Miltenyi Biotec

CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit
human

Order no. 130-091-301

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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.

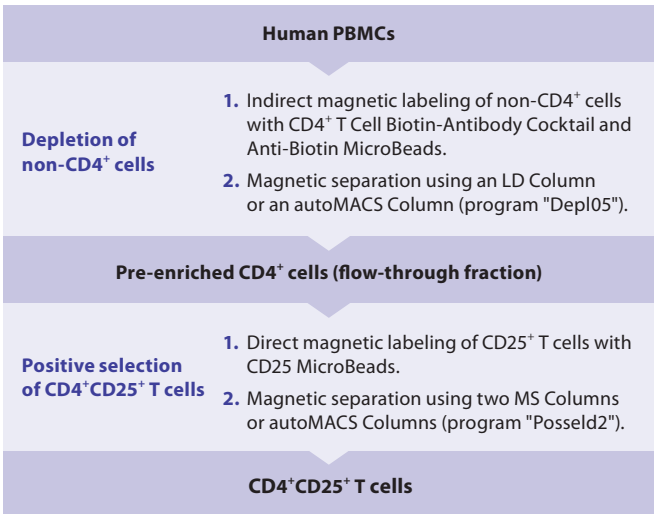
1. Description

This product is for research use only.

Components	**1 mL CD4$^+$ T Cell Biotin-Antibody Cocktail, human:** Cocktail of biotin-conjugated monoclonal anti-human antibodies against CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, and CD235a (Glycophorin A). **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-CD25 antibody (isotype: mouse IgG1).
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$^+$ regulatory T cells is performed in a two-step procedure. First, the non-CD4$^+$ 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$^+$ regulatory T cells are directly labeled with CD25 MicroBeads 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$^+$ 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$^+$ regulatory T cells must be 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. Regulatory CD4$^+$CD25$^+$ T cells seem to suppress harmful immunological reactions to self or foreign antigens.

Miltenyi Biotec B.V. & Co. KG
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macsde@miltenyi.com
www.miltenyibiotec.com

1.3 Applications

- Isolation of $CD4^+CD25^+$ regulatory T cells from human peripheral blood mononuclear cells (PBMCs) for further phenotypical or functional characterization.
- For the isolation of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells for *in vitro* suppression assays, for example, by using the Treg Suppression Inspector.

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 LD Column. The subsequent positive selection of $CD4^+CD25^+$ T cells can be performed on two MS Columns. Positive selection and depletion can also be performed by using the autoMACS Pro Separator.

Column	Max. number of labeled cells	Max. number of total cells	Separator
Depletion			
LD	10^8	5×10^8	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II
Positive selection			
MS	10^7	2×10^8	MiniMACS, OctoMACS, VarioMACS, SuperMACS II
Depletion or positive selection			
autoMACS	2×10^8	4×10^9	autoMACS Pro

▲ **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 (VIT4)-VioBlue®, CD127-PE, and CD25-APC, clone 4E3. For more information about fluorochrome-conjugated antibodies refer to www.miltenyibiotec.com/antibodies.
- (Optional) Anti-FoxP3-APC or Anti-FoxP3-PE 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.

For details refer to the protocols section at www.miltenyibiotec.com/protocols.

When working with tissues, prepare a single-cell suspension using the gentleMACS™ Dissociator.

For details refer to www.gentlemacs.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. 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 90 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 20 µL of Anti-Biotin MicroBeads per 10^7 cells.
7. Mix well and incubate for an additional 10 minutes in the refrigerator (2–8 °C).
8. Proceed to magnetic separation (2.3).

   ▲ **Note:** A minimum of 500 µL is required for magnetic separation. If necessary, add buffer to the cell suspension.

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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$^+$ 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 Column

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details refer to 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 labeling of CD4$^+$CD25$^+$ T cells.

Magnetic separation with the autoMACS® Pro Separator

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

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

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 one of the following programs:

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

2.4 Magnetic labeling of CD4$^+$CD25$^+$ regulatory 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 90 µL of buffer.
3. Add 10 µL of CD25 MicroBeads.
4. Mix well and incubate for 15 minutes in the dark in the refrigerator (2–8 °C).
5. (Optional) Add staining antibodies, e.g., 10 µL of CD25-PE and, e.g., 10 µL of CD4-FITC, or CD4-APC, and incubate for 5 minutes in the dark in the refrigerator (2–8 °C).

   ▲ **Note:** For the analysis of CD25 it is highly recommended to use REA945 or clone 4E3.
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 10^8 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$^+$ regulatory T cells

▲ 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$^+$ cells, the eluted fraction must 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

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

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

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$^+$ regulatory T cell fraction.

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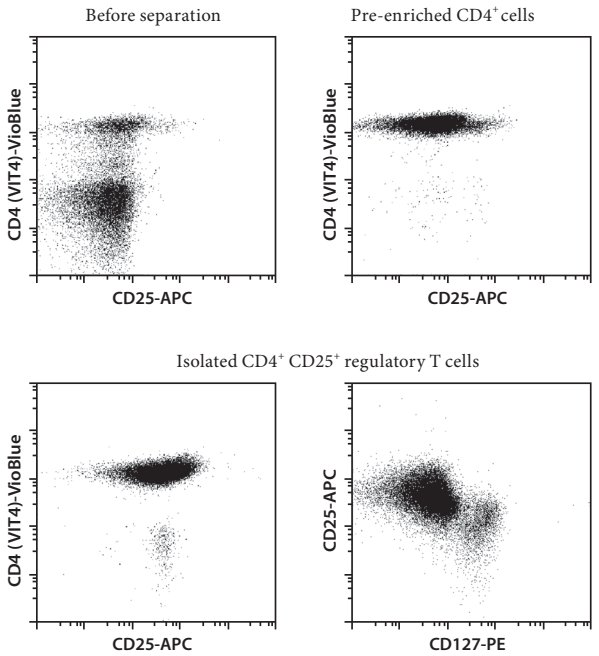
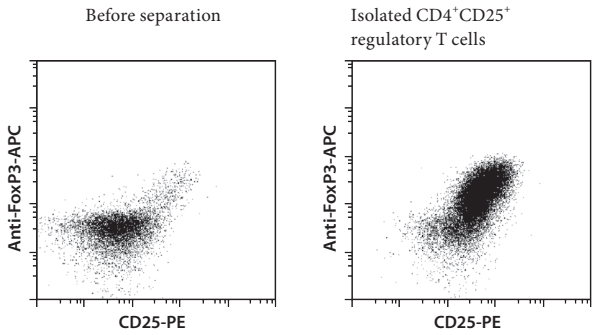
3. Example of a separation using the CD4$^+$ CD25$^+$ Regulatory T Cell Isolation Kit

CD4$^+$CD25$^+$ regulatory T cells were isolated from human PBMCs by using the CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit, an LD and two MS Columns, a MidiMACS™ and a MiniMACS™ Separator. The cells were fluorescently stained with CD4 (VIT4)-VioBlue® (# 130-094-153), CD25-APC, clone 4E3 (# 130-092-858), and CD127-PE (# 130-094-889) (A), or CD4 (VIT4)-VioBlue, CD25-PE, clone 4E3 (# 130-091-024), 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 (VIT4)-VioBlue dot plot).

A)



B)



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Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com/local to find your nearest Miltenyi Biotec contact.

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