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HTAPP_Depletion of CD45+ cells from single-cell suspension for single-cell RNA-seq

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Protocol status: Working
We use this protocol and it's working

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Abstract

In some tumor specimens the proportion of malignant cells is relatively low and the that of immune cells high. In these cases, we considered strategies to deplete CD45+ immune cells as a way to both enrich for epithelial cells without using specific epithelial cells markers, and to maintain the stromal cells.

The protocol described here is used for the depletion of CD45+ expressing immune cells from human tumor-derived single-cell suspensions. It is adapted from the CD45 MicroBeads MACS Separation protocol from Miltenyi Biotec.

For the Human Tumor Atlas Pilot Project (HTAPP), CD45 depletion was used to increase the recovery of malignant epithelial cells from lung non-small cell adenocarcinoma samples in which the proportion of these types of cells was low.

Description of this protocol and guidance for testing and selecting methods for processing different tumor and sample types can be found in ***Slyper et al***.

Guidelines

- Keep sample on ice and use cold reagents unless noted otherwise.
- Report information as indicated in the protocol.

Materials

MATERIALS

- UltraPure™ BSA (50 mg/mL) **Thermo Fisher Scientific Catalog #** AM2616
- PBS pH 7.4 **Thermo Fisher Scientific Catalog #**10010049
- BSA **Cell Signaling Technology Catalog #**9998S
- Trypan Blue Solution 0.4% Sterile-filtered **Sigma Aldrich Catalog #**T8154
- Flex-Tube® 1.5 mL PCR clean colorless **Eppendorf Catalog #**022364120
- Tips RT-LTS-A-1000µL-/F-768/8 **Rainin Catalog #**30389212
- Centrifuge 5430 R refrigerated with Rotor FA-45-30-11 incl. rotor lid keypad 120 V/50–60 Hz (US) **Eppendorf Catalog #**022620601
- Tips RT-LTS-A-10µL-/F/L-960/10 **Rainin Catalog #**30389226
- Tips RT-LTS-A-200µL-/F/L-960/10 **Rainin Catalog #**30389240
- Falcon® 15 mL High Clarity PP Centrifuge Tube Conical Bottom with Dome Seal Screw Cap Sterile 50/Rack 500/Case **Corning Catalog #**352097
- NanoEnTek Inc. Disposable Hemocytometer **Westnet Catalog #**C-CHIP
- MiniMACS Separator **Miltenyi Biotec Catalog #**130-042-102
- MACS MultiStand **Miltenyi Biotec Catalog #**130-042-303
- MS Columns **Miltenyi Biotec Catalog #**130-042-201
- CD45 MicroBeads human **Miltenyi Biotec Catalog #**130-045-801
- Trypan Blue solution **Millipore Sigma Catalog #**T8154
- Stericup Quick Release-GP Sterile Vacuum Filtration System **Millipore Sigma Catalog #**S2GPU02RE
- Sorvall Legend RT Refrigerated Tabletop Centrifuge **Thermo Scientific**

Safety warnings

Follow general lab safety and institutional guidelines when working with sharps and human derived samples.

Before start

- Set centrifuges to 4˚C.
- Label four 15 mL conical tubes as "Supernatant", "Wash", "CD45-" and "CD45- Supernatant", and two 1.5 mL Eppendorf tubes as "CD45-". Optionally, if you plan to also collect the CD45+ fraction for further analysis, label two 15 mL conical tubes as "CD45+" and "CD45+ Supernatant", and one 1.5 mL Eppendorf tube as "CD45+". Keep tubes on ice. Supernatants are collected to enable, if needed, the recovery by centrifugation of cells that have failed to pellet or were accidentally aspirated (e.g., if the final yield is too low).
- Prepare MACS buffer: PBS with 0.5% BSA and 2 mM EDTA (using Cell Signaling Technology BSA #9998S). Sterile filter, degas, and keep on ice. This solution can be prepared in advance and stored at 4˚C for several weeks.
- Prepare PBS with 0.4% BSA (using Thermo Fisher Scientific UltraPure BSA #AM2616) and keep on ice. This solution can be prepared in advance and stored at 4˚C for several weeks.

Initial Quality Control

1 Mix 5 µL of single-cell suspension with 5 µL Trypan blue and load on hemocytometer.

2 Count and record the number of viable single-cells, dead single-cells, cell doublets, and whether debris are present, then calculate additional quality control metrics below. Take picture if possible.

		Initial Cell Suspension
	Number of Viable Single Cells Counted	
	Number of Dead Single Cells Counted	
	Number of Cell Clumps or Doublets Counted	
	Concentration of Viable Single Cells (cells/µL)	
	Concentration of Dead Single Cells (cells/µL)	
	Concentration of Cell Clumps or Doublets (doublets/µL)	
	Volume of Single Cell Suspension (µL)	
	Total Number of Viable Single Cells	
	Proportion of Single Cells that are Viable (%)	
	Proportion of Cell Clumps or Doublets (%)	
	Description of debris (if any)	

Note

Insert Picture:

Magnetic Labeling

3 Centrifuge cell suspension in a 1.5 mL Eppendorf tube at 500 g for 4 minutes in 4˚C pre-cooled centrifuge.

500 x g, 4°C, 00:04:00

4 Carefully transfer supernatant to the 15 mL “Supernatant” tube kept on ice without disturbing the pellet.

On ice

5 If the cell pellet contains less than 10^7 cells, resuspend in 80 µL MACS buffer. If the cell pellet contains more than 10^7 cells, resuspend in 80 µL MACS buffer per 10^7 cells.

On ice

Note

Volume of MACS Buffer Used (µL):

6 Add 20 µL CD45 microbeads per 80 µL MACS buffer.

On ice

Note

Volume of Microbeads Used (µL):

7 Incubate in 4°C refrigerator for 15 minutes.

00:15:00 Labeling

4 °C Refrigerator

8 During the incubation, insert an MS column into the MiniMACS separator set on the MACS MultiStand. Prime the column with 500 µL MACS buffer, collecting the effluent in the 15 mL "Wash" tube.

Room temperature

9 Following incubation (Step 7), add 1 mL cold MACS buffer per 100 µL suspension.

On ice

Note

Volume of MACS Buffer Used (µL):

10 Centrifuge suspension at 500 g for 4 minutes in 4˚C pre-cooled centrifuge.

500 x g, 4°C, 00:04:00

11 Carefully transfer supernatant to the 15 mL “Supernatant” tube kept on ice without disturbing the pellet.
On ice

12 If the cell pellet contains less than 10^8 cells, resuspend in 500 µL MACS buffer. If the cell pellet contains more than 10^8 cells, resuspend in 500 µL MACS buffer per 10^8 cells.
On ice

Note

Volume of MACS Buffer Used (µL):

Collection of the CD45- fraction

13 Position the 15 mL "CD45-" tube under the MS column, discarding the "Wash" tube.
Room temperature

14 Transfer cell suspension (up to 2×10^8 cells) to the primed MS column on the MiniMACS separator and collect the flow-through in the 15 mL "CD45-" tube.
Room temperature

15 Wash the MS column by adding 500 µL MACS buffer and collecting the flow-through in the 15 mL "CD45-" tube. Repeat wash two more times.
Room temperature

16 Transfer the "CD45-" tube on ice.
On ice

[Optional] Collection of the CD45+ fraction

17 Remove the MS column from the MiniMACS separator and position it on top of the 15 mL "CD45+" tube.
Room temperature

18 Add 1 mL of MACS Buffer to the column and immediately elute the CD45+ cells by firmly pushing the plunger into the column.
Room temperature

19 Transfer the "CD45+" tube on ice.

On ice

Cell Concentration

20 Transfer the CD45- fraction to two 1.5 mL "CD45-" Eppendorf tubes on ice. (If a CD45+ fraction has been collected, transfer it to one 1.5 mL "CD45+" Eppendorf tube on ice.)

On ice

21 Centrifuge cell suspensions at 500 g for 4 minutes in 4˚C pre-cooled centrifuge.
Tip: If the number of cells is expected to be small (e.g., less than 50,000 cells), higher recovery may be obtained by centrifugation for 8 sec at 4˚C using short spin setting, with centrifugal force ramping up to but not exceeding 11,000 g (do not spin for a longer duration or at a higher centrifugal force, as this would result in cell death).

500 x g, 4°C, 00:04:00

22 Carefully transfer supernatant from the "CD45-" tubes to the 15 mL “CD45- Supernatant” tube kept on ice without disturbing the pellet. (If a CD45+ fraction has been collected, transfer the supernatant from the "CD45+" tube to the 15 mL "CD45+ Supernatant" tube kept on ice, without disturbing the pellet.)

On ice

23 Resuspend and combine both CD45- cell pellets in a total volume of 50 µL cold PBS with 0.4% BSA. (If a CD45+ fraction has been collected, resuspend the CD45+ pellet in 50 µL cold PBS with 0.4% BSA.)

On ice

Final Quality Control

24 Mix 5 µL of single-cell suspension with 5 µL Trypan blue and load on hemocytometer.

25 Count and report the number of viable single cells, dead single cells, cell doublets or clumps, and whether debris are present, then calculate additional quality control metrics below. Take picture if possible.

		CD45- Fraction	CD45+ Fraction (if collected)
	Number of Viable Single Cells Counted		
	Number of Dead Single Cells Counted		

	Number of Cell Clumps or Doublets Counted		
	Concentration of Viable Single Cells (cells/µL)		
	Concentration of Dead Single Cells (cells/µL)		
	Concentration of Cell Clumps or Doublets (doublets/µL)		
	Volume of Single Cell Suspension (µL)		
	Total Number of Viable Single Cells		
	Proportion of Single Cells that are Viable (%)		
	Proportion of Cell Clumps or Doublets (%)		
	Description of debris (if any)		

Note

Insert Picture for CD45- fraction:

Insert Picture for CD45+ fraction (if collected):

Loading on 10x

26 If necessary, adjust the concentration of the CD45- fraction before proceeding to loading on 10x Genomics Single-Cell RNA-seq system, following 10x Genomics recommendations.
*Tip: 8,000-10,000 live cells are typically loaded per channel. Optimal cell recovery is achieved for concentrations between 800 and 1,200 cells/µL but deviations from that range are acceptable (see **10x Technical Note** on this topic). Furthermore, it is recommended that viability be higher than 60% and the proportion of cell clumps lower than 5%.*
On ice

27 Report the information listed below about loading on 10x Genomics Single-Cell RNA-seq system, including the number and concentration of cells per channel.

Note

Time of Loading:

Person Loading:

Single-Cell RNA-seq Kit Used:

Concentration of Viable Cells Loaded (cells/μL):

Number of Cells Loaded per Channel:

Number of Channels Loaded: