Miltenyi Biotec

Double-negative T Cell Isolation Kit
human

Order no. 130-092-614

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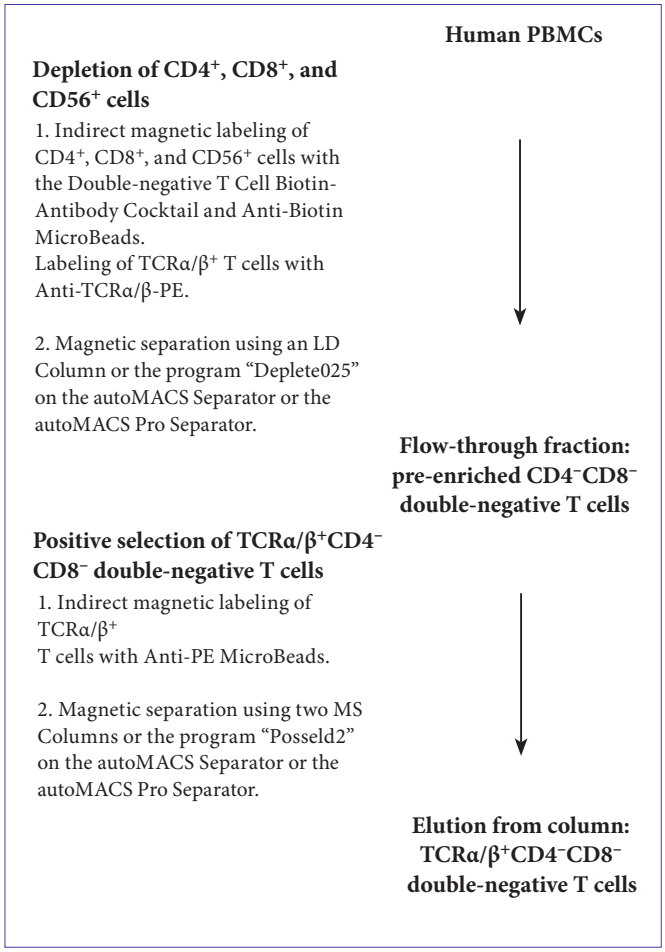
1. Description

Components	**2 mL Double-negative T Cell Biotin-Antibody Cocktail, human:** Cocktail of biotin-conjugated monoclonal mouse anti-human antibodies against human CD4 (isotype: mouse IgG1), CD8 (isotype: mouse IgG2a), and CD56 (isotype: mouse IgG1). **2×2 mL Anti-Biotin MicroBeads:** MicroBeads conjugated to monoclonal anti-biotin antibodies (isotype: mouse IgG1). **2 mL Anti-TCRα/β-PE, human:** Monoclonal anti-human TCRα/β antibodies conjugated to R-phycoerythrin (PE) (clone: BW242/412; isotype: mouse IgG2b). **1 mL Anti-PE MicroBeads:** MicroBeads conjugated to monoclonal anti-PE antibodies (isotype: mouse IgG1).
Capacity	For 2×10^9 total cells, up to 20 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 double-negative T cells (CD4$^-$CD8$^-$CD56$^-$CD3$^+$ TCRα/β$^+$) is performed in a two-step procedure. To remove all unwanted TCRα/β$^+$ cells, firstly the CD4$^+$, CD8$^+$, and CD56$^+$ cells are indirectly magnetically labeled by using a cocktail of biotin-conjugated antibodies against CD4, CD8, and CD56. These cells are subsequently magnetically labeled with Anti-Biotin MicroBeads for depletion. Anti-TCRα/β-PE is added simultaneously with the Anti-Biotin MicroBeads. The labeled cells are then depleted by separation over a MACS® Column.

In the second step, the flow-through fraction with the pre-enriched double-negative T cells is incubated with Anti-PE MicroBeads. During subsequent magnetic separation, the TCRα/β$^+$CD4$^-$CD8$^-$ double-negative T cells are retained within the column and eluted after removing the column from the magnetic field. To achieve highest purities, the positively selected cell fraction containing the TCRα/β$^+$CD4$^-$CD8$^-$ double-negative T cells is separated over a second column.



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1.2 Background information

The down-regulation of immune responses by regulatory T cells plays a key role in the induction of tolerance. To date, several regulatory T cell populations with specific combinations of cell surface markers have been characterized. Recently, it was reported that so called double-negative T cells – a unique subset[1] of T cells characterized by being $CD4^-CD8^-CD3^+TCR\alpha/\beta^+$ – also have the ability to specifically down-regulate immune responses towards allo-antigens both in humans and mice.[2]

Double-negative T cells are found in lymphoid as well as in non-lymphoid tissues. They constitute about 1–3% of peripheral $CD3^+$ cells. It has been described that $CD4^-CD8^-CD3^+TCR\alpha/\beta^+$ double-negative T cells can take up allo-MHC peptide complexes from antigen-presenting cells. In a process requiring cell-to-cell contact and Fas/FasL interaction, they are then able to specifically trap and kill $CD4^+$ or $CD8^+$ T cells that recognize the presented allo-MHC peptides.[2]

1.3 Applications

- Specific isolation of $TCR\alpha/\beta^+CD4^-CD8^-$ double-negative T cells from peripheral blood mononuclear cells (PBMCs) for phenotypical and functional characterization.

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. Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- MACS Columns and MACS Separators: Depletion of $CD4^+$, $CD8^+$, and $CD56^+$ cells is performed on an LD Column. The subsequent positive selection of $CD4^-CD8^-$ double-negative T cells according to the expression of $TCR\alpha/\beta^+$ is performed on two MS Columns. Depletion and positive selection can also be performed by using the autoMACS or 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
Positive selection			
MS	10^7	2×10^8	MiniMACS, OctoMACS, VarioMACS, SuperMACS
Depletion and positive selection			
autoMACS	2×10^8	4×10^9	autoMACS, autoMACS Pro

▲ **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 antibody for flow cytometric analysis, e.g., CD4-FITC (# 1430-080-501) or CD4-APC (# 130-091-232); CD8-FITC (# 130-080-601) or CD8-APC (# 130-091-076); Anti-Biotin-FITC (# 130-090-857) or Anti-Biotin-APC (# 130-090-856); and CD56-APC (# 130-090-843). For more information about other fluorochrome-conjugates see www.miltenyibiotec.com.
- (Optional) Propidium iodide (PI) 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™. For details see the General Protocols section of the respective separator user manual. The General Protocols are also available at 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 $CD4^+$, $CD8^+$, and $CD56^+$ 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 separation. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove cell clumps which may clog the column. Wet filter with buffer before use.

▲ 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 **Double-negative 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. Add 100 µL of **Anti-TCRα/β-PE** per 10^8 cells.
9. Mix well and incubate for 15 minutes in the refrigerator in the dark (2–8 °C).
10. Wash cells by adding 10–20 mL of buffer and centrifuge at 300×g for 10 minutes at 2–8 °C. Aspirate supernatant completely.

11. Resuspend up to 10^8 cells in 500 µL of buffer.

▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.

12. Proceed to magnetic separation (2.3).

2.3 Magnetic separation: Depletion of CD4$^+$, CD8$^+$, and CD56$^+$ 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. Collect total effluent; this is the enriched CD4$^-$CD8$^-$ double-negative T 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 TCRα/β$^+$CD4$^-$CD8$^-$ double-negative T cells.

Depletion with the autoMACS™ Separator or the autoMACS™ Pro Separator

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

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

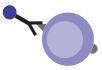
▲ Program choice depends on the isolation strategy, the strength of magnetic labeling, and the frequency of magnetically labeled cells. For details refer to the Cell separation programs section in the respective user manual.

Depletion 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 below the uptake port and the fraction collection tubes at port neg1 and port pos1.
3. For a standard separation choose the following program:
   Depletion: "Depl025"
   Collect negative fraction from outlet port neg1; this is the enriched CD4$^-$CD8$^-$ double-negative T cell fraction.
4. Proceed to 2.4 for the isolation of TCRα/β$^+$CD4$^-$CD8$^-$ double-negative T cells.

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 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; this is the enriched CD4$^-$CD8$^-$ double-negative T cell fraction.
4. Proceed to 2.4 for the isolation of TCRα/β$^+$CD4$^-$CD8$^-$ double-negative T cells.



2.4 Magnetic labeling of TCRα/β$^+$CD4$^-$CD8$^-$ double-negative 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 an **initial** starting cell number of up to 10^8 cells. For larger initial cell numbers, scale up volumes accordingly.

▲ Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.

1. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 450 µL of buffer.
3. Add 50 µL of **Anti-PE MicroBeads**.
4. Mix well and incubate for 15 minutes in the refrigerator in the dark (2–8 °C).
5. (Optional) Add staining antibodies, e.g., 50 µL Anti-Biotin-FITC (# 130-090-857) or Anti-Biotin-APC (# 130-090-856), and incubate for 5 minutes in the refrigerator in the dark (2–8 °C).
6. Wash cells by adding 5–10 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend cells in 500 µL of buffer.
8. Proceed to magnetic separation (2.5).

2.5 Magnetic separation: Positive selection of TCRα/β$^+$CD4$^-$CD8$^-$ double-negative 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 MS Column data sheet.
2. Prepare column by rinsing with 500 µL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with 3×500 µL of buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer three times. Only add new buffer 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 (TCRα/β$^+$CD4$^-$CD8$^-$ double negative T cells) by firmly pushing the plunger into the column.
7. To increase purity of TCRα/β$^+$CD4$^-$CD8$^-$ double negative T 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.

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Positive selection with the autoMACS™ Separator or the autoMACS™ Pro Separator

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

▲ Buffers used for operating the autoMACS Separator or the autoMACS Pro 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 Cell separation programs section in the respective user manual.

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 below 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. This is the enriched TCRα/β$^+$CD4$^-$CD8$^-$ double-negative T cell fraction.

Positive selection with the autoMACS™ Pro Separator

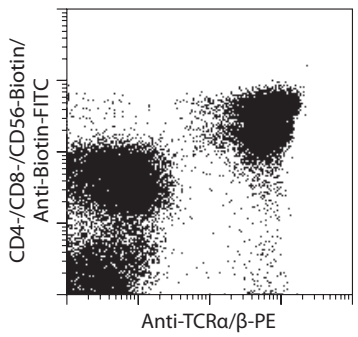
1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and fraction collection tubes in rows B and C.
3. For a standard separation choose the following program:

   Positive selection: "Posseld2"
   Collect positive fraction in row C of the tube rack. This is the enriched TCRα/β$^+$CD4$^-$CD8$^-$ double-negative T cell fraction.

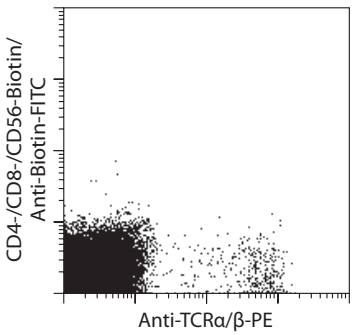
3. Example of a separation using the Double-negative T Cell Isolation Kit

TCRα/β$^+$CD4$^-$CD8$^-$ double-negative T cells were isolated from human PBMCs by using the Double-negative T Cell Isolation Kit, one LD and two MS Columns, a MidiMACS™ and a MiniMACS™ Separator. Cells labeled with the Double-negative T Cell Isolation Kit are additionally fluorescently stained with Anti-Biotin-FITC (# 130-090-857). Cell debris and dead cells are excluded from the analysis based on scatter signals and PI fluorescence.

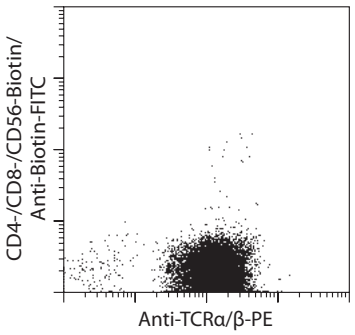
PBMCs before separation



Pre-enriched double-negative T cells after depletion of CD4$^+$, CD8$^+$, and CD56$^+$ cells



Isolated TCRα/β$^+$CD4$^-$CD8$^-$ double-negative T cells



4. References

1. Londei, M. *et al.* (1989) Definition of a population of CD4$^-$CD8$^-$ T cells that express the αβ T-cell receptor and respond to interleukins 2, 3, and 4. Proc. Natl. Acad. Sci. USA. 86: 8502–8506.
2. Zhang, Z.-X. *et al.* (2000) Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. Nat. Med. 6: 782–789.

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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