10x Genomics®

Sample Preparation Demonstrated Protocol

Enrichment of CD3+ T Cells from Dissociated Tissues for Single Cell RNA Sequencing and Immune Repertoire Profiling

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Table of Contents

Demonstrated Protocol

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1. Overview

This Demonstrated Protocol outlines best practices for enriching the percentage of Cluster of Differentiation 3 positive (CD3+) T cells obtained from dissociated tumors in preparation for use in 10x Genomics® Single Cell Protocols.

While this Protocol is demonstrated with tissue matched dissociated primary tumor cells and peripheral blood mononuclear cells (PBMCs) from a clear cell renal carcinoma (CCRC) patient, it has also been demonstrated with dissociated primary tumor cells from colorectal cancer (CRC) and renal cell carcinoma (RCC) patients, and with lymph node cells from melanoma patients. This Protocol may also be used as a basis for enrichment of the CD3+ T cell population from other dissociated tissues as well as other primary cells in preparation for use in 10x Genomics® Single Cell Protocols. Modifications to this Protocol may be necessary for other sample types (*e.g.* media type, resuspension buffer, centrifugation speed and time).

2. Getting Started

2.1. Tips & Safety

Best practices for handling any cell line 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	-
Rainin	Tips LTS 200UL Filter RT-L200FLR	17007961
	Tips LTS 1ML Filter RT-L1000FLR	17007954
	Pipet-Lite LTS Pipette L-200XLS+	17014391
	Pipet-Lite LTS Pipette L-1000XLS+	17014382
Corning Cellgro	Phosphate-Buffered Saline (PBS) 1X without calcium & magnesium	21-040-CV
	RPMI 1640	10-040-CM
Sigma-Aldrich	Phosphate-Buffered Saline (PBS) with 10% Bovine Albumin	SRE0036
Miltenyi Biotec	CD3 Microbeads (Human)	130-050-101
	MS Columns	130-042-201
	LS Columns	130-042-401
	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
Bel-Art	Flowmi™ Cell Strainer, 40 µm	H13680-0040
Thermo Fisher Sci	UltraPure™ Bovine Serum Albumin	AM2616
	Trypan Blue Stain (0.4%) for use with the Countess® Automated Cell Counter	T10282
	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
Seradigm	Fetal Bovine Serum	1500-500

*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 chilled (4°C) Wash Buffer: 0.5% v/v Fetal Bovine Serum (FBS), 2 mM EDTA in calcium- and magnesium-free 1X Phosphate-Buffered Saline (PBS), pH 7.2.

b) Prepare Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS.

c) Prepare tissue culture medium (RPMI 1640) containing 10% FBS for the final resuspension.

2.4. Cell Preparation & Sourcing

a) Dissociated tumor cells from a CCRC patient and matched PBMCs samples, in addition to tumor cells from CRC and RCC patients, and lymph node cells from melanoma patients were acquired from Conversant Bio. The cells were thawed, washed and counted per the Conversant Bio protocol, except the steps describing the DNAse I treatment of the cells were omitted. Conversant Bio protocols can be found at www.conversantbio.com. Cells were washed and resuspended in an appropriate tissue culture medium containing FBS, counted, and the percent viability determined.

b) Cells were prepared in DMEM, 10% FBS as follows:

Cell Type	Concentration	Cell Count
CCRC tumor (matched)	~1 x 10^6 cells/ml	3.5 x 10^6 cells
PBMCs (matched)	~1 x 10^6 cells/ml	11 x 10^6 cells
CRC	~1 x 10^6 cells/ml	6.5 x 10^6 cells
RCC	~1 x 10^6 cells/ml	3.4 x 10^6 cells
Melanoma	~1 x 10^6 cells/ml	30 x 10^6 cells

c) If the viability of the prepared cell suspension is <60%, centrifuge the cells at low speed (150 rcf for 10 min) and remove the supernatant to remove dead cells and debris. If the viability of the cell suspension is still low, remove dead cells by following Sample Preparation Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093) washing the cells twice in the appropriate medium before starting the enrichment.

d) If necessary, use a cell strainer to remove any cell debris and large clumps.

3. CD3+ Cell Enrichment Protocol

NOTE

This Protocol was demonstrated using sample sizes compatible with Miltenyi Biotec MS columns (1×10^4 – 1×10^7 labeled cells in 1×10^6 – 2×10^8 total cells). If using sample sizes compatible with Miltenyi Biotec LS columns (1×10^5 – 1×10^8 labeled cells in 1×10^7 – 2×10^9 total cells), consult the manufacturer's instructions.

a) Centrifuge the cell sample at **300 rcf** for **10 min** at **4°C**.

b) Remove supernatant without disturbing the pellet. Add **80 µl** chilled Wash Buffer. Using a **regular-bore** pipette tip, gently pipette mix 10 – 15 times or until cells are completely suspended.

c) Add **20 µl** CD3 MicroBeads. Using a **regular-bore** pipette tip, gently pipette mix 10 – 15 times.

d) Incubate for **15 min** at 4 – 8°C by placing the tube in a refrigerator.

e) Rinse the MS column with **500 µl** chilled Wash Buffer while the cells are incubating with the CD3 MicroBeads.

NOTE

If using the LS column, consult the manufacturer's instructions.

f) After incubation is complete, dilute the cell suspension containing the CD3 MicroBeads with **1.5 ml** chilled Wash Buffer. Mix by gently inverting the tube 5 times.

g) Centrifuge the cell suspension containing the CD3 MicroBeads at **300 rcf** for **10 min**.

h) Remove the supernatant without disturbing the pellet. Add **500 µl** chilled Wash Buffer. Using a **regular-bore** pipette tip, gently pipette mix 10 – 15 times or until cells are completely suspended.

i) Place a 2 ml tube labeled "CD3-" under the MS column and apply the cell suspension to the prepared MS column. The positively selected CD3+ cells will be retained on the column while the CD3- cells pass through the column.

CRITICAL!

Do not apply the plunger supplied with the column, otherwise positively selected CD3+ cells will be collected in the effluent.

j) Collect the effluent containing the unlabeled CD3- cells.

k) Wash the MS column with **1.5 ml** chilled Wash Buffer and collect the combined effluent containing the CD3- cells.

l) Place a 2 ml tube labeled "CD3+" under the MS column.

m) Remove the column from the magnet. Add **1 ml** chilled Wash Buffer to the MS column. Immediately flush out the magnetically labeled CD3+ cells by firmly applying the plunger supplied with the column.

n) Centrifuge both the CD3+ and CD3- cell fractions at **300 rcf** for **10 min**.

o) Remove supernatant without disturbing pellet. Add **200 µl** tissue culture medium (RPMI 1640) containing 10% FBS, or an appropriate volume, to achieve a target cell concentration of ~1×10^6 cells/ml. Using a **regular-bore** pipette tip, gently pipette mix 10 – 15 times or until cells are completely suspended.

NOTE

Do not invert the tube in this step, as cells can stick to the sides of the tube, changing the cell concentration.

p) Once the target cell concentration of ~1×10^6 cells/ml is obtained, place the cells on ice.

q) Proceed with the 10x Genomics® Single Cell Protocol.

4. Results

The results obtained following this Protocol are outlined below. The percent of CD3+ cells were determined via sequencing, analysis with Cell Ranger™ 1.3.1 and Seurat, and visualization with Loupe™ Cell Browser. Cells maintained percent viability and remained well singulated following this CD3 enrichment Protocol.

Sample	% CD3+ Cells Before	% CD3+ Cells After
Clear cell renal carcinoma primary tumor (matched)	26%	65%
PBMCs (matched)	27%	95%
Colorectal cancer primary tumor	27%	89%
Renal cell carcinoma primary tumor	37%	88%
Melanoma lymph node	30%	94%