Miltenyi Biotec

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

Order no. 130-091-041

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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 $CD4^+CD25^+$ Regulatory T Cell Biotin-Antibody Cocktail, mouse:
Cocktail of biotin-conjugated monoclonal anti-mouse antibodies against CD8a, CD11b, CD45R, CD49b, and Ter-119.

2 mL Anti-Biotin MicroBeads:
MicroBeads conjugated to monoclonal anti-biotin antibody (isotype: mouse IgG1).

1 mL CD25-PE, mouse:
Monoclonal anti-mouse CD25 antibody conjugated to R-Phycoerythrin (PE) (isotype: rat IgM).

1 mL Anti-PE MicroBeads:
MicroBeads conjugated to monoclonal anti-PE antibodies (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.

1.1 Principle of the MACS® Separation

The isolation of the mouse $CD4^+CD25^+$ regulatory T cells is performed in a two-step procedure. First, the non-$CD4^+$ T 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 the two labeling steps no washing steps are required. In parallel, the cells are labeled with CD25-PE. The magnetically labeled cells are subsequently depleted by separation over a MACS® Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled non-$CD4^+$ T cells are retained in the column, while the unlabeled $CD4^+$ T cells run through.
In the second step, the $CD25^+$ PE-labeled cells are magnetically 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. The magnetically labeled $CD4^+CD25^+$ cells are retained in the column, while the unlabeled $CD4^+CD25^-$ cells run through and can be used for suppression assays as responder T cells.
After removing the column from the magnetic field, the magnetically retained $CD4^+CD25^+$ cells can be eluted as the positively selected cell fraction. To increase the purity, the positively selected cell fraction containing the $CD4^+CD25^+$ cells must be separated over a second column.

Mouse splenocytes: Depletion of non-$CD4^+$ T cells

1. Indirect magnetic labeling of non-$CD4^+$ T cells with $CD4^+CD25^+$ Regulatory T Cell Biotin-Antibody Cocktail and Anti-Biotin MicroBeads.
2. Labeling of $CD25^+$ cells with CD25-PE.
3. Magnetic separation using an LD Column or an autoMACS Column (program "Depl025").

$CD4^+$ T cells (flow-through fraction): Positive selection of $CD4^+CD25^+$ cells

1. Indirect magnetic labeling of $CD25^+$ cells with Anti-PE MicroBeads.
2. Magnetic separation using two MS Columns or an autoMACS Column (program "Posseld2").

$CD4^+CD25^+$ regulatory T cells (eluted fraction)
$CD4^+CD25^-$ T cells (flow-through fraction)

1.2 Background information

The $CD4^+CD25^+$ Regulatory T Cell Isolation Kit has been developed for the isolation of mouse $CD4^+CD25^+$ regulatory T cells from single-cell suspensions of spleen or lymph nodes without centrifugational washing steps during the separation.

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CD4+CD25+ immunoregulatory T cells have been shown to actively suppress immune responses against autologous and foreign antigens *in vivo* and *in vitro*. CD25, the IL-2Rα chain, is also expressed on activated CD8+ T cells, dendritic cells (DCs), and B cells.

The kit contains a cocktail of lineage specific biotin-conjugated antibodies against CD8, CD11b, CD45R, CD49b, Ter-119, and Anti-Biotin MicroBeads for depletion of non-CD4+ T cells, as well as CD25-PE and Anti-PE MicroBeads for subsequent positive selection of CD4+CD25+ regulatory T cells.

1.3 Applications

- Isolation of CD4+CD25+ regulatory T cells from single-cell suspensions of spleen or lymph nodes for:
  - Treg expansion by using, e.g., the Treg Expansion Kit, mouse (# 130-095-925),
  - *in vitro* Treg suppression assays in combination with CD4+ CD25– cell fraction as T responder/effector cells,
  - co-culture experiments with DCs to study priming of DCs for tolerance induction *in vitro* and after adoptive transfer of primed DCs *in vivo*,[1]
  - adoptive transfer experiments (e.g. from UV-exposed mice) to analyze the role of regulatory T cells during induction and elicitation of hapten-specific tolerance.[2]

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 mouse serum albumin, mouse 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 T cells can be performed on an LD Column. The subsequent positive selection of CD4+CD25+ 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
Positive selection			
MS	10^7	2×10^8	MiniMACS, OctoMACS
Depletion			
LD	10^8	5×10^8	MidiMACS, QuadroMACS
Positive selection or depletion			
autoMACS	2×10^8	4×10^9	autoMACS Pro

- (Optional) Fluorochrome-conjugated antibodies for flow cytometric analysis, e.g., CD4-FITC (# 130-102-541) and CD25-PE (# 130-102-593). For more information about antibodies refer to 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) Pre-Separation Filters, 30 µm (# 130-041-407) to remove cell clumps.

2. Protocol

2.1 Sample preparation

Prepare a single-cell suspension from spleen or lymph nodes using manual methods or the gentleMACS™ Dissociators.

For details refer to www.gentleMACS.com/protocols.

▲ **Note:** The Kit is not optimized for the isolation of regulatory T cells from blood and thymus.

▲ Red blood cell lysis or density gradient centrifugation is not necessary, since the CD4+CD25+ T Cell Biotin-Antibody Cocktail contains Anti-Ter-119 antibody.

2.2 Magnetic labeling of non-CD4+ cells and fluorescent labeling of CD25+ 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.

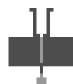
▲ Volumes for magnetic labeling given below are for up to 10^7 total cells. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^7 total cells, use twice the volume 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 cells 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 the CD4+CD25+ Regulatory T Cell Biotin-Antibody Cocktail per 10^7 total cells.
5. Mix well and incubate for 10 minutes in the refrigerator (2−8 °C).
6. Add 30 µL of buffer, 20 µL of Anti-Biotin MicroBeads, and 10 µL of CD25-PE antibody per 10^7 total cells.
7. Mix well and incubate for additional 15 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.



2.3 Magnetic separation: Depletion of non-CD4+ T cells

▲ 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 the 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 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 magnetic labeling of CD25+ T cells.

Depletion 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:

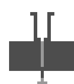
   Depletion: Depl025
   Collect negative fraction in row B of the tube rack.
4. Proceed to 2.4 for the magnetic labeling of CD25+ T cells.

2.4 Magnetic labeling of CD25+ 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 5 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 90 µL of buffer.
3. Add 10 µL of Anti-PE MicroBeads.
4. Mix well and incubate for 15 minutes in the refrigerator (2−8 °C).
5. Proceed to magnetic separation (2.5).

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



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 refer to 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 CD4+CD25− cells.
4. Wash column with 2×500 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 as soon as the column reservoir is empty.
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette 1 mL of buffer onto the column. Immediately flush out the magnetically labeled cells (CD4+CD25+ cells) by firmly pushing the plunger into the column.

   ▲ **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.
7. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new MS Column.

Positive selection 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+ T cell fraction.

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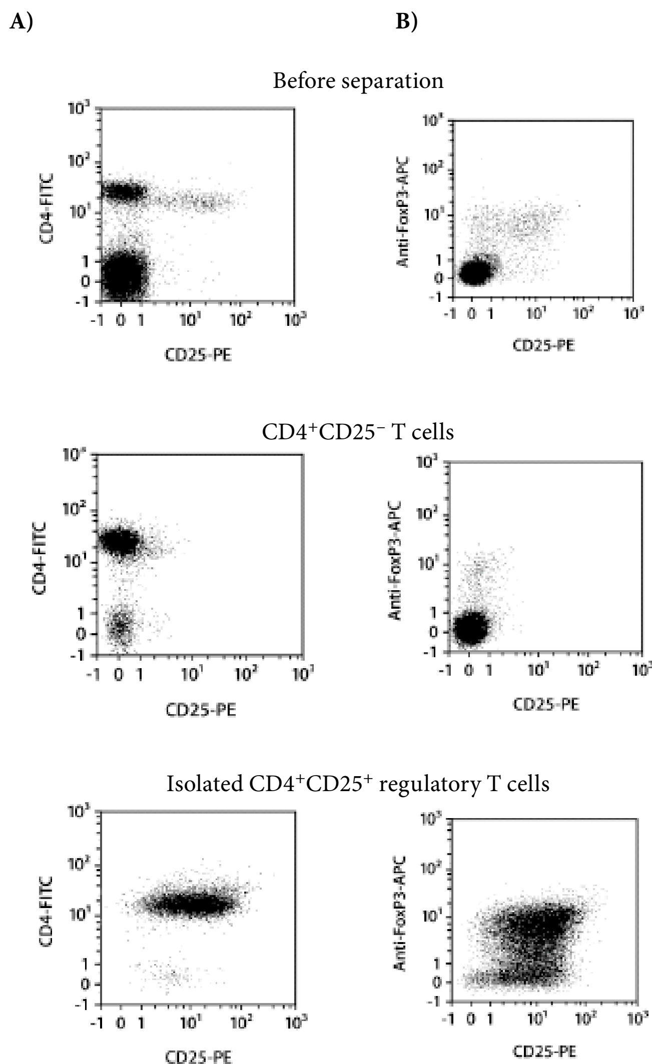
3. Example of a separation using the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit

$CD4^+CD25^+$ regulatory T cells were isolated from mouse spleen cell suspension by using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, an LD and two MS Columns, a MidiMACS™ Separator and a MiniMACS™ Separator. The cells were fluorescently stained with CD25-PE and CD4-FITC (A) or Anti-FoxP3-APC (B) and analyzed by flow cytometry using the MACSQuant® Analyzer. Cell debris and dead cells were excluded from analysis based on scatter signals and propidium iodide fluorescence.



4. References

1. Fallarino, F. *et al.* (2003) Modulation of tryptophan catabolism by regulatory T cells. Nat. Immunol. 4(12): 1206–1212.

2. Schwarz, A. *et al.* (2004) Ultraviolet radiation-induced regulatory T cells not only inhibit the induction but can suppress the effector phase of contact hypersensitivity. J. Immunol. 172: 1036–1043.

Refer to **www.miltenyibiotec.com** for all data sheets and protocols.

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