

Contents

1. Description
 - 1.1 Principle of the MACS[®] Separation
 - 1.2 Background information
 - 1.3 Applications
 - 1.4 Reagent and instrument requirements
2. Protocol
 - 2.1 Sample preparation
 - 2.2 Magnetic labeling of non-CD4⁺ and CD127^{high} cells
 - 2.3 Magnetic separation: Depletion of non-CD4⁺ and CD127^{high} cells
 - 2.4 Magnetic labeling of CD4⁺CD25⁺CD127^{dim/-} regulatory T cells
 - 2.5 Magnetic separation: Positive selection of CD4⁺CD25⁺CD127^{dim/-} regulatory T cells
3. Example of a separation using the CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation Kit II
4. References

1. Description

Components	<p>1 mL CD4⁺CD25⁺CD127^{dim/-} T Cell Biotin-Antibody Cocktail II, human: Cocktail of biotin-conjugated monoclonal anti-human antibodies against CD8, CD19, CD123, and CD127.</p> <p>2 mL Anti-Biotin MicroBeads: MicroBeads conjugated to monoclonal anti-biotin antibody (isotype: mouse IgG1).</p> <p>1 mL CD25 MicroBeads II, human: MicroBeads conjugated to monoclonal anti-CD25 antibody (isotype: mouse IgG1).</p>
Capacity	For 10 ⁹ total cells, up to 100 separations.
Product format	All components are supplied in buffer containing stabilizer and 0.05% sodium azide.
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

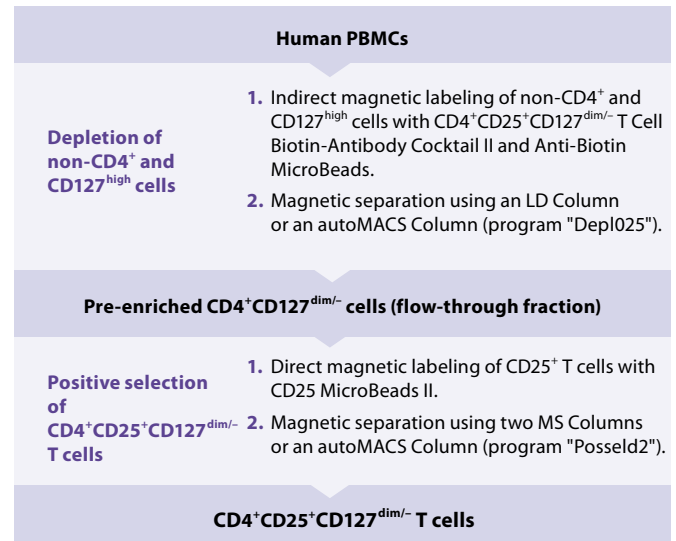
1.1 Principle of the MACS[®] Separation

The isolation of CD4⁺CD25⁺CD127^{dim/-} regulatory T cells is performed in a two-step procedure. First, the non-CD4⁺ and CD127^{high} cells are indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies, as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labeling reagent. In between and after the two

labeling steps no washing steps are required. The labeled 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⁺CD25⁺CD127^{dim/-} regulatory T cells are directly labeled with CD25 MicroBeads II and isolated by positive selection from the pre-enriched CD4⁺ T 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 magnetically retained CD4⁺CD25⁺CD127^{dim/-} regulatory T cells can be eluted as the positively selected cell fraction. To increase the purity, the positively selected cell fraction containing the CD4⁺CD25⁺CD127^{dim/-} regulatory T cells is separated over a second column.



1.2 Background information

Regulatory CD4⁺ T cells are suppressor cells that neutralize other immune cells by various mechanisms.¹ Their characteristic marker is the transcription factor FoxP3. CD4⁺CD25⁺ regulatory T cells were originally discovered in mice, but a population with identical phenotype has also been identified in humans.²⁻⁶ CD25 is the interleukin-2-receptor α-chain, which is not only expressed by regulatory T cells but also by activated effector T cells.

CD127, the α-chain of the IL-7 receptor, is expressed on most mature T cells and plays an important role in their proliferation and differentiation.⁷ However, on regulatory T cells CD127 is absent and its expression inversely correlates with FoxP3 expression.^{8,9} Thus, CD127 can be used as an additional marker to discriminate between human regulatory and activated T cells.

1.3 Applications

- Isolation of CD4⁺CD25⁺CD127^{dim/-} regulatory 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 Ca²⁺ or Mg²⁺ are not recommended for use.

- MACS Columns and MACS Separators: Depletion of non-CD4⁺ and CD127^{high} cells can be performed on an LD Column. The subsequent positive selection of CD4⁺CD25⁺CD127^{dim/-} 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
Positive selection			
MS	10 ⁷	2×10 ⁸	MiniMACS, OctoMACS, VarioMACS, SuperMACS II
Depletion			
LD	10 ⁸	5×10 ⁸	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II

Positive selection or depletion

▲ **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.

- (Optional) Fluorochrome-conjugated antibodies for flow cytometric analysis, e.g., CD4-FITC (# 130-080-501), CD25-APC (# 130-092-858), and CD127-PE (# 130-094-889) For more information about fluorochrome-conjugated antibodies see www.miltenyibiotec.com/antibodies.
- (Optional) Anti-FoxP3-APC (# 130-093-013) or Anti-FoxP3-PE (# 130-093-014) and FoxP3 Staining Buffer Set (# 130-093-142).
- (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 refer to 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⁺ and CD127^{high} 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 10⁷ total cells. When working with fewer than 10⁷ 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 2×10⁷ 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. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

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 10⁷ total cells.
4. Add 10 μL of CD4⁺CD25⁺CD127^{dim/-} T Cell Biotin-Antibody Cocktail II per 10⁷ total cells.
5. Mix well and incubate for 5 minutes in the refrigerator (2–8 °C).
6. Add 30 μL of buffer and 20 μL of Anti-Biotin MicroBeads per 10⁷ total cells.
7. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).
8. Adjust the volume to a minimum of 500 μL of buffer.

▲ **Note:** Resuspend up to 10⁸ cells in 500 μL of buffer. For higher cell numbers, scale up buffer volume accordingly. For higher cell numbers, scale up buffer volume accordingly.
9. Proceed to magnetic separation (2.3).



2.3 Magnetic separation: Depletion of non-CD4⁺ and CD127^{high} cells

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of CD4⁺CD25⁺CD127^{dim/-} cells. For details refer to table in section 1.4.

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

Depletion with LD Columns

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details see LD Column data sheet.
2. Prepare column by rinsing with 2 mL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with 2×1 mL of buffer. Collect total effluent; this is the unlabeled pre-enriched CD4⁺ cell fraction. Perform washing steps by adding buffer two times. Only add new buffer when the column reservoir is empty.
5. Proceed to 2.4 for the isolation of CD4⁺CD25⁺CD127^{dim/-} 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: Depl025

Collect negative fraction in row B of the tube rack.

4. Proceed to 2.4 for the isolation of CD4⁺CD25⁺CD127^{dim/-} 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: Depl025

Collect negative fraction from outlet port neg1.

4. Proceed to 2.4 for the isolation of CD4⁺CD25⁺CD127^{dim/-} cells.



2.4 Magnetic labeling of CD4⁺CD25⁺CD127^{dim/-} regulatory T cells

▲ Volumes for magnetic labeling given below are for an initial starting cell number of up to 10⁷ 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 90 µL of buffer per 10⁷ total cells.
3. Add 10 µL of CD25 MicroBeads II per 10⁷ total cells.
4. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
5. (Optional) Add staining antibodies, e.g., 10 µL of CD25-APC (# 130-092-858), 10 µL of CD4-FITC (# 130-080-501), and 10 µL of CD127-PE, and incubate for 5 minutes in the dark in the refrigerator (2–8 °C).
6. Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend up to 10⁸ cells in 500 µL of buffer.
▲ Note: For higher cell numbers, scale up buffer volume accordingly.
8. Proceed to magnetic separation (2.5).



2.5 Magnetic separation: Positive selection of CD4⁺CD25⁺CD127^{dim/-} regulatory 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.

7. To increase purity of CD4⁺CD25⁺CD127^{dim/-} cells, the eluted fraction can be 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: Posseld2

Collect positive fraction in row C of the tube rack. This is the enriched CD4⁺CD25⁺CD127^{dim/-} 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 pos2.
3. For a standard separation choose one of the following programs:

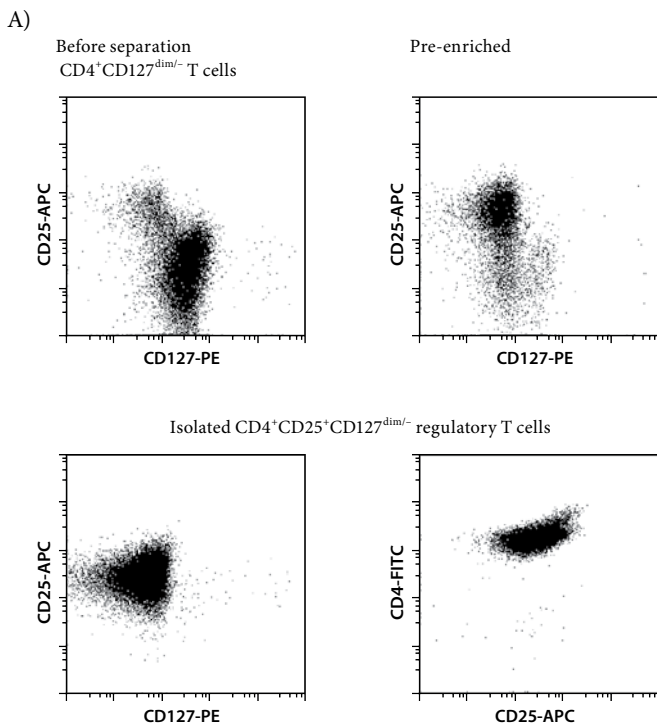
Positive selection: Posseld2

Collect positive fraction from outlet port pos2. This is the enriched CD4⁺CD25⁺CD127^{dim/-} T cell fraction.

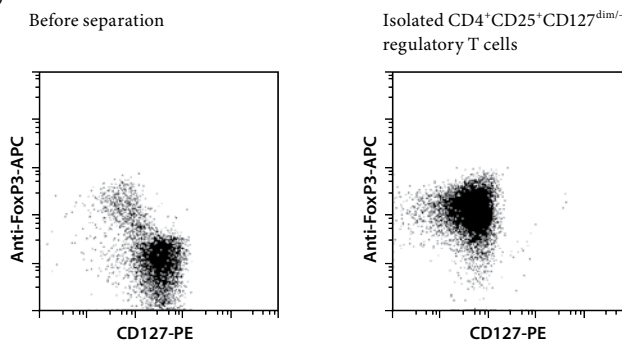
3. Example of a separation using the CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation Kit II

CD4⁺CD25⁺CD127^{dim/-} regulatory T cells were isolated from human PBMCs by using the CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation Kit II, an LD and two MS Columns, a MidiMACS[™] and a MiniMACS[™] Separator. The cells were fluorescently stained with CD4-FITC (# 130-080-501), CD25-APC (# 130-092-858), and CD127-PE (# 130-094-889) (A), or CD4-FITC, CD127-PE and Anti-Fox-P3-APC (# 130-093-013) (B) 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.

Gating was performed according to CD4-expression (except for the CD25-APC versus CD4-FITC dot plot).



B)



4. References

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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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