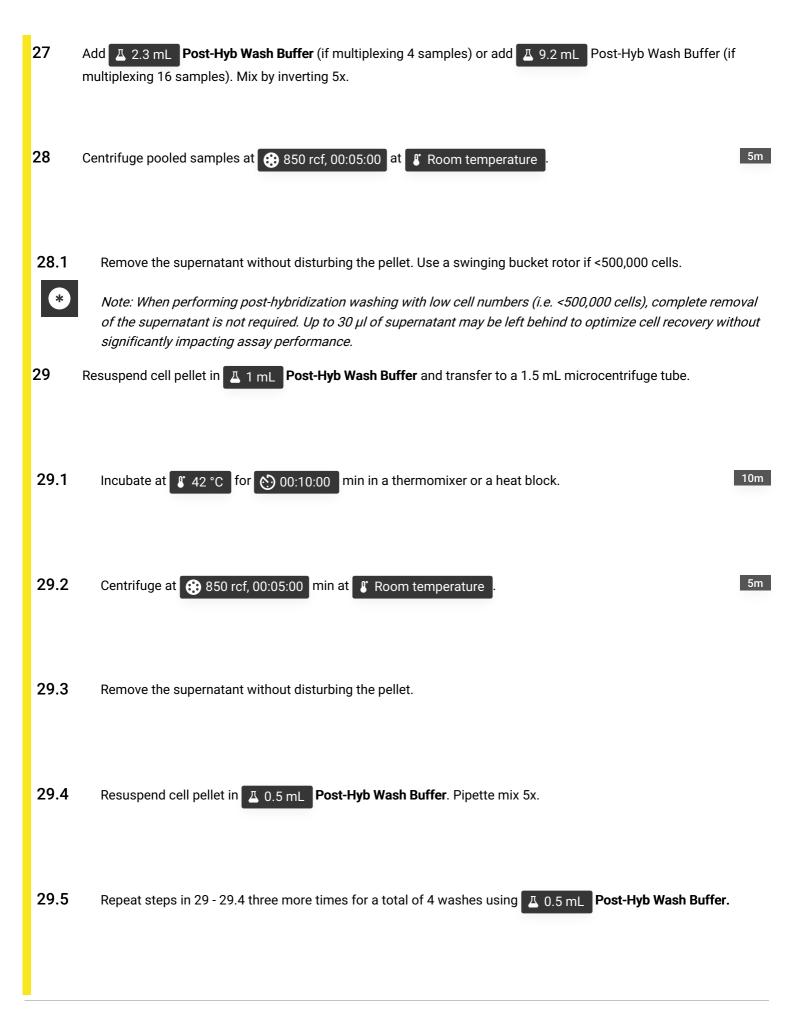
- Remove tubes from thermal cycler (8-tube strips) after overnight incubation.
- 23 Dilute each sample by adding Δ 190 μL of Post-Hyb Wash Buffer prepared in step 21 and pipette mix 5x.
- Count cells in *duplicate* using below protocol and keep in mind that concentrations provided by the counter assume 1mL volume, but the actual volume is half.



25 Enter cell concentrations (cells/µl) and sample volume in the Chromium Fixed RNA Profiling for Multiplexed Samples - Pooling Workbook to determine the volume required to normalize cell concentrations:

CG000565\_ChromiumFixedRNAProfiling\_MultiplexedSamples\_PoolingWorkbook\_RevA\_TEMPLATE.xlsx1 4MB

Pool an equal number of cells from different hybridization reactions into a 5-ml (for 4 pooling samples) or 15-ml (for pooling 16 samples) centrifuge tube.



During the final centrifuge step in 29.5, prepare Post-Hyb Resuspension Buffer. Pipette mix 10x and maintain at

A	В	С
Post-Hyb Resuspension Buffer (Add reagents in the order listed)	PN	1 Pool + 10% (μl)
Nuclease-free water	-	1567.5
Conc. Post-Hyb Buffer	2000533	82.5
Total		1650.0

Resuspend cell pellet in an appropriate volume of chilled Post-Hyb Resuspension Buffer. The buffer volume will depend upon the starting number of cells in the pool (table below). Pipette mix 20x to resuspend and breakup any cell clumps and maintain on ice.

A	В
Starting Total Cell Number in Pool	Post-Hyb Resuspension Buffer (µI)
<1 x 10^6	550
1 x 10^6 - 4 x 10^6	800
5 x 10^6 - 8 x 10^6	1050
9 x 10^6 - 12 x 10^6	1300
13 x 10^6 - 16 x 10^6	1550

Volumes reflect a 50µl overage to account for the subsequent counting step.

- Pass the sample through a 30 μm filter (Sysmex CellTrics or Miltenyi Biotec Pre-Separation Filters) into a new 1.5-ml/2-ml microcentrifuge tube and place on ice.
- Count cells in *duplicate* using below protocol and keep in mind that concentrations provided by the counter assume 1mL volume, but the actual volume is half.
- 33.1 If the sample concentration is not sufficient to achieve the desired targeted cell recovery, concentrate the sample as follows:
  - Centrifuge a known volume of sample at 850 rcf for 5 min at room temperature.
  - Carefully remove only a fraction of the supernatant, and pipette thoroughly to resuspend the cell pellet in the remaining volume. The amount of supernatant removed should be proportional to the desired increase in concentration.

- For example, to increase the concentration 4-fold from a starting volume of 400 μl, centrifuge, then remove 300 μl supernatant, and finally resuspend the cell pellet in the remaining 100 μl (400/100 = 4).
- Recount to confirm final concentration.

# 34.1 Equilibrate Single Cell TL v1 Gel Beads to Room temperature 00:30:00 min before loading the chir 30m 34.2 Equilibrate Reducing Agent B to Room temperature Nortex, verify no precipitate, centrifuge briefly. 34.3 Thaw GEM Reagent Mix at Room temperature Nortex, verify no precipitate, centrifuge briefly. Keep 34.4 Keep GEM Enzyme Mix at -20 °C until ready to use. Centrifuge briefly before

adding to the mix.

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

Α		В	С
GE in	EM Master Mix (Add reagents the order listed)	PN	1X* (µI)
GE	EM Reagent Mix	2000491	20.9
Re	educing Agent B	2000087	1.7
GE	EM Enzyme Mix	2000490	12.4
Тс	otal		35.0

<sup>\*1</sup>X = 1 well reaction. If loading more wells scale volumes accordingly.

36 Consult the attached tables to determine the correct ratio of sample to Post-Hyb Resuspension Buffer based on cell

concentration and targeted cell recovery.

### Cell Suspension Volume Calculator for Multiplexing 4 or 16 Samples.pdf3.7MB

- Add Δ 35 μL of prepared **GEM Master Mix** into each tube containing diluted sample and immediately proceed to the next step.
- 38 Assemble Chromium Next GEM Chip Q as follows:
  - 1. Close the holder lid. Attach the gasket by holding the tongue (curved end, to the right) and hook the gasket on the left-hand tabs of the holder. Gently pull the gasket toward the right and hook it on the two right-hand tabs.
  - 2. DO NOT touch the smooth side of the gasket.
  - 3. Open the chip holder.
  - 4. Remove the chip from the sealed bag. Use the chip within  $\leq$  24 h.
  - 5. Align notch on the chip (upper left corner) and the open holder with the gasket attached.
  - 6. Slide the chip to the left until the chip is inserted under the guide on the holder. Depress the right hand side of the chip until the spring-loaded clip engages.
  - 7. Keep the assembled unit with the attached gasket open until ready for and while dispensing reagents into the wells.
  - 8. DO NOT touch the smooth side of the gasket.
  - 9. After loading reagents, close the chip holder. DO NOT press down on the top of the gasket.
- 39 Load Chromium Next GEM Chip Q as follows.



When loading the chip, raising and depressing the pipette plunger should take ~5 sec. When dispensing, raise the pipette tips at the same rate as the liquid is rising, keeping the tips slightly submerged.

- **39.1** Add 50% glycerol solution to each unused well.
  - Д 70 µL in each unused well in **Row 1**.
  - Д 50 µL in each unused well in **Row 2**.
  - $\bot$  45  $\mu$ L in each unused well in **Row 3**.

DO NOT add 50% glycerol to the bottom row of unused wells.

### 39.2 Prepare Gel Beads:

35s

- Snap the tube strip holder with the Gel Bead strip onto a 10x Vortex Adapter. Vortex ♦ 00:00:30 sec.
- Centrifuge Gel Bead strip for ~ 00:00:05
   sec. 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.

### **39.3** Load Row 1:

- With the pipette set to 70µl, gently pipette the GEM Master Mix + Sample 15x.

**39.4** Load Row 2:



- Puncture the foil seal of the Gel Bead tubes. Slowly aspirate Д 50 µL Gel Beads.
- Dispense into the wells in Row 2 without introducing bubbles.
- Wait ( 00:01:00 min.
- **39.5** Load Row 3:
  - Dispense  $\bot$  45  $\mu$ L **Partitioning Oil** into the wells in Row 3 from a reagent reservoir.
- 39.6 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.
- 40 Run the Chromium iX:
  - 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 (~5.5 min), Chromium X/iX will chime.

### Immediately proceed to the next step.

### 41 Transfer GEMs:

20s

- 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 1-2. Abnormally high volume in any well indicates a clog.
- Slowly aspirate Д 100 µL **GEMs** from the lowest points of the recovery wells in Row 3 (top of chip) 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 ~ 600:00:20 sec, dispense GEMs into the tube strip on ice with the pipette tips against the sidewalls of the tubes.
- 42 Incubate in a thermal cycler with the following protocol:



A	В	С
Step	Temperature	Time
1	25C	60 min
2	60C	45 min
3	80C	20 min
Hold	4C	Hold

Lid Temperature: 80C

Reaction Volume: 100µl Run Time: ~125 min

Store at 4 °C for up to 1 week, or proceed to: