Miltenyi Biotec

CD4+CD25+CD45RA+ Regulatory T Cell Isolation Kit

human

Order no. 130-093-631

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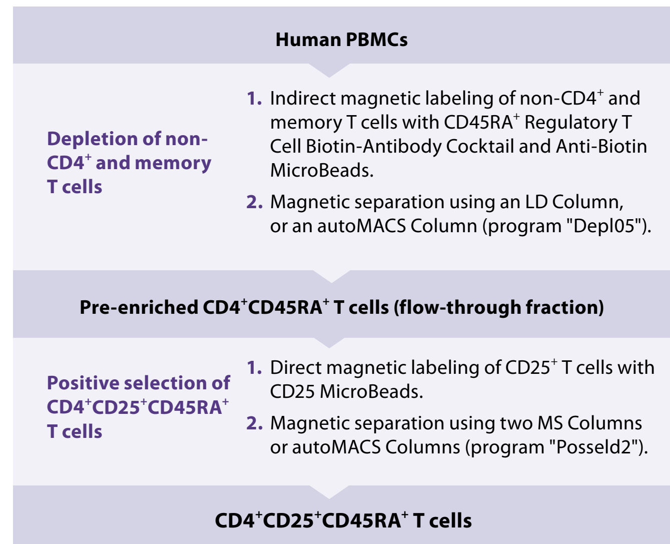
1. Description

Components	**2 mL CD45RA+ Regulatory T Cell Biotin-Antibody Cocktail, human:** Cocktail of biotin-conjugated monoclonal antibodies against CD8, CD14, CD16, CD19, CD36, CD45RO, CD56, CD123, TCRγδ, and CD235a (Glycophorin A). **2×2 mL Anti-Biotin MicroBeads:** MicroBeads conjugated to monoclonal anti-biotin antibody (isotype: mouse IgG1). **1 mL CD25 MicroBeads, human:** MicroBeads conjugated to monoclonal anti-human CD25 antibodies (isotype: mouse IgG1).
Capacity	For 2×10^9 total cells.
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+CD45RA+ regulatory T cells is performed in a two-step procedure. First, non-CD4+ and memory T cells are indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies and Anti-Biotin MicroBeads. The labeled cells are subsequently depleted by separation over a MACS® Column.

In the second step, CD4+CD25+CD45RA+ T cells are directly labeled with CD25 MicroBeads and isolated by positive selection from the pre-enriched CD4+CD45RA+ T cell fraction. The magnetically labeled CD4+CD25+CD45RA+ T cells are retained on the column and eluted after removal of the column from the magnetic field. To achieve highest purities, the positively selected cell fraction containing the CD4+CD25+CD45RA+ T cells is separated over a second column.



1.2 Background information

A subpopulation of CD4+CD25+ T cells appears to act as regulatory T cells upon activation through their T cell receptor.[1] CD4+CD25+ regulatory T cells were originally discovered in mice, but a population with identical phenotypic and functional properties has also been characterized in humans.[2–7] Regulatory CD4+CD25+ T cells seem to suppress harmful immunological reactions to self or foreign antigens. Regulatory T cells isolated from the CD45RA+ naive T cell compartment were shown to be optimal for *in vitro* expansion.[8,9] These expanded regulatory T cells maintained the FoxP3+ phenotype and their suppressive activity, whereas CD45RA− regulatory T cells from the memory T cell compartment lost FoxP3 expression and were only modestly suppressive.[8,9]

1.3 Applications

- Isolation of CD4+CD25+CD45RA+ regulatory T cells from human peripheral blood mononuclear cells (PBMCs) for further phenotypical or functional characterization and expansion.

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Miltenyi Biotec GmbH
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macs@miltenyibiotec.de
www.miltenyibiotec.com

Miltenyi Biotec Inc.
12740 Earhart Avenue, Auburn, CA 95602, USA
Phone 800 FOR MACS, +1 530 888 8871, Fax +1 530 888 8925
macs@miltenyibiotec.com

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$^+$ and memory T cells is performed on an LD Column. The subsequent positive selection of CD4$^+$CD25$^+$CD45RA$^+$ T cells is performed on two MS Columns. Depletion and positive selection 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			
LD	10^8	5×10^8	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
Positive selection			
MS	10^7	2×10^8	MiniMACS, OctoMACS, VarioMACS, SuperMACS
Depletion or positive selection			
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.

- (Optional) Fluorochrome-conjugated antibodies for flow cytometric analysis, e.g., CD4-FITC (# 130-080-501), CD25-PE (# 130-091-024), FoxP3-APC (# 130-093-013), CD45RA-PE (# 130-092-248), or CD45RA-APC (# 130-092-249). 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 (# 130-041-407) to remove cell clumps.

2. Protocol

2.1 Sample preparation

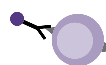
When working with anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™.

▲ **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$^+$ and memory 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.

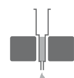
▲ Volumes for magnetic labeling given below are for up to 10^8 total cells. When working with fewer than 10^8 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^8 total cells, use twice the volume 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, # 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 400 µL of buffer per 10^8 total cells.
4. Add 100 µL of CD45RA$^+$ Regulatory T Cell Biotin-Antibody Cocktail per 10^8 total cells.
5. Mix well and incubate for 10 minutes in the refrigerator (2−8 °C).
6. Add an additional 300 µL of buffer per 10^8 total cells.
7. Add 200 µL of Anti-Biotin MicroBeads per 10^8 total cells.
8. Mix well and incubate for additional 15 minutes in the refrigerator (2−8 °C).
9. Wash cells by adding 10–20 mL of buffer per 10^8 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
10. Resuspend up to 10^8 cells in 500 µL of buffer.

    ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
11. Proceed to magnetic separation (2.3).



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

▲ 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.

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4. Collect unlabeled cells that pass through and wash column with 2×1 mL of buffer. Collect total effluent; this is the unlabeled 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 $CD4^+CD25^+CD45RA^+$ 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.

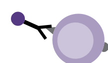
▲ Program choice depends on the isolation strategy, the strength of magnetic labeling, and the frequency of magnetically labeled cells. For details refer to the section describing the cell separation programs in the respective user manual.

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: "Depl05"
   Collect negative fraction in row B of the tube rack.
4. Proceed to 2.4 for the isolation of $CD4^+CD25^+CD45RA^+$ T cells.

Magnetic separation with the autoMACS™ Separator

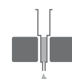
1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube at the uptake port and the fraction collection tubes at port neg1.
3. For a standard separation choose the following program:
   Depletion: "Depl05"
   Collect negative fraction from outlet port neg1.
4. Proceed to 2.4 for the isolation of $CD4^+CD25^+CD45RA^+$ T cells.



2.4 Magnetic labeling of $CD4^+CD25^+CD45RA^+$ regulatory T cells

▲ Volumes for magnetic labeling given below are for an initial starting cell number of up to 10^8 cells. For larger initial cell numbers, scale up volumes accordingly.

1. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 450 µL of buffer per 10^8 total cells.
4. Add 50 µL of CD25 MicroBeads per 10^8 total cells.
5. Mix well and incubate for 15 minutes in the refrigerator (2−8 °C).
6. Wash cells by adding 5−10 mL of buffer per 10^8 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend up to 10^8 cells in 500 µL of buffer.
8. Proceed to magnetic separation (2.5).



2.5 Magnetic separation: Positive selection of $CD4^+CD25^+CD45RA^+$ 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 see the 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 with3×500 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 the purity of $CD4^+CD25^+CD45RA^+$ cells, the eluted fraction can be enriched over a second MS Column. Repeat the magnetic separation procedure as described in steps 1 to 7 by using a new column.

Positive selection 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.

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.

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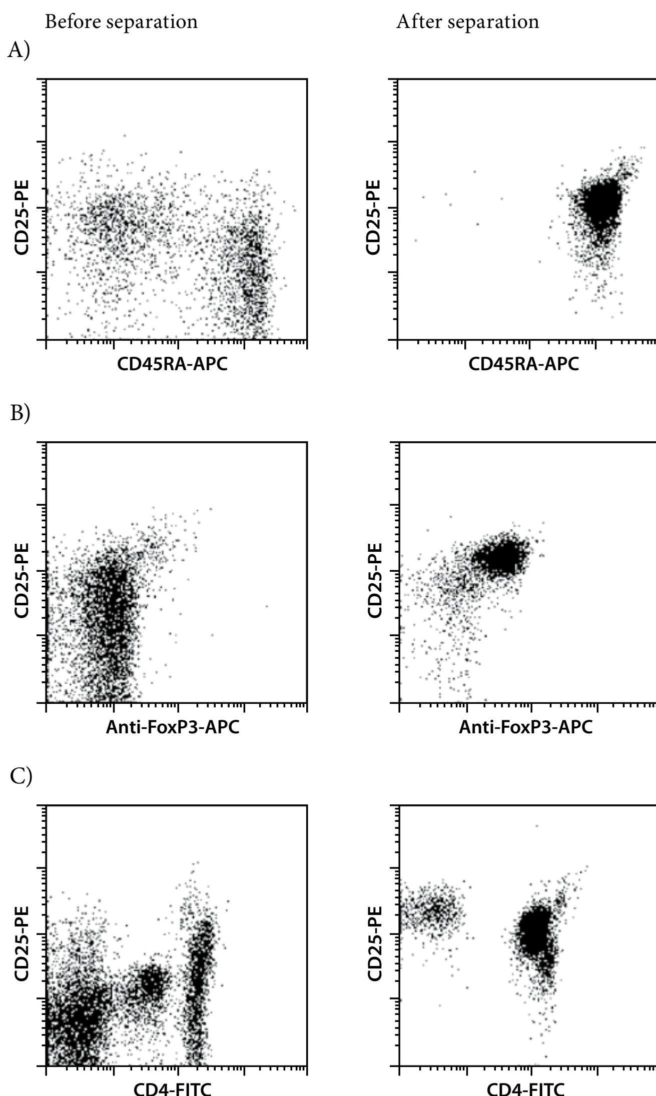
Magnetic separation with the autoMACS™ Separator

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 the following program:

   Positive selection: "Posseld2"
   Collect positive fraction from outlet port pos2.

3. Example of a separation using the CD4+CD25+CD45RA+ Regulatory T Cell Isolation Kit

CD4+CD25+CD45RA+ regulatory T cells were isolated from human PBMCs by using the CD4+CD25+CD45RA+ Regulatory T Cell Isolation Kit, an LD, and two MS Columns, a MidiMACS™ Separator, and a MiniMACS™ Separator. The cells are fluorescently stained with CD4-FITC, CD25-PE, and either CD45RA-APC (A) or FoxP3-APC (B), gated on CD4+ cells. In C, cells are stained with CD4-FITC and CD25-PE, gated on lymphocytes. Cell debris and dead cells were excluded from the analysis based on scatter signals and PI fluorescence. Dot plots show stainings gated on viable lymphocytes (C) or viable CD4+ lymphocytes (A, 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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