

Isolation of CD4⁺CD294 (CRTH2)⁺ Th2 cells from human PBMCs

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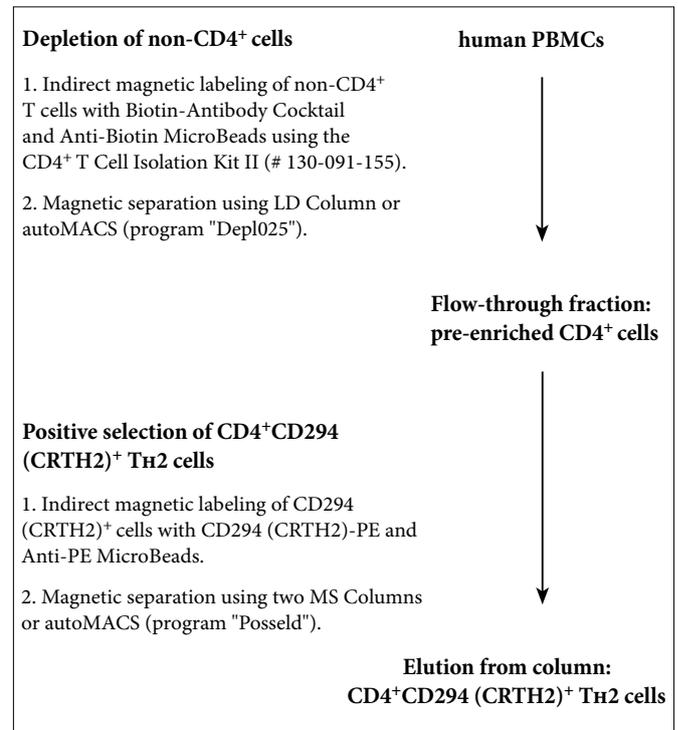
1. Description

1.1 Principle of MACS[®] separation

The isolation of CD4⁺CD294 (CRTH2)⁺ Th2 cells can be performed in a two-step procedure. After depletion of non-CD4⁺ cells using the CD4⁺ T Cell Isolation Kit II (# 130-091-155), the CD4⁺CD294 (CRTH2)⁺ cells are positively isolated using the CD294 (CRTH2) MicroBead Kit (# 130-091-274).

First, non-CD4⁺ T cells are labeled with a cocktail of biotin-conjugated antibodies and Anti-Biotin MicroBeads. The magnetically labeled cells are subsequently depleted by separation over a MACS[®] Column.

In a second step the CD294 (CRTH2)⁺ cells are fluorescently and indirectly magnetically labeled with CD294 (CRTH2)-PE and Anti-PE MicroBeads for positive selection. The magnetically labeled CD4⁺CD294 (CRTH2)⁺ cells are retained on a MACS Column and eluted after removal of the column from the magnetic field. To achieve highest purities, the positively selected cell fraction containing the CD4⁺CD294 (CRTH2)⁺ Th2 cells is separated over a second column.



1.2 Background and product applications

CD4, the co-receptor for MHC class II-restricted T cell activation, is expressed by T helper cells. T helper cells are divided into different functional subtypes, such as T helper type 1 (Th1) cells, which are responsible for inflammatory immune reactions, and Th2 cells supporting humoral immune responses. CD294 (CRTH2) (chemoattractant receptor of Th2 cells) is a receptor for prostaglandin D₂ and is involved in migration of leukocytes. CD294 (CRTH2) is expressed on Th2 cells, but is not present on Th1 cells.^{1,2} Also, the CD294 (CRTH2) antigen is highly expressed on peripheral blood basophils and eosinophils.³ It is also expressed by a small population of CD8⁺ T cells^{1,2} and is discussed to be present on a subpopulation of dendritic cells⁴.

Example of applications

- Isolation of CD4⁺CD294 (CRTH2)⁺ Th2 cells from human PBMC for further phenotypical characterization or functional analysis.

1.3 Reagent and instrument requirements

- CD4⁺ T Cell Isolation Kit II (# 130-091-155) containing a Biotin-Antibody Cocktail and Anti-Biotin MicroBeads.
- CD294 (CRTH2)⁺ MicroBead Kit (# 130-091-274) containing CD294 (CRTH2)-PE, Anti-PE MicroBeads and FcR Blocking Reagent.

- **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).

▲ **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 gelatine, human serum, or fetal calf serum. Buffers or media containing Ca²⁺ or Mg²⁺ 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⁺CD294 (CRTH2)⁺ Th2 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
Positive selection or depletion			
autoMACS	2×10 ⁸	4×10 ⁹	autoMACS

▲ **Note:** Column adapters are required to insert certain columns into VarioMACS or SuperMACS. For details, see MACS Separator data sheets.

- (Optional) Additional staining reagents, such as CD4-FITC (# 130-080-501) or CD4-APC (# 130-091-232).
- (Optional) Propidium iodide (PI) or 7-AAD for flow cytometric exclusion 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, PBMC should be isolated by density gradient centrifugation (see "General Protocols" in the User Manuals or visit www.miltenyibiotec.com).

▲ **Note:** Remove platelets after density gradient separation: resuspend cell pellet in buffer and centrifuge at 200xg for 10–15 minutes at 20 °C. Carefully remove supernatant. Repeat washing step and carefully remove supernatant.



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 10⁷ cells. When working with fewer than 10⁷ 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 2×10⁷ 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 cells at 300xg for 10 minutes. Pipette off supernatant completely.
3. Resuspend cell pellet in 40 μL of buffer per 10⁷ cells.
4. Add 10 μL of **Biotin-Antibody Cocktail** per 10⁷ cells.
5. Mix well and incubate for 10 minutes at 4–8 °C.
6. Add additional 30 μL of buffer and 20 μL of **Anti-Biotin MicroBeads** per 10⁷ cells.
7. Mix well and incubate for additional 15 minutes at 4–8 °C.
8. Wash cells by adding 1–2 mL of buffer and centrifuge at 300xg for 10 minutes at 4 °C. Pipette off supernatant completely.
9. Resuspend up to 10⁸ cells in 500 μL of buffer.
 - ▲ **Note:** For larger cell numbers, scale up buffer volume accordingly.
 - ▲ **Note:** For depletion with LD Columns, resuspend cell pellet in 500 μL of buffer for up to 1.25×10⁸ cells.
10. Proceed to magnetic separation (2.3).



2.3 Magnetic separation: Depletion of non-CD4⁺ cells

Depletion with LD Column

1. Place LD Column in the magnetic field of a suitable MACS Separator (see "Column data sheets").
2. Prepare column by rinsing with 2 mL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells which 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⁺CD294 (CRTH2)⁺ Th2 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 "Depl025".
3. Collect unlabeled fraction (outlet port "neg1"). This is the pre-enriched CD4⁺ T cell fraction.
4. Proceed to 2.4 for the isolation of CD4⁺CD294 (CRTH2)⁺ Th2 cells.



2.4 Magnetic labeling of CD294 (CRTH2)⁺ Th2 cells

▲ Volumes for magnetic labeling of pre-enriched CD4⁺ T cells given below are for an initial starting cell number of up to 10⁷ cells. For larger initial cell numbers, scale up volumes accordingly.

1. Centrifuge the cells at 300xg for 10 minutes. Pipette off supernatant completely.
2. Resuspend cell pellet in 80 µL of buffer.
3. Add 20 µL of **FcR Blocking Reagent** and 10 µL of **CD294 (CRTH2)-PE**.
4. (Optional) Add additional staining antibodies, e.g. 10 µL of CD4-FITC (# 130-080-501) or CD4-APC (# 130-091-232).
5. Mix well and incubate for 10 minutes at 4–8 °C.
6. Wash cells by adding 1–2 mL of buffer and centrifuge at 300xg for 10 minutes. Pipette off supernatant completely.
7. Resuspend cell pellet in 80 µL of buffer.
8. Add 20 µL of **Anti-PE MicroBeads**.
9. Mix well and incubate for additional 15 minutes at 4–8 °C.
10. Wash cells by adding 1–2 mL of buffer and centrifuge at 300xg for 10 minutes. Pipette off supernatant completely.
11. Resuspend up to 10⁸ cells in 500 µL of buffer.
12. Proceed to magnetic separation (2.5).



2.5 Magnetic separation of CD4⁺CD294 (CRTH2)⁺ Th2 cells

Positive selection of CD294 (CRTH2)⁺ cells with MS Columns

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

1. Place MS Column in the magnetic field of a suitable MACS Separator (see "Column data sheets").
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 3x500 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⁺CD294 (CRTH2)⁺ Th2 cells) by firmly applying the plunger supplied with the column.
7. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.

Magnetic separation 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. For a standard separation (positive selection), choose separation program "Posseld"

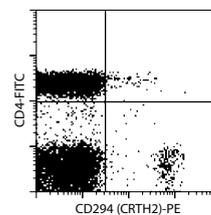
▲ **Note:** Program choice depends on the isolation strategy, the strength of magnetic labeling and the frequency of magnetically labeled cells. For details see autoMACS User Manual: "autoMACS Cell Separation Programs".

3. When using the program "Posseld", collect positive fraction (outlet port "pos2"). This is the purified CD294 (CRTH2)⁺ Th2 cell fraction.

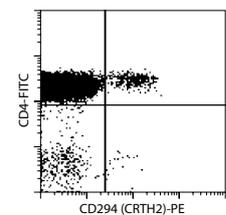
3. Example of a separation using the CD4⁺ T Cell Isolation Kit II and the CD294 (CRTH2) MicroBead Kit

CD4⁺CD294 (CRTH2)⁺ Th2 cells were isolated from human PBMCs by using the CD4⁺ T Cell Isolation Kit II and CD294 (CRTH2) MicroBead Kit, an LD and two MS Columns, a MidiMACS™ Separator and a MiniMACS™ Separator. The cells are fluorescently stained with CD294 (CRTH2)-PE and CD4-FITC. Cell debris and dead cells were excluded from the analysis based on scatter signals and PI fluorescence.

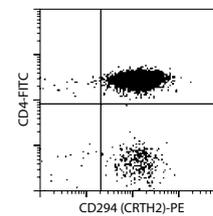
PBMCs before separation



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



Enriched CD4⁺CD294 (CRTH2)⁺ Th2 cells



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