CD25$^+$CD49d$^-$ Regulatory T Cell Isolation Kit
human

Order no. 130-094-551

Contents

Human PBMCs

Depletion of CD8$^+$CD49d$^+$ T cells
1. Direct magnetic labeling of CD8$^+$ and CD49d$^+$ cells with a cocktail of CD8 and CD49d MicroBeads.
2. Magnetic separation using an LD Column or an autoMACS Column (program "Depl05").

Pre-enriched CD49d$^-$ T cells (flow-through fraction)

Positive selection of CD25$^+$CD49d$^-$ regulatory T cells
1. Direct magnetic labeling of CD25$^+$ T cells with CD25 MicroBeads II.
2. Magnetic separation using two MS Columns or autoMACS Columns (program "PosseId2").

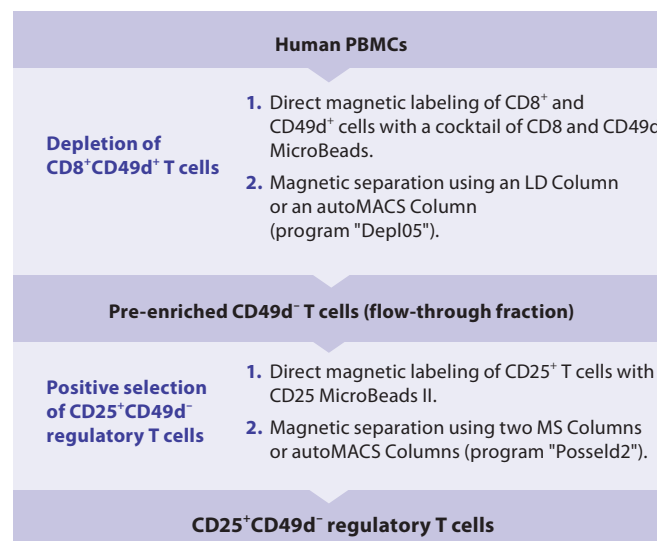**CD25$^+$CD49d$^-$ regulatory T cells**

1. Description

Components	**2 mL CD8$^+$CD49d$^+$ T Cell Depletion MicroBead Cocktail, human:** MicroBeads conjugated to monoclonal CD8 and CD49d antibodies. **1 mL CD25 MicroBeads II, 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 CD25$^+$CD49d$^-$ regulatory T cells is performed in a two-step procedure. First, CD8$^+$ and CD49d$^+$ cells are directly magnetically labeled with a cocktail of CD8 and CD49d MicroBeads. The labeled cells are subsequently depleted by separation over a MACS® Column.

In the second step, CD25$^+$CD49d$^-$ regulatory T cells are directly labeled with CD25 MicroBeads II and isolated by positive selection from the pre-enriched T cell fraction. The magnetically labeled CD25$^+$CD49d$^-$ regulatory 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 CD25$^+$CD49d$^-$ T cells is separated over a second column.

1.2 Background information

Regulatory 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.

CD49d is the α-chain of the integrin VLA-4 and is present on the majority of proinflammatory effector T cells mediating their migration into inflamed tissues. Within the CD25$^+$ T cell subset CD49d has been described to discriminate between FoxP3$^+$ regulatory T cells and contaminating effector T cells, which only transiently express FoxP3 and/or CD25$^+$.[7] Thus, the depletion of CD49d$^+$ T cells is a useful tool to improve the purity of CD25$^+$ regulatory T cells with the removal of contaminating effector T cells.

1.3 Applications

- Isolation of CD25$^+$CD49d$^-$ regulatory T cells from human peripheral blood mononuclear cells (PBMCs) for further phenotypical or functional characterization.

- The isolation of CD25$^+$CD49d$^-$ regulatory T cells from cord blood is not recommended.

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 $CD49d^+CD8^+$ cells is performed on an LD Column. The subsequent positive selection of $CD25^+$ 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 and 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), or CD49d-Biotin (# 130-093-280) and Anti-Biotin-APC (# 130-090-856). 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 $CD8^+CD49d^+$ 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, # 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 80 µL of buffer per 10^7 total cells.

4. Add 20 µL of **$CD8^+CD49d^+$ T Cell Depletion MicroBead Cocktail** per 10^7 total cells.

5. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).

6. Wash cells by adding 1–2 mL of buffer per 10^7 cells 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 (2.3).

2.3 Magnetic separation: Depletion of $CD8^+CD49d^+$ 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.

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 $CD49d^-$ 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 $CD25^+$ T cells.

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page 2/5

140-002-683.03

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.

Depletion 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. Choose the program: "Depl05"

   Collect negative fraction in row B of the tube rack. This is the pre-enriched CD49d⁻ T cell fraction.

4. Proceed to 2.4 for the isolation of CD25⁺ 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. Choose the program: "Depl05".

   Collect negative fraction from outlet port neg1. This is the pre-enriched CD49d⁻ T cell fraction.

4. Proceed to 2.4 for the isolation of CD25⁺ T cells.

2.4 Magnetic labeling of 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 90 µL of buffer per 10^7 total cells.

3. Add 10 µL of **CD25 MicroBeads II** per 10^7 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 CD4-FITC (# 130-080-501) and 10 µL of CD25-PE (# 130-091-024), and incubate for 5 minutes in the dark in the refrigerator (2–8 °C).

6. Wash cells by adding 1–2 mL of buffer per 10^7 cells 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 CD25⁺CD49d⁻ regulatory T cells

Positive selection with MS Columns

▲ To achieve highest purities, perform two consecutive column runs.

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

1. Place MS Column in the magnetic field of a suitable MACS Separator. For details see 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 the purity of CD25⁺CD49d⁻ 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

▲ 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.

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. Choose the program: "Posseld2"

   Collect positive fraction in row C of the tube rack. This is the enriched CD25⁺CD49d⁻ T cell fraction.

Positive selection 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.

140-002-683.03

3. Choose the program: "Posseld2"

Collect positive fraction from outlet port pos2. This is the enriched CD25$^+$CD49d$^-$ T cell fraction.

## 3. Example of a separation using the CD25$^+$CD49d$^-$ Regulatory T Cell Isolation Kit

CD25$^+$CD49d$^-$ regulatory T cells were isolated from human PBMCs using the CD25$^+$CD49d$^-$ Regulatory T Cell Isolation Kit, an LD Column, two MS Columns, a MidiMACS™ Separator, and a MiniMACS™ Separator. Cells were fluorescently stained with CD4-FITC (# 130-080-501), CD25-PE (# 130-091-024), CD49d-Biotin (# 130-093-280) and Anti-Biotin-APC (# 130-090-856) (A), or CD4-FITC, CD25-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.

A)

Before isolation
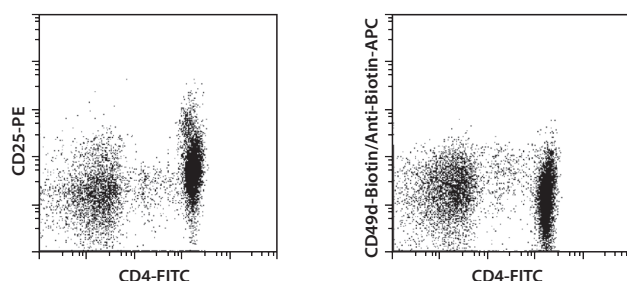
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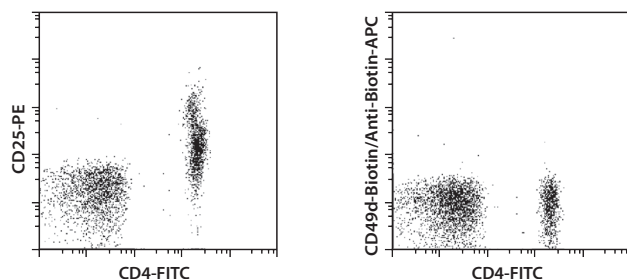
Isolated CD25$^+$CD49$^-$ regulatory T cells
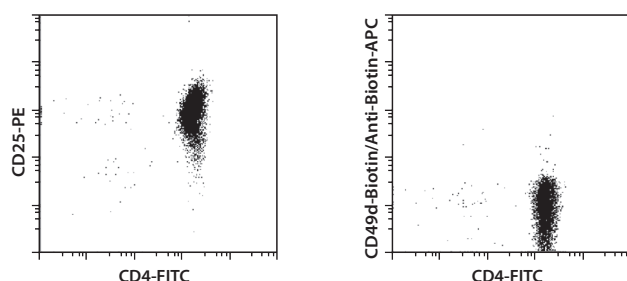
B)

Gated on CD4$^+$ T cells
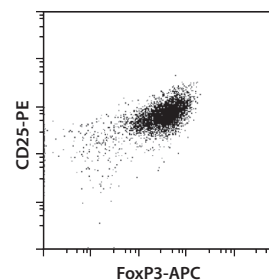
4. References

1. Sakaguchi *et al.* (2008) Regulatory T cells and immune tolerance. Cell 133: 775-787.

2. Levings, M. K. *et al.* (2001) CD4$^+$CD25$^+$ regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. J. Exp. Med. 193: 1295–1302.

3. Dieckmann, D. *et al.* (2001) Ex vivo isolation and characterisation of CD4$^+$CD25$^+$ T cells with regulatory properties from human blood. J. Exp. Med. 193: 1303–1310.

4. Jonuleit, H. *et al.* (2001) Identification and Functional Characterisation of Human CD4$^+$CD25$^+$ T Cells with Regulatory Properties Isolated from Peripheral Blood. J. Exp. Med. 193: 1285–1294.

5. Taams, L. S. *et al.* (2001) Human anergic/suppressive CD4$^+$CD25$^+$ T cells: a highly differentiated and apoptosis-prone population. Eur. J. Immunol. 31: 1122–1131.

6. Stephens, L. A. *et al.* (2001) Human CD4$^+$CD25$^+$ thymocytes and peripheral T cells have immune suppressive activity in vitro. Eur. J. Immunol. 31: 1247–1254.

7. Kleinewietfeld, M. *et al.* (2009) CD49d provides access to 'untouched' human Foxp3$^+$ Treg free of contaminating effector cells. Blood. 113: 827–836.

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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page 4/5

140-002-683.03

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page 5/5