Isolation of CD8⁺CD279⁺ T cells from human PBMCs

Contents

1. Description

1.1 Principle of the MACS® Separation

This special protocol describes the isolation of CD8⁺CD279⁺ T cells from human peripheral blood mononuclear cells (PBMCs) using a two-step magnetic purification procedure. After initial selection for CD8 expression, purified cells are magnetically labeled for a second time to select for CD279 expression. After the second step, the resulting cell population will be CD8⁺CD279⁺ T cells.

Sequential magnetic purification of the target cells is facilitated by using the CD8 MultiSort Kit, human (# 130-055-201). In parallel the cells are labeled with CD279-PE. After a MicroBead removal step the cells are labeled for a second time with Anti-PE MicroBeads (# 130-048-801).

1.2 Background information

CD279, also known as PD-1 (programmed death-1), is expressed on T cells, B cells, NK cells, activated myeloid cells, and dendritic cells. The ligands PD-L1 (B7-H1;CD274) and PD-L2 (B7-DC; CD273) belong to the B7 immunglobulin superfamily.[1]
PD-1 and its ligands mediate inhibitory signals that regulate the balance between T cell activation and tolerance in infections, autoimmunity, and tumor.
PD-1 upregulation on human immunodificiency virus (HIV)-specific CD8⁺ T cells correlates with high viral load.[2] Differential expression of PD-1 on HIV-, cytomegalo virus (CMV)-, ebstein-barr-virus (EBV)-, and varizella zosta virus (VZV)-specific T cells from the same subjects provides evidence for differential regulation of virus-specific T cell function during chronic viral infections.[1,3]

In Hepatitis C Virus (HCV) infection PD-1 expression was reported to be high on HCV-specific CD4⁺ and CD8⁺ T cells early in acute infection, but also to be continued in resolved and chronic stages of infection.[4] Tumor antigen-specific CD8 T cells infiltrating the tumor were shown to express high levels of PD-1 and to be functionally impaired.[5]

1.3 Applications

● Isolation of CD8⁺CD279⁺ T cells from human peripheral blood mononuclear cells (PBMCs) for further phenotypical or functional characterization.

1.4 Reagent and instrument requirements

● Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS® BSA Stock Solution (# 130-091-376) 1:20 with autoMACS® Rinsing Solution (# 130-091-222). Keep buffer cold (2–8 °C). Degas buffer before use, as air bubbles could block the column.

    ▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as human serum albumin, human serum, or fetal bovine serum (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

● MACS Columns and MACS Separators: Enrichment of CD8⁺ cells can be performed on an MS or LS Column. The enrichment of CD8⁺CD279⁺ T cells can be performed on two MS Columns. Positive selection and depletion can also be performed by using the autoMACS Pro Separator or the autoMACS Separator.

Column	Max. number of labeled cells	Max. number of total cells	Separator
Depletion and positive selection			
MS	10^7	$2×10^8$	MiniMACS, OctoMACS, VarioMACS, SuperMACS II
LS	10^8	$2×10^9$	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II
autoMACS	$2×10^8$	$4×10^9$	autoMACS Pro, autoMACS

    ▲ **Note:** Column adapters are required to insert certain columns into the VarioMACS™ or SuperMACS™ II Separators. For details refer to the respective MACS Separator data sheet.

● CD8 MultiSort Kit, human (# 130-055-201)

● Anti-PE MicroBeads (# 130-048-801)

● CD279-PE, human (# 130-096-164)

● (Optional) Fluorochrome-conjugated antibodies for flow cytometric analysis, e.g., CD8-FITC (# 130-080-601) and CD3-APC (# 130-091-373). For more information about fluorochrome-conjugated antibodies refer to www.miltenyibiotec.com/antibodies.

● (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD for flow cytometric exclusion of dead cells.

- (Optional) Dead Cell Removal Kit (# 130-090-101) for the depletion of dead cells.
- (Optional) Pre-Separation Filters, 30 μm (# 130-041-407) to remove cell clumps.

## 2. Protocol

2.1 Sample preparation

When working with anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™.

▲ **Note:** To remove platelets after density gradient separation, resuspend cell pellet in buffer and centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully aspirate supernatant. Repeat washing step.

When working with tissues or lysed blood, prepare a single-cell suspension using standard methods.

For details see the protocols section at www.miltenyibiotec.com/protocols.

▲ Dead cells may bind non-specifically to MACS MicroBeads. To remove dead cells, we recommend using density gradient centrifugation or the Dead Cell Removal Kit (# 130-090-101).
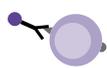
2.2 Magnetic labeling of CD8⁺ T cells

▲ Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ Volumes for magnetic labeling given below are for up to 10^7 total cells. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^7 total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic labeling. Pass cells through 30 μm nylon mesh (Pre-Separation Filters, 30 μm # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

▲ The recommended incubation temperature is 2–8 °C. Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.

1. Determine cell number.

2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.

3. Resuspend cell pellet in 70 μL of buffer per 10^7 total cells.

4. Add 20 μL of CD8 MultiSort MicroBeads and 10 μL of CD279-PE per 10^7 total cells.

5. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).

6. (Optional) Add staining antibodies, e.g., 10 μL of CD8-FITC (# 130-080-601) and 10 μL of CD3-APC (# 130-091-373), and incubate for 5 minutes in the dark in the refrigerator (2–8 °C).

7. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.

8. Resuspend up to 10^8 cells in 500 μL of buffer.

▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.

9. Proceed to magnetic separation (2.3).

2.3 Magnetic separation: Enrichment of CD8⁺ T cells

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of CD8⁺ T cells. For details see table in section 1.4.

▲ Always wait until the column reservoir is empty before proceeding to the next step.

Magnetic separation with MS or LS Columns

1. Place column in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.

2. Prepare column by rinsing with the appropriate amount of buffer:

    MS: 500 μL   LS: 3 mL

3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.

4. Wash column with the appropriate amount of buffer. Collect unlabeled cells that pass through and combine with the flow-through from step 3.

    MS: 3×500 μL   LS: 3×3 mL

▲ **Note:** Perform washing steps by adding buffer aliquots only when the column reservoir is empty.

5. Remove column from the separator and place it on a suitable collection tube.

6. Pipette the appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

    MS: 1 mL   LS: 5 mL

7. Proceed to Removal of MultiSort MicroBeads and magnetic labeling of CD8⁺CD279⁺ cells (2.4).

Magnetic separation with the autoMACS® Pro Separator or the autoMACS® Separator

▲ Refer to the respective user manual for instructions on how to use the autoMACS® Pro Separator or the autoMACS Separator.

▲ Buffers used for operating the autoMACS Pro Separator or the autoMACS Separator should have a temperature of ≥ 10 °C.

▲ Program choice depends on the isolation strategy, the strength of magnetic labeling, and the frequency of magnetically labeled cells. For details refer to the section describing the cell separation programs in the respective user manual. Program recommendations below refer to separation of human PBMCs.

Magnetic separation with the autoMACS® Pro Separator

1. Prepare and prime the instrument.

2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.

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3. For a standard separation choose the following program:
   Positive selection: Possel
   Collect positive fraction in row C of the tube rack.

4. Proceed to Removal of MultiSort MicroBeads and magnetic labeling of CD8+CD279+ cells (2.4).

Magnetic separation with the autoMACS® Separator

1. Prepare and prime the instrument.

2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube at the uptake port and the fraction collection tubes at port neg1 and port pos1.

3. For a standard separation choose the following program:
   Positive selection: Possel
   Collect negative fraction from outlet port neg1.

4. Proceed to Removal of MultiSort MicroBeads and magnetic labeling of CD8+CD279+ cells (2.4).
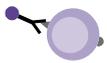
2.4 Removal of MultiSort MicroBeads and magnetic labeling of CD8+CD279+ T cells

▲ Volumes for magnetic labeling given below are for an initial starting cell number of up to 10^7 total cells. For higher initial cell numbers, scale up all volumes accordingly.

▲ The recommended incubation temperature is 2–8 °C. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

1. Remove a sample for analysis by flow cytometry and proceed with the remaining magnetically labeled fraction.

2. Add 20 µL of MultiSort Release Reagent per 1 mL of cell suspension.

3. Mix well and incubate for 10 minutes in the refrigerator in the dark (2–8 °C).

4. Repeat the magnetic separation procedure as described in 2.3. Separate cells over a new column of the same type (MS or LS Column) or use the same autoMACS Pro Separator or autoMACS Separator program to be used in the second parameter separation.

5. Wash cells from the released fraction carefully by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.

6. Resuspend cells in 60 µL of buffer per 10^7 total cells .

7. Add 10 µL Anti-PE MicroBeads and 30 µL of MultiSort Stop Reagent per 10^7 total cells and mix well.

8. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).

5. Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.

6. Resuspend up to 10^8 cells in 500 µL of buffer.
   ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.

7. Proceed to magnetic separation (2.5).

2.5 Magnetic separation: Positive selection of CD8+CD279+ T cells

Positive selection with MS Columns

▲ To achieve highest purities, perform two consecutive column runs.

1. Place MS Column in the magnetic field of a suitable MACS Separator. For details refer to MS Column data sheet.

2. Prepare column by rinsing with 500 µL of buffer.

3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.

4. Wash column with 3×500 µL of buffer. Collect unlabeled cells that pass through and combine with the effluent from step 3.
   ▲ **Note:** Perform washing steps by adding buffer aliquots only when the column reservoir is empty.

5. Remove column from the separator and place it on a suitable collection tube.
   ▲ **Note:** To perform a second column run, you may elute the cells directly from the first onto the second, equilibrated column instead of a collection tube.

6. Pipette 1 mL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

Positive selection with the autoMACS® Pro Separator or the autoMACS® Separator

**Magnetic separation with the autoMACS® Pro Separator**

1. Prepare and prime the instrument.

2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.

3. For a standard separation choose the following program:
   Positive selection: Possel
   Collect positive fraction in row C of the tube rack. This is the enriched CD8+CD279+ T cell fraction.

Magnetic separation with the autoMACS® Separator

1. Prepare and prime the instrument.

2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube at the uptake port and the fraction collection tubes at port neg1 and port pos1.

3. For a standard separation choose one of the following programs:
   Positive selection: Possel
   Collect positive fraction from outlet port pos1. This is the enriched CD8+CD279+ T cell fraction.

3. Example of a separation of CD8+CD279+ T cells from human PBMCs

CD8+CD279+ T cells were isolated from human PBMCs by using the CD8 MultiSort Kit, CD279-PE, Anti-PE MicroBeads, MS Columns, and a MiniMACS™ Separator. The cells were fluorescently stained and analyzed by flow cytometry using the MACSQuant® Analyzer. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.

Before separation

Pre-enriched CD8+ T cells

Isolated CD8+CD279+ T cells
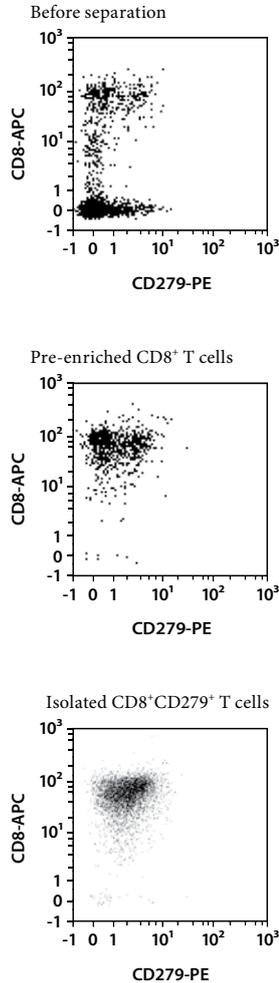
4. References

1. Keir, M. E. *et al.* (2008) PD-1 and its ligands in tolerance and immunity. Annu. Rev. Immunol. 26: 677–704.

2. Trautman, L. *et.al.* (2006) Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. Nature Medicine 12: 1198–1202.

3. Kassu, A. *et.al.* (2010) Regulation of virus-specific CD4+ T cell function by multiple costimulatory receptors during chronic HIV infection. J. Immunol. 185: 3007–3018.

4. Kasprowicz, V. *et. al.* (2008) High level of PD-1 expression on hepatitis C virus (HCV)-specificCD8 and CD4 T cells during acute HCV infection, irrespective of clinical outcome. J. Virol. 82: 3154–3160.

5. Ahmadzadeh, M. *et. al.* (2009) Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. Blood 114: 1537–1544.

All protocols and data sheets are available at **www.miltenyibiotec.com**.

Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with running water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

Warranty

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