10x Genomics®

Sample Preparation Demonstrated Protocol

Tumor Dissociation for Single Cell RNA Sequencing

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Manual Part Number

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Demonstrated Protocol

Tumor Dissociation for Single Cell RNA Sequencing

1. Overview

This demonstrated protocol outlines best practices for dissociation of mouse melanoma, colon tumor, and breast tumor for use in 10x Genomics® Single Cell protocols.

While this protocol is demonstrated with freshly obtained (not frozen) mouse melanoma, colon tumor, and breast tumor, it may be used for dissociating other tumor types for use in 10x Genomics Single Cell protocols. Modifications to this demonstrated protocol may be necessary for other tumor types (e.g. dissociation time, resuspension buffer, enzyme concentration, centrifugation speed and time).

2. Getting Started

2.1. Tips & Safety

Best practices for handling any cell type includes using sterile techniques, nuclease-free reagents and consumables, minimizing pipetting steps, and using wide-bore pipette tips when possible to minimize cell damage. Transfer pipettes may be used to remove supernatant after centrifugation to minimize disturbance of the cell pellet.

To determine accurate cell counts, best practices include sampling the cell suspension at least twice and at least two counts on each sample (i.e. a minimum of four counts in total, based on two independent draws from the cell suspension). Consult Technical Note *Guidelines on Accurate Target Cell Counts* (Document CG000091) for more information.

CRITICAL!

Human and animal cells carry potentially hazardous pathogens. Primary tumor cells should be handled under BSL-2 conditions. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage and disposal of biological materials.

2.2. General Materials

Supplier	Description	Part Number (US)
-	Refrigerated Benchtop Centrifuge for 15 ml and 50 ml tubes	-
	Microcentrifuge for 2 ml LoBind tubes	-
	Petri dish	
Rainin	Tips LTS 200UL Filter RT-L200FLR	17007961
	Tips LTS 1ML Filter RT-L1000FLR	17007954
	Tips LTS W-O 200UL Fltr RT-L200WFLR	17014294
	Tips LTS W-O 1ML Fltr RT-L1000WFLR	17014297
	Pipet-Lite LTS Pipette L-200XLS+	17014391
	Pipet-Lite LTS Pipette L-1000XLS+	17014382
Miltenyi Biotec	MACS Multistand	130-042-303
	OctoMACS™ Separator *(for use with MS columns)*	130-042-109
	QuadroMACS™ Separator *(for use with LS columns)*	130-090-976
	MACS® Dead Cell Removal Kit	130-090-101
	MS Columns	130-042-201
	LS Columns	130-042-401
	Tumor Dissociation Kit, mouse	130-096-730
	Red Blood Cell Lysis Solution (10X)	130-094-183
	MACS® SmartStrainers (70 µm)	130-098-462
	gentleMACS™ Octo Dissociator with Heaters	130-096-427
	gentleMACS™ C Tubes	130-093-237
Thermo Fisher Sci	Dulbecco's Phosphate-Buffered Saline (DPBS), No Calcium, No Magnesium	14190144
	UltraPure™ Bovine Serum Albumin (BSA, 50 mg/ml)	AM2616
	Nuclease-Free Water (not DEPC-Treated)	AM9937
	Trypan Blue Stain (0.4%)	T10282
	UltraPure™ Bovine Serum Albumin (BSA, 50 mg/ml)	AM2616
	Countess® II FL Automated Cell Counter	AMQAF1000
	Countess® II Automated Cell Counting Chamber Slides	C10228
Eppendorf	DNA LoBind Tubes, 2.0 ml*	022431048
ATCC	Dulbecco's Modified Eagle's Medium (DMEM)	30-2002
Corning Cellgro	RPMI 1640	10-040-CM
VWR	10 ml Serological Pipette	89130-898
	Sterile Polypropylene Centrifuge Tubes, 15 ml	21008-103
	Sterile Polypropylene Centrifuge Tubes, 50 ml	21008-178
Integra	PIPETBOY acu 2	155018

*No substitutions are allowed. Items have been validated by 10x Genomics® and are required for Single Cell workflow, training and system operations.

2.3. Preparation – Buffers

a) Prepare Enzyme D: Add **3 ml** RPMI 1640 or DMEM to resuspend lyophilized Enzyme D (Tumor Dissociation Kit). Aliquot and store at **–20°C** for up to **6 months** after reconstitution.

b) Prepare Enzyme R: Add **2.7 ml** RPMI 1640 or DMEM to resuspend the lyophilized Enzyme R (Tumor Dissociation Kit). Aliquot and store at **–20°C** for up to **6 months** after reconstitution.

NOTE

Thoroughly mix reconstituted Enzyme R immediately before withdrawing the required reaction volume.

c) Prepare Enzyme A: Add **1 ml** Buffer A to resuspend the lyophilized Enzyme A (Tumor Dissociation Kit). Aliquot and store at **–20°C** for up to **6 months** after reconstitution.

d) Chill 1X DPBS at **4°C**.

e) Prepare **10 ml** chilled (4°C) Wash Buffer: 1X DPBS containing 0.04% BSA.

f) Prepare **10 ml** 1X Red Blood Cell Lysis Solution: Mix **1 ml** 10X Red Blood Cell Lysis Solution with **9 ml** double distilled water (ddH_2O).

CRITICAL!

Do not use deionized water to prepare 1X Red Blood Cell Lysis Solution. Store at 4°C. Discard unused solution at the end of the day.

2.4. Specific Tissue Sourcing & Materials

NOTE

The protocol was demonstrated with fresh CT26 colon tumors, B16F10 Mouse melanoma tumors, and 4T1-e274 breast tumors from Charles River Laboratories (www.criver.com). The tumors were not frozen. Materials were stored according to manufaturer's recommendations prior to starting the protocol. Fresh colon tumor tissue was shipped on cold packs and used immediately upon receipt.

Tissue	Description	Supplier	Part Number
CT26 mouse colon tumors	Whole colon tumor in Miltenyi tissue storage solution. Tumor size: 300–500 mm^3	Charles River Laboratories	CT26
B16F10 mouse melanoma tumors	Whole colon tumor in Miltenyi tissue storage solution. Tumor size: 300–500 mm^3	Charles River Laboratories	B16F10
4T1-e274 mouse breast tumors	Whole breast tumor in Miltenyi tissue storage solution. Tumor size: 300–500 mm^3	Charles River Laboratories	-

3. Mouse Tumor Dissociation Protocol

3.1. Tumor Dissociation

NOTE

This protocol was demonstrated using fresh tissue of ~300–500 mm^3 size. Frozen tissue was not used for this protocol.

a) Wash the tumor tissue in a 50-ml centrifuge tube by adding **10 ml** chilled 1X DPBS.

b) Place in a petri dish and cut the tumor tissue to small pieces of ~2–4 mm^3.

c) Prepare enzyme mix in a gentleMACS™ C Tube by adding **2.35 ml** RPMI 1640 or DMEM, **100 µl** Enzyme D, **50 µl** Enzyme R, and **12.5 µl** Enzyme A.

d) Transfer the tumor tissue pieces to the C Tube containing the enzyme mix.

e) Tightly close the C Tube and attach upside down to a sleeve of a gentleMACS Octo Dissociator with Heaters.

CRITICAL!

The tissue in the C Tube should be close to the stator of the dissociator.

NOTE

Select the dissociator program based on tumor tissue texture (soft, medium or hard tissue).

f) Run the program **37C_m_TDK_1** for melanoma and colon tumor (soft and medium tissue respectively). Run the program **37C_m_TDK_2** for breast tumor (hard tissue).

g) At the end of the run, detach the C Tube from the dissociator.

h) Centrifuge at **300 rcf** for **30 sec** at **room temperature**.

i) Remove the supernatant without disturbing the cell pellet.

j) Add **10 ml** RPMI 1640 or DMEM and gently pipette mix to resuspend the cell pellet.

k) Filter the cell suspension through a prewetted 70-µm MACS® SmartStrainer placed on a 50-ml centrifuge tube.

l) Wash the strainer with **10 ml** RPMI 1640 or DMEM and collect the wash in the tube with the cell suspension.

m) Centrifuge the cell suspension at **300 rcf** for **7 min** at **room temperature**.

n) Remove supernatant without disturbing the cell pellet. Proceed immediately to step 3.2 for Red Blood Cell Lysis.

3.2. Red Blood Cell Lysis

NOTE

The red blood cells present in the dissociated tumor cell suspension are lysed by the Red Blood Cell Removal Solution. Prolonged exposure to this solution could lyse the tumor cells.

a) Add **1 ml** chilled 1X Red Blood Cell Removal Solution to the cell pellet from step 3.1n and gently pipette mix to resuspend the cells.

CRITICAL!

Do not vortex.

b) Incubate for **10 min** at **4°C**

c) Add **10 ml** chilled Wash Buffer.

d) Centrifuge at **4°C** at **300 rcf** for **10 min**.

e) Remove supernatant without disturbing the cell pellet.

f) Add **5 ml** chilled Wash Buffer and gently pipette mix to resuspend the cell pellet.

g) Determine the cell concentration using a Countess® II FL Automated Cell Counter or hemocytometer.

NOTE

Stain the cells with Trypan Blue and assess viability using the Countess II FL Automated Cell Counter/microscopy. If the percentage of viable cells <70%, follow Demonstrated Protocol Removal of Dead Cells Protocol (Document CG000093) to increase the percentage of viable cells. Multiple rounds of dead cell removal may be performed to obtain a high viability sample.

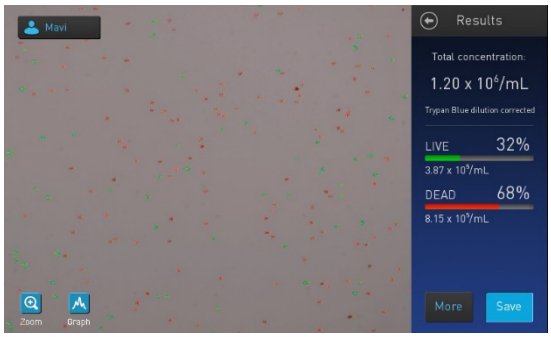
h) Add appropriate volume of chilled Wash buffer to the cell suspension and gently pipette mix to achieve the target cell concentration of **700–1200 cells/µl** ($7x10^5$–$1.2x10^6$ cells/ml).

i) Proceed immediately with the 10x Genomics® Single Cell protocol.

4. Results

After tumor dissociation, Trypan Blue staining of the sample showed a high percentage (68%) of dead cells. To reduce the number of dead cells, after tumor dissociation, the protocol outlined in Demonstrated Protocol *Removal of Dead Cell Protocol* (Document CG000093) was followed. Post dead cell removal, the dead cell fraction was reduced from 68% to 12%, thus increasing the percentage of viable cells in the sample. Results may vary between different tumor types.

Dissociated Tumor Cell Viability



Before Dead Cell Removal

Live = Green = 32%

Dead = Red =68%

Post Dead Cell Removal

Live = Green = 88%

Dead = Red = 12%

5. Troubleshooting

5.1. Troubleshooting Sample Preparation

Problem	Possible Reason	Solution
High fraction of non-viable **cells** after tissue dissociation	• Poor tissue quality • Protocol not optimized for the specific tissue type • Fragile sample type • Rough cell handling (fast pipetting, use of regular-bore pipette tips)	• Optimize cell/tissue dissociation protocol for improved sample quality • Reduce fraction of dead cells following Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093) • Gently handle cell suspensions by following best practices • Reduce cell processing time
	• High fraction of cellular debris in final resuspension	• Add 1–2 additional wash steps • Filter cell suspension with the appropriate strainer • Use flow cytometry to sort sample • Clean sample by density centrifugation following Demonstrated Protocol *Isolation of Nuclei for Single Cell RNA Sequencing* (Document CG000124, step3.6)
<700 cells/µl after final resuspension	• Low input tissue amount prior to dissociation • Overly dilute cell suspension	• Concentrate cell suspension to achieve target concentration of 700–1200 cells/µl

5.2. Troubleshooting Partitioning & Library Preparation

Problem	Possible Reason	Solution
Low/no cDNA yield	• Low quality input material	• Reduce fraction of dead cells and debris following Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093) • Obtain intact fresh tissue and avoid frozen tissue
	• Overly dilute cell suspension	• Concentrate cell suspension to achieve target concentration of 700–1200 cells/µl
	• Aggregation of cells and/or possible clog during partitioning	• The percentage of BSA in Wash Buffer can be increased to 1% to reduce aggregation and cell clumping • Cell suspensions should always be kept on ice • Use regular-bore pipette tips during final resuspension of cells • Filter cell suspension with the appropriate strainer • Cells combined with the Single Cell Master Mix should be gently pipette mixed 5–10 times with a regular-bore pipette tip and samples immediately loaded with the same pipette tip into the chip

5.3. Troubleshooting Data Analysis

Problem	Possible Reason	Solution
Low (<50%) "Fraction Reads in Cells"	• High fraction of ambient RNA in cell suspension	• Reduce fraction of dead cells and debris in the input material following Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093) • Add 1–2 additional wash steps when preparing the input material • Run the sample through Density centrifugation following Demonstrated Protocol *Isolation of Nuclei for Single Cell RNA Sequencing* (Document CG000124, step3.6)
Low library complexity (low number of genes/UMI's per cell)	• Low cDNA yield • Low quality input material	• Reduce fraction of dead cells and debris in the input material following Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093) • Obtain intact fresh tissue and avoid frozen tissue • Optimize cell/tissue dissociation protocol for improved sample quality • Filter fixed cell suspension with the appropriate strainer • Use flow cytometry to sort sample • Add RNAse inhibitor to the Wash Buffer to prevent RNA degradation