Isolation of CD4$^+$CD279$^+$ cells from human PBMCs

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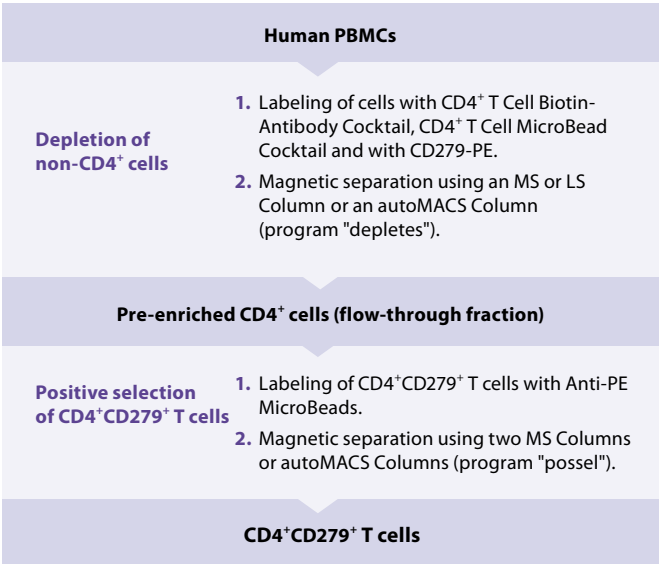

1. Description

1.1 Principle of the MACS® Separation

This special protocol describes the isolation of CD4$^+$CD279$^+$ T cells in a two-step procedure by using the CD4$^+$ T Cell Isolation Kit, human (# 130-096-533), which contains a Biotin-Antibody Cocktail and CD4$^+$ T Cell MicroBead Cocktail, CD279-PE (# 130-096-164), and Anti-PE MicroBeads (# 130-048-801).

First, the non-CD4$^+$ cells are labeled with a cocktail of biotin-conjugated antibodies and the CD4$^+$ T Cell MicroBead Cocktail. In parallel, the cells are labeled with CD279-PE. The non-target cells are subsequently depleted by separation over a MACS® Column, which is placed in the magnetic field of a MACS Separator. In the second step, the CD4$^+$CD279$^+$ cells are directly labeled with Anti-PE MicroBeads and isolated by positive selection from the pre-enriched CD4$^+$ cell fraction by separation over a MACS Column, which is placed in the magnetic field of a MACS Separator.

After removing the column from the magnetic field, the CD4$^+$CD279$^+$ cells can be eluted from the column as the positively selected cell fraction. To increase the purity, the positively selected cell fraction containing the CD4$^+$CD279$^+$ cells is separated over a second column.

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] Also a correlation between PD-1 expression on HIV-specific CD4 T cells and viral load has been reported.[3] 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.[1,4]

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.[5]

1.3 Applications

- Isolation of CD4$^+$CD279$^+$ T cells from human peripheral blood mononuclear cells (PBMCs) for further phenotypical or functional characterization.

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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: Depletion of non-$CD4^+$ cells can be performed on an MS or LS Column. The subsequent positive selection of $CD4^+CD279^+$ T cells can be performed on two MS Columns. Positive selection and depletion can also be performed by using the autoMACS Pro 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™ Separators. For details see the respective MACS Separator data sheet.

- $CD4^+$ T Cell Isolation Kit, human (# 130-096-533)
- Anti-PE MicroBeads (# 130-048-801)
- CD279-PE (# 130-096-164)
- (Optional) Fluorochrome-conjugated antibodies for flow cytometric analysis, e.g., CD4-FITC (# 130-080-501) and CD3-APC (# 130-091-373). For more information about fluorochrome-conjugated antibodies see www.miltenyibiotec.com.
- (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 non-$CD4^+$ 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 40 µL of buffer per 10^7 total cells.
4. Add 10 µL of $CD4^+$ T Cell Biotin-Antibody Cocktail per 10^7 total cells.
5. Mix well and incubate for 5 minutes in the refrigerator (2–8 °C).
6. Add 30 µL of buffer per 10^7 total cells.
7. Add 20 µL of $CD4^+$ T Cell MicroBead Cocktail and 10 µL of CD279-PE per 10^7 total cells.
8. Mix well and incubate for an additional 10 minutes in the refrigerator (2–8 °C).
9. (Optional) Add staining antibodies, e.g., 10 µL of CD4-FITC (# 130-080-501) and 10 µL of CD3-APC (# 130-091-373), and incubate for 5 minutes in the dark in the refrigerator (2–8 °C).
10. Adjust the volume to a minimum of 500 µL of buffer.
11. Proceed to magnetic separation (2.3).

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2.3 Magnetic separation: Depletion of non-CD4$^+$ cells

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of CD4$^+$ 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, representing the unlabeled pre-enriched CD4$^+$ cell fraction.
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. (Optional) Elute retained cells outside of the magnetic field. This fraction represents the magnetically labeled non-CD4$^+$ T cells.

Depletion 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.
3. For a standard separation choose the following program:

   Depletion: "depletes"
   Collect negative fraction in row B of the tube rack.
4. Proceed to 2.4 for the isolation of CD4$^+$CD279$^+$ T cells.

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:

   Depletion: "depletes"
   Collect negative fraction from outlet port neg1.
4. Proceed to 2.4 for the isolation of CD4$^+$CD279$^+$ T cells.

2.4 Magnetic labeling of CD4$^+$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.

1. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 80 µL of buffer per 10^7 total cells.
3. Add 20 µL of Anti-PE MicroBeads per 10^7 total cells.
4. 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 CD4$^+$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 see 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.

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7. To increase purity of $CD4^+CD279^+$ T cells, the eluted fraction is enriched over a second MS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new 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 $CD4^+CD279^+$ T cell fraction.

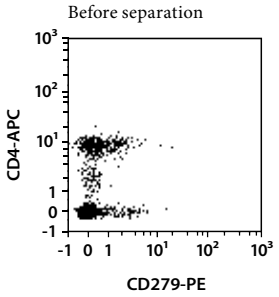
Magnetic separation with the autoMACS® Separator

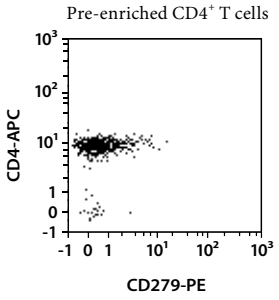
1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube at the uptake port and the fraction collection tubes at port neg1 and port pos1.
3. For a standard separation choose one of the following programs:

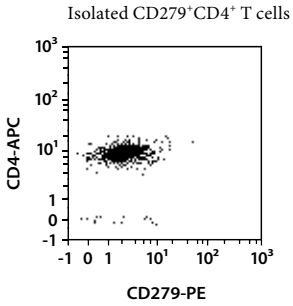
   Positive selection: "possel"
   Collect positive fraction from outlet port pos1. This is the enriched $CD4^+CD279^+$ T cell fraction.

3. Example of a separation of $CD4^+CD279^+$ T cells from human PBMCs

$CD4^+CD279^+$ T cells were isolated from human PBMCs by using the $CD4^+$ T Cell Isolation 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.







4. References

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6. 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.

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