Miltenyi Biotec

CD4+CD25+ Regulatory T Cell Isolation Kit

non-human primate

Order no. 130-092-984

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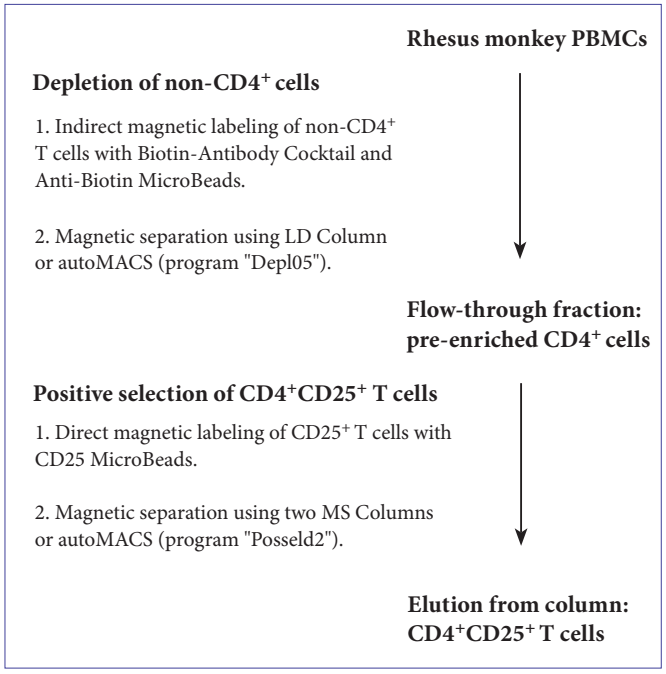
1. Description

Components	**1 mL CD4+CD25+ T Cell Biotin-Antibody Cocktail, non-human primate:** Cocktail of biotin-conjugated monoclonal anti-human antibodies against CD8, CD11b, CD16, CD20, CD56, and CD66abce. **2 mL Anti-Biotin MicroBeads:** MicroBeads conjugated to monoclonal anti-biotin antibody (isotype: mouse IgG1). **2 mL CD25 MicroBeads, non-human primate:** MicroBeads conjugated to monoclonal anti-CD25 antibody (isotype: mouse IgG1).
Size	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.

This product is applicable for the separation of cells from rhesus monkey (*Macaca mulatta*). The antibodies have been tested to cross-react with cynomolgus monkey (*Macaca fascicularis*). Cross-reactivity has not been tested with other non-human primates.

1.1 Principle of MACS® Separation

The isolation of CD4+CD25+ regulatory T cells is performed in a two-step procedure. First, non-CD4+ 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+ T cells are directly labeled with CD25 MicroBeads and isolated by positive selection from the pre-enriched CD4+ T cell fraction. The magnetically labeled CD4+CD25+ 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+ T cells is separated over a second column.



1.2 Background and product applications

CD4 is the coreceptor for MHC class II-restricted T cell activation. CD25 is the low affinity interleukin-2-receptor. 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 and non-human primates. Regulatory CD4+CD25+ T cells seem to suppress harmful immunological reactions to self or foreign antigens.

Example application

- Isolation of CD4+CD25+ regulatory T cells from rhesus monkey peripheral blood mononuclear cells (PBMCs) for further phenotypical or functional characterization.

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1.3 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 (4–8 °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 calf serum. Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- MACS Columns and MACS Separators: Depletion of non-$CD4^+$ cells is performed on an LD Column. The subsequent positive selection of $CD4^+CD25^+$ T cells is performed on two MS Columns. Depletion and positive selection can also be performed by using 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 and positive selection			
autoMACS	2×10^8	4×10^9	autoMACS

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

- (Optional) Fluorochrome-conjugated antibody for flow cytometric analysis, e.g., CD4-FITC (# 130-080-501), CD4-APC (# 130-091-232), CD25-PE (# 130-091-024), or CD25-APC (# 130-092-858).
- (Optional) PI (propidium iodide) or 7-AAD for flow cytometric exclusion of dead cells without cell fixation. For cell fixation and flow cytometric exclusion of dead cells, the Fixation and Dead Cell Discrimination Kit (# 130-091-163) is recommended.
- (Optional) Pre-Separation Filters (# 130-041-407) to remove cell clumps.

2. Protocol

2.1 Sample preparation

When working with rhesus monkey anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by density gradient centrifugation, e.g., using Ficoll-Paque™. For details see section General Protocols in the user manuals or visit www.miltenyibiotec.com/protocols.

▲ **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.



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 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 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 separation. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove cell clumps which may clog the column.

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 cells.
4. Add 10 µL of **Biotin-Antibody Cocktail** per 10^7 cells.
5. Mix well and refrigerate for 10 minutes (4–8 °C).
6. Add 30 µL of buffer and 20 µL of **Anti-Biotin MicroBeads** per 10^7 cells.
7. Mix well and refrigerate for additional 15 minutes (4–8 °C).
8. Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes at 4–8 °C. Aspirate supernatant completely.
9. Resuspend up to 10^8 cells in 500 µL of buffer.
   ▲ **Note:** For larger cell numbers, scale up buffer volume accordingly.
10. Proceed to magnetic separation (2.3).



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

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. Perform washing steps by adding buffer successively once the column reservoir is empty. Collect total effluent. This contains the unlabeled pre-enriched $CD4^+$ T cell fraction.
5. Proceed to 2.4 for the isolation of $CD4^+CD25^+$ T cells.

Depletion with the autoMACS™ Separator

▲ Refer to the autoMACS™ user manual for instructions on how to use the autoMACS Separator.

1. Prepare and prime autoMACS Separator.
2. Place tube containing the magnetically labeled cells in the autoMACS Separator. Choose separation program "Depl05".

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3. Collect unlabeled fraction (outlet port "neg1"). This is the pre-enriched $CD4^+$ T cell fraction.
4. Proceed to 2.4 for the enrichment 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 cells. For larger initial cell numbers, scale up volumes accordingly.

1. Centrifuge cells at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 80 µL of buffer.
3. Add 20 µL of **CD25 MicroBeads**.
4. Mix well and refrigerate for 15 minutes (4–8 °C).
5. (Optional) Add staining antibodies. Add 10 µL of CD25-PE (# 130-091-024) and e.g., 10 µL of CD4-FITC (# 130-080-501) or CD4-APC (# 130-091-232), and refrigerate for 5 minutes (4–8 °C).
6. Wash cells by adding 200–400 µL 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.
8. Proceed to magnetic separation (2.5).

2.5 Magnetic separation: Positive selection of $CD4^+CD25^+$ 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 respective MACS Column data sheet.
2. Prepare column by rinsing with 500 µL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells which pass through and wash column with 3×500 µL of buffer. Perform washing steps by adding buffer three times once 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 fraction with magnetically labeled cells ($CD4^+CD25^+$ T cells) by firmly pushing the plunger into the column.
7. Repeat the magnetic separaion procedure as described in steps 1 to 6 by using a new MS Column.

Positive selection with the autoMACS™ Separator

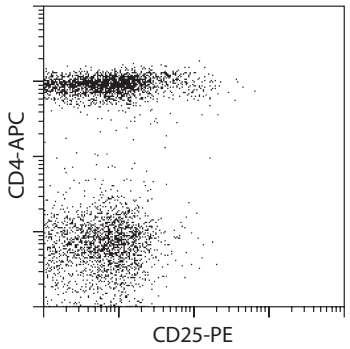
▲ Refer to the autoMACS™ user manual for instructions on how to use the autoMACS Separator.

1. Prepare and prime autoMACS Separator.
2. Place tube containing the magnetically labeled cells in the autoMACS Separator. Choose separation program "Posseld2".
3. Collect positive fraction (outlet port "pos2"). This is the enriched $CD4^+CD25^+$ T cell fraction.

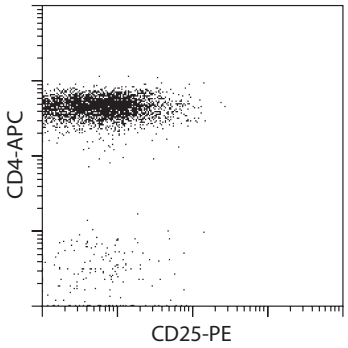
3. Example of a separation using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit

$CD4^+CD25^+$ regulatory T cells were isolated from rhesus monkey PBMCs by using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, an LD Column and two MS Columns, a MidiMACS™ Separator and a MiniMACS™ Separator. The cells are fluorescently stained with CD25-PE and CD4-APC. Cell debris and dead cells were excluded from the analysis based on scatter signals and PI fluorescence.

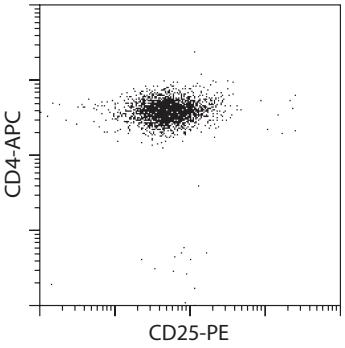
PBMCs before separation



Pre-enriched $CD4^+$ T cells after depletion of non-$CD4^+$ cells



Isolated $CD4^+CD25^+$ regulatory T cells



4. Reference

1. Sakaguchi, S. *et al.* (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). J. Immunol. 155: 1151–1164.

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.

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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