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MANUSCRIPT CITATION:

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

Created: Dec 14, 2023

Fixed RNA - FFPE Resection Tissue (gentleMACS dissociation)

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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 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**

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PROTOCOL integer ID: 92347

- Chromium Next GEM Chip Q Single Cell Kit, 48 rxns **10x Genomics Catalog #1000418**

OR

- Chromium Next GEM Chip Q Single Cell Kit, 16 rxns **10x 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 L-glutamine **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 °C .
Set heat block to 42 °C .
Chill PBS in 4 °C if not already.

Isolation of Cells from FFPE Tissue Sections for Chromium Fixed RNA... 10m

20m

1 **Buffer Preparation (~ 00:20:00 min):**

> **Note**
>
> All buffers should be prepared fresh.

1.1 Prepare Dissociation Enzyme Mix; incubate at 37 °C for 00:10:00 min before proceeding with dissociation: 10m

	A	B	C	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	B	C	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).

2 Transfer either one 50 µm or two 25 µm FFPE scrolls to a gentleMACS C tube keeping the scrolls intact.

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.

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

3.1 Remove without breaking the scrolls.

3.2 Repeat Step 3 and 3.1.

4 Add 3 mL 100% Ethanol; incubate for 00:00:30 sec. 30s

4.1 Remove without breaking the scrolls.

5 Repeat sequentially (00:00:30 sec each) with 1 mL 100% Ethanol, 1 mL 70% Ethanol, 1 mL Ethanol. 30s

6 Add 1 mL Nuclease-free water; incubate for 30 sec.

6.1 Remove without breaking the scrolls.

7 Add 1 mL chilled PBS; maintain on ice.

7.1 When ready to begin gentleMACS Octo Dissociator, remove PBS.

8 Add 2 mL Dissociation Enzyme Mix; secure cap, attach to gentleMACS with heater attached and run the following program (duration: ~ 00:48:00 min): 48m

	A	B
	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. 1m

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. 5m

11.1 Remove supernatant.

12 Add 500 µL Tissue Resuspension Buffer; resuspend pellet.

13 Count cells using below protocol and keep in mind that concentrations provided by the counter assume 1mL volume, but the actual volume is half.

Protocol

NAME

Counting Cells Using Cellaca MX

CREATED BY

Ksenija Sabic

PREVIEW

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).

Probe Hybridization

1h 50m

15 **Reagent Preparation (~ 00:20:00 min):** 20m

15.1 Thaw **Hyb Buffer B** at 42 °C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use 1h
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 01:00:00 hour.

15.2 Thaw **Enhancer** for 00:10:00 min at 65 °C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use. 20m

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 00:10:00 min.

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

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

	A	B	C	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 42 °C for 00:05:00 min. 5m

15.6 Set thermal cycler to the following program:

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

Saved as 'hybridization'
Lid Temperature: 42 °C
Reaction Volume: 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. 5m

16.1 Remove the supernatant.

17 Resuspend each pellet in 80 µL prepared **Hyb Mix** (from step 15.4) and transfer to a pcr tube strip. Keep sample at room temperature. *DO NOT place on ice.*

18 Add 20 µL unique single Human WTA Probes to the 80 µl mixture of Hyb Mix and fixed sample and gently pipette mix 10x with pipette set at 80 µl. Record the Human/Mouse WTA Probes name and part number used for each sample.

19 Incubate sample for 16:00:00 to 24:00:00 hours in preset thermal cycler program. 1d 16h

Incubation time should be consistent across all samples in an experiment.

Post-Hybridization Pool & Wash 40m

20 **Reagent Preparation (~ 00:20:00 min):** 20m

20.1 Thaw **Enhancer** for 00:10:00 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. 20m
Once thawed, Enhancer can be kept at 42 °C for up to 00:10:00 min.

20.2 Thaw **Conc. Post-Hyb Buffer** at Room temperature and keep on ice.

21

Note

This protocol utilizes the Pooled Wash Workflow, for the Individual Wash Workflow consult the original 10x protocol.

Prepare Post-Hyb Wash Buffer. Vortex briefly and keep at Room temperature. DO NOT keep at 4C.

These volumes are sufficient for 1 well with 10% overage (4 or 16 samples). If loading more than 1 well, adjust volumes accordingly.

	A	B	C	D

	A	B	C	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.

22 Remove tubes from thermal cycler (8-tube strips) after overnight incubation.

23 Dilute each sample by adding 190 µL of Post-Hyb Wash Buffer prepared in step 21 and pipette mix 5x.

24 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.

Protocol

NAME

Counting Cells Using Cellaca MX

CREATED BY

Ksenija Sabic

PREVIEW

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.xlsx
4MB

26 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.

27 Add 2.3 mL **Post-Hyb Wash Buffer** (if multiplexing 4 samples) or add 9.2 mL Post-Hyb Wash Buffer (if multiplexing 16 samples). Mix by inverting 5x.

28 Centrifuge pooled samples at 850 rcf, 00:05:00 at Room temperature . 5m

28.1 Remove the supernatant without disturbing the pellet. Use a swinging bucket rotor if <500,000 cells.

Note: When performing post-hybridization washing with low cell numbers (i.e. <500,000 cells), complete removal of the supernatant is not required. Up to 30 µl of supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.

29 Resuspend cell pellet in 1 mL **Post-Hyb Wash Buffer** and transfer to a 1.5 mL microcentrifuge tube.

29.1 Incubate at 42 °C for 00:10:00 min in a thermomixer or a heat block. 10m

29.2 Centrifuge at 850 rcf, 00:05:00 min at Room temperature . 5m

29.3 Remove the supernatant without disturbing the pellet.

29.4 Resuspend cell pellet in 0.5 mL **Post-Hyb Wash Buffer**. Pipette mix 5x.

29.5 Repeat steps in 29 - 29.4 three more times for a total of 4 washes using 0.5 mL **Post-Hyb Wash Buffer.**

30 During the final centrifuge step in 29.5, prepare Post-Hyb Resuspension Buffer. Pipette mix 10x and maintain at 4 °C:

	A	B	C
	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

31 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	B
	Starting Total Cell Number in Pool	Post-Hyb Resuspension Buffer (µl)
	<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.

32 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.

33 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.*

GEM Generation and Barcoding

1h

34 **Reagent Preparation (~ 00:30:00 min):** 30m

34.1 Equilibrate **Single Cell TL v1 Gel Beads** to Room temperature 00:30:00 min before loading the chip 30m

34.2 Equilibriate **Reducing Agent B** to Room temperature. Vortex, verify no precipitate, centrifuge briefly.

34.3 Thaw **GEM Reagent Mix** at Room temperature. Vortex, verify no precipitate, centrifuge briefly. Keep On ice.

34.4 Keep GEM Enzyme Mix at -20 °C until ready to use. Centrifuge briefly before adding to the mix.

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

	A	B	C
	GEM Master Mix (Add reagents in the order listed)	PN	1X* (µl)
	GEM Reagent Mix	2000491	20.9
	Reducing Agent B	2000087	1.7
	GEM Enzyme Mix	2000490	12.4
	Total		35.0

**1X = 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.pdf 3.7MB

37 Add 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 ≤ 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.

- 70 µL in each unused well in **Row 1**.
- 50 µL in each unused well in **Row 2**.
- 45 µL 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.
- Using the same pipette tips, dispense 70 µL **GEM Master Mix + Sample** into the bottom center of wells in **Row 1** without introducing bubbles.

39.4 Load Row 2: 1m

- Puncture the foil seal of the Gel Bead tubes. Slowly aspirate 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 45 µL **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 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 ~ 00:00:20 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	B	C
	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:**