Miltenyi Biotec

CD4$^+$ Recent Thymic Emigrant Isolation Kit
human

Order no. 130-094-299

Contents

1. Description
   - 1.1 Principle of the MACS® Separation
   - 1.2 Background information
   - 1.3 Applications
   - 1.4 Reagent and instrument requirements
2. Protocol
   - 2.1 Sample preparation
   - 2.2 Magnetic labeling of non-CD4$^+$ and memory CD4$^+$ T cells
   - 2.3 Magnetic separation: Depletion of non-CD4$^+$ and memory CD4$^+$ T cells
   - 2.4 Magnetic labeling of CD4$^+$ RTEs
   - 2.5 Magnetic separation: Positive selection of CD4$^+$ RTEs
3. Example of a separation using the CD4$^+$ Recent Thymic Emigrant Isolation Kit
4. Reference

1. Description

Components

1 mL CD4$^+$ RTE Biotin-Antibody Cocktail, human:
Cocktail of biotin-conjugated monoclonal antibodies against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγ/δ, HLA-DR, and CD235a (Glycophorin A).

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

2 mL CD31 MicroBeads, human:
MicroBeads conjugated to monoclonal anti-human CD31 antibodies (isotype: mouse IgG1).

Capacity For 10^9 total cells.

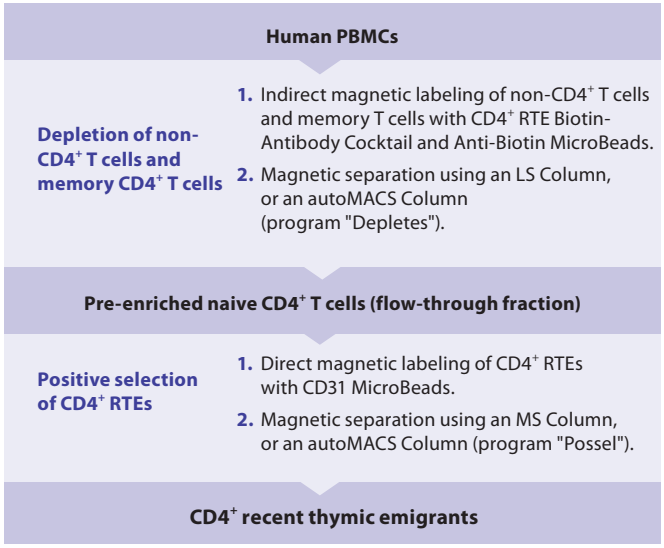
Product format All components are supplied in buffer containing stabilizer and 0.05% sodium azide.

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$^+$ recent thymic emigrants (RTEs) is performed in a two-step procedure. First, non-CD4$^+$ and memory CD4$^+$ T cells are indirectly magnetically labeled with a cocktail of biotinylated antibodies and Anti-Biotin MicroBeads. The labeled cells are subsequently depleted over a MACS® Column.

In the second step, CD4$^+$ RTEs (CD4$^+$CD45RA$^+$CD31$^+$) are directly labeled with CD31 MicroBeads and isolated by positive selection from the pre-enriched naive CD4$^+$ T cell fraction (CD4$^+$CD45RA$^+$ T cells). The magnetically labeled CD4$^+$ RTEs are retained within the column and eluted after removal of the column from the magnetic field.



1.2 Background information

The CD4$^+$ Recent Thymic Emigrant Isolation Kit is designed for the isolation of CD4$^+$CD45RA$^+$CD31$^+$ recent thymic emigrants from human peripheral blood mononuclear cells (PBMCs).

RTEs are a subpopulation of naive T cells characterized by their content of T cell receptor excision circles (TRECs)—stable DNA episomes formed during T cell receptor rearrangement. TRECs are not replicated during mitosis and dilute out with each cellular division. The determination of a cell's TREC content has been used for the quantitation of thymic T cell production. The output of RTEs from the thymus and the homeostatic proliferation of T cells comprise the total naive T cell pool.

CD4$^+$ RTEs play a critical role in immunoreconstitution, for example, after bone marrow transplantation or chemotherapy where the repopulation of the T cell repertoire is essential. The number of CD4$^+$ RTEs is thought to be an indicator for the recovery of the immune system and provides information on thymic function.

CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1) has been described as a marker to identify the CD4$^+$ RTEs among the naive CD4$^+$ T cell population, as its expression correlates with the TREC content of CD4$^+$ T cells.[1] CD4$^+$ T cells lacking CD31 expression harbor little or no TRECs, suggesting that they already proliferated in the periphery.

Therefore, the magnetic isolation of CD4$^+$ RTEs is possible by direct isolation of CD31$^+$ cells from previously enriched, untouched naive CD4$^+$ T cells.

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1.3 Application

- Isolation of $CD4^+CD45RA^+CD31^+$ recent thymic emigrants from human peripheral blood mononuclear cells (PBMCs).

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 non-$CD4^+$ T cells is performed on an LS Column. The subsequent positive selection of $CD4^+$ RTE cells is performed on an MS Column. Positive selection or depletion 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			
LS	10^8	2×10^9	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-PE (# 130-091-231), CD45RA-FITC (# 130-092-247), and CD31-APC (# 130-092-652). For more information about other fluorochrome conjugates 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.

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 non-$CD4^+$ and memory $CD4^+$ 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 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 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.

▲ 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 40 µL of buffer per 10^7 total cells.
4. Add 10 µL of $CD4^+$ RTE Biotin-Antibody Cocktail per 10^7 total cells.
5. Mix well and incubate for 10 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 cell pellet in 80 µL of buffer per 10^7 total cells.
8. Add 20 µL of Anti-Biotin MicroBeads per 10^7 total cells.
9. Mix well and incubate for an additional 15 minutes in the refrigerator (2–8 °C).
10. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. 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).

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

▲ Choose an appropriate MACS Column and MACS Separator according to the number of labeled cells and the number of total cells. For details see table in section 1.4.

Depletion with LS Columns

1. Place LS Column in the magnetic field of a suitable MACS Separator. For details see LS Column data sheet.
2. Prepare column by rinsing with 3 mL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with 3×3 mL of buffer. Collect total effluent; this is the unlabeled pre-enriched naive CD4$^+$ T cell fraction. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty.
5. Proceed to 2.4 for further isolation of CD4$^+$ RTEs.

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.

Magnetic separation 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:
   Depletion: "Depletes"
   Collect negative fraction in row B of the tube rack. This fraction represents the unlabeled pre-enriched naive CD4$^+$ T cell fraction.
4. Proceed to 2.4 for the isolation of CD4$^+$ RTE 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. For a standard separation choose the following program:
   Depletion: "Depletes"
   Collect negative fraction from outlet port neg1. This fraction represents the unlabeled pre-enriched naive CD4$^+$ T cell fraction.
4. Proceed to 2.4 for the isolation of CD4$^+$ RTE cells.

2.4 Magnetic labeling of CD4$^+$ RTEs

▲ Volumes for magentic 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. 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 CD31 MicroBeads 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 starting 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$^+$ RTEs

▲ Choose an appropriate MACS Column and MACS Separator according to the number of labeled cells and the number of CD4$^+$ RTEs. For details see table in section 1.4.

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.
6. Pipette 1 mL of buffer onto the column. Immediately flush out the magnetically labeled CD31$^+$ RTEs by firmly pushing the plunger into the column.

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

Magnetic separation 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: "Possel"

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Collect positive fraction in row C of the tube rack. This fraction represents the magnetically labeled CD4$^+$ RTE cell fraction.

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 and port pos1.
3. For a standard separation choose the following program:

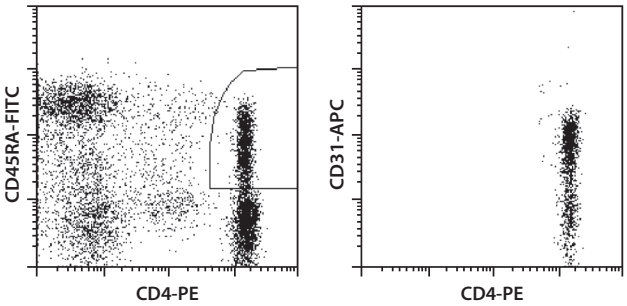
   Positive selection: "Possel"
   Collect positive fraction from outlet port pos1. This fraction represents the magnetically labeled CD4$^+$ RTE cell fraction.

3. Example of a separation using the CD4$^+$ Recent Thymic Emigrant Isolation Kit

Isolation of CD4$^+$ RTEs from PBMCs using the CD4$^+$ Recent Thymic Emigrant Isolation Kit, an LS Column, and an MS Column. To evaluate the separation of CD4$^+$ RTEs, PBMCs before separation, the pre-enriched naive CD4$^+$ T cells, and the isolated CD4$^+$ RTEs are fluorescently stained with CD4-PE (# 130-091-231), CD45RA-FITC (# 130-092-247), and CD31-APC (# 130-092-652). Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.

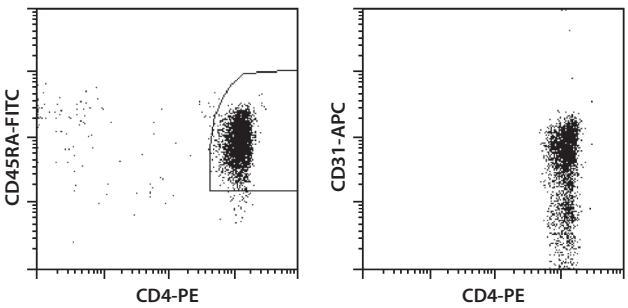
Before separation

Gated on CD4$^+$CD45RA$^+$ cells



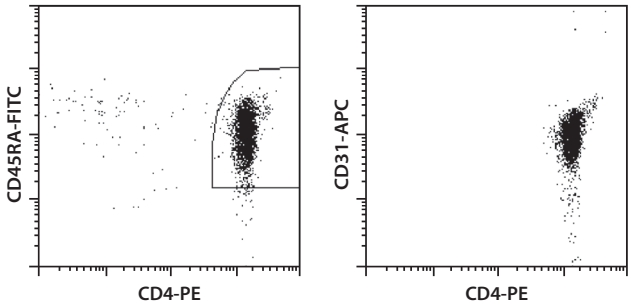
Pre-enriched naive CD4$^+$ T cells

Gated on CD4$^+$CD45RA$^+$ cells



Isolated CD4$^+$ RTEs

Gated on CD4$^+$CD45RA$^+$ cells



4. Reference

1. Kimmig, S. *et al.* (2002) Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. J. Exp. Med. 195: 789–794.

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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