Miltenyi Biotec

REAlease® CD3 MicroBead Kit
human

Order no. 130-117-038

Contents

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 REAlease CD3-Biotin, human** **5 mL REAlease Anti-Biotin MicroBeads (CD3, human)** **4 mL REAlease Bead Release Reagent (50×)** **4 mL REAlease Release Reagent** **4 mL REAlease Stop Reagent**
Capacity	For 10^9 total cells, up to 100 separations.
Product format	REAlease Stop Reagent is supplied in buffer containing 0.05% sodium azide. All other reagents 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 REAlease MACS Separation

The REAlease Technology relies on recombinantly engineered antibody fragments to label specific cell surface markers. The fragments are developed to have low affinity for epitopes. However, when fragments are multimerized as a REAlease Biotin Complex (i.e., REAlease CD3-Biotin) they bind to epitopes with high avidity. REAlease Technology can control the multimer/monomer state of antibody fragments. With this technology a controlled label release is possible where monomerized antibody fragments dissociate from the cell surface, enabling users to obtain bead- and label-free cells.

First, the target cells in a peripheral blood mononuclear cell (PBMC) population are labeled with REAlease CD3-Biotin (REAlease Biotin Complex). Subsequently, REAlease Anti-Biotin MicroBeads (CD3, human) bind to REAlease Biotin Complex. Then, the cell suspension is loaded onto a MACS Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled cells are retained within the column. The unlabeled non-target cells flow through; this cell fraction is thus depleted of $CD3^+$ T cells. After removing the column from the magnetic field, the target cells are eluted using the REAlease Bead Release Reagent, which simultaneously removes the MicroBeads from the cell. Finally, during the subsequent incubation with the REAlease Release Reagent, the REAlease Biotin Complex monomerizes and dissociates from the cell surface leaving the cells free of all labels.

1.2 Background information

The REAlease CD3 MicroBead Kit has been developed for positive selection $CD3^+$ T cells from PBMCs. CD3 is expressed on all T cells and $CD56^+$ NKT cells, and is associated with the T cell receptor. Most of the human peripheral blood lymphocytes (70–80%) and thymocytes (65–85%) are $CD3^+$. The epitope recognized by the REAlease CD3-Biotin complex is located on the CD3ε chain.

The REAlease CD3 MicroBead Kit is an indirect magnetic labeling system that allows to obtain cells free of MicroBeads and REAlease Biotin Complex.

1.3 Applications

- Isolation of specific $CD3^+$ cell subsets. The selected $CD3^+$ cells can be sorted according to a second marker of interest, e.g., CD56 (NKT cells).
- Isolation of $CD3^+$ cell subset that needs to be label-free.

Miltenyi Biotec B.V. & Co. KG
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macsde@miltenyi.com
www.miltenyibiotec.com

1.4 Reagent and instrument requirements

- Separation 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). Use buffer at room temperature (+19 °C to +25 °C). Store buffer cold (2–8 °C). Degas buffer before use, as air bubbles could block the column.

  ▲ **Note:** 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.

- REAlease Bead Release buffer: Prepare a 1:50 dilution of REAlease Bead Release Reagent (50×), e.g., for 1 mL add 20 µL of REAlease Bead Release Reagent to 980 µL of separation buffer.

  ▲ **Note:** Use freshly prepared buffer the same day. Store at room temperature.

  ▲ **Note:** Prepare 1 mL per MS Column and 5 mL per LS Column.

- MACS Columns and MACS Separators: $CD3^+$ cells can be enriched by using MS or LS Columns.

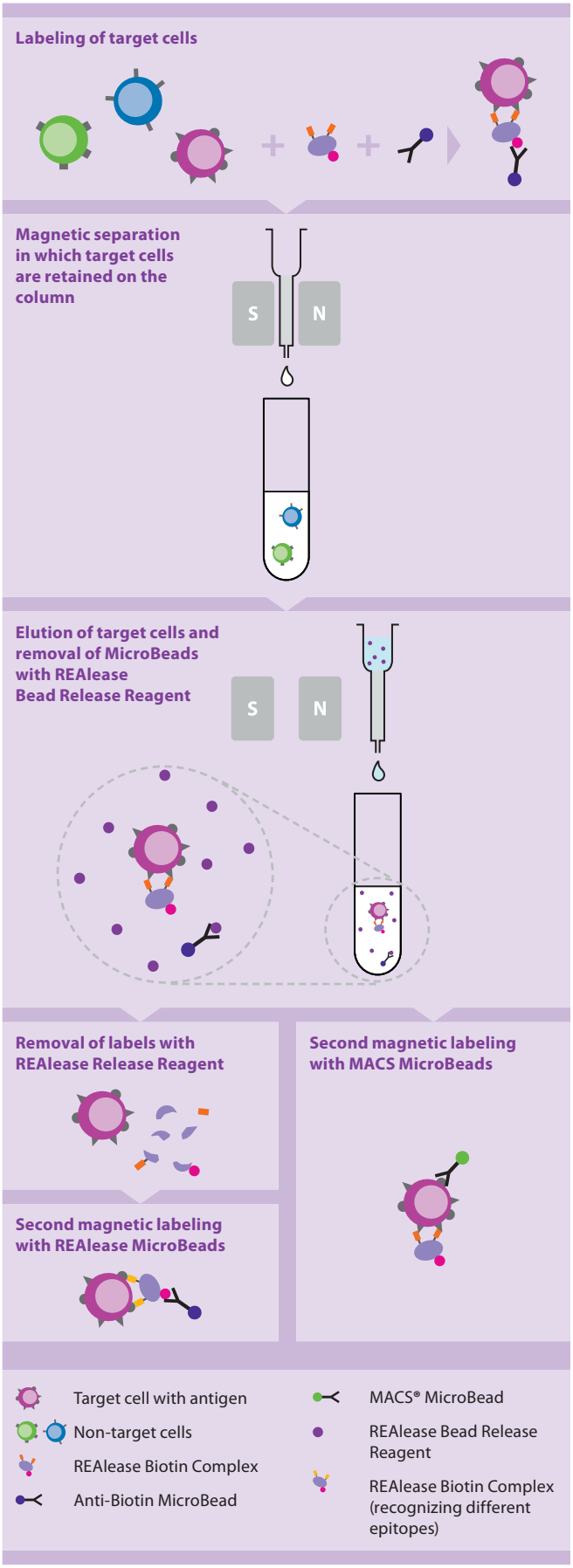
Column	Max. number of labeled cells	Max. number of total cells	Separator
Positive selection			
MS	10^7	$2×10^8$	MiniMACS, OctoMACS, SuperMACS II
LS	10^8	$2×10^9$	MidiMACS, QuadroMACS, SuperMACS II

▲ **Note:** Column adapters are required to insert certain columns into SuperMACS™ II Separators. For details refer to the respective MACS Separator data sheet.

- (Optional) Fluorochrome-conjugated CD3 antibodies for flow cytometric analysis, e.g., CD3-FITC. For more information about antibodies refer to www.miltenyibiotec.com/antibodies.
- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD Staining Solution (# 130-111-568) for flow cytometric exclusion of dead cells.
- (Optional) Pre-Separation Filters (30 µm) (# 130-041-407) to remove cell clumps.

2. Protocol

2.1 Protocol overview



2.2 Sample preparation

When working with anticoagulated peripheral blood or buffy coat, 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 refer to the protocol "Isolation of mononuclear cells from human peripheral blood by density gradient centrifugation" at www.miltenyibiotec.com.

2.3 Magnetic labeling

▲ The recommended incubation temperature is at room temperature (+19 °C to +25 °C).

▲ 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.

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 separation buffer per 10^7 total cells.
4. Add 10 µL of REAlease CD3-Biotin per 10^7 total cells.
5. Mix well and incubate for 5 minutes.
6. Add 50 µL of REAlease Anti-Biotin MicroBeads (CD3, human) per 10^7 total cells.
7. Mix well and incubate for 5 minutes.
8. (Optional) Add staining antibodies, e.g., CD3-FITC, and incubate for 5 minutes in the dark in the refrigerator (2–8 °C).

   ▲ **Note:** These staining antibodies cannot be removed from the cells.
9. Dilute up to 5×10^7 cells in a total volume of 500 µL with separation buffer.

   ▲ **Note:** For volumes larger than 500 µL a dilution is not needed.
10. Proceed to magnetic separation (2.4).

2.4 Magnetic separation and removal of magnetic labeling

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of $CD3^+$ cells. For details refer to the table in section 1.4.

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

▲ The recommended incubation temperature is at room temperature (+19 °C to +25 °C).

Magnetic separation with MS or LS Columns

1. Place column in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.
2. Prepare column by rinsing with the appropriate amount of separation buffer:

   MS: 500 µL LS: 3 mL
3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
4. Wash column with the appropriate amount of separation buffer. Collect unlabeled cells that pass through and combine with the flow-through from step 3.

   MS: 3×500 µL LS: 3×3 mL

   ▲ **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 the appropriate amount of REAlease Bead Release buffer (prepared by REAlease Bead Release Reagent (50×), refer to chapter 1.4) onto the column. Immediately flush out target cells by firmly pushing the plunger into the column.

   MS: 1 mL LS: 5 mL
7. Mix well and incubate for 10 minutes.
8. Cells are now free from MicroBeads and ready for analysis or downstream applications.
9. (Optional) Proceed either to
   - 2.5 Removal of REAlease Complex and second magnetic labeling with REAlease MicroBeads

     or proceed to
   - 2.6 Second magnetic labeling with MACS MicroBeads.

2.5 (Optional) Removal of the REAlease Complex and second magnetic labeling with REAlease MicroBeads

▲ The recommended incubation temperature is at room temperature (+19 °C to +25 °C).

▲ For second magnetic labeling with MACS Anti-Biotin MicroBeads proceed through all steps of chapter 2.5.

2.5.1 Removal of the REAlease Complex

1. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in appropriate amount of separation buffer:

   MS: 1 mL LS: 5 mL
3. Add an appropriate amount of REAlease Release Reagent:

   MS: 20 µL LS: 100 µL
4. Mix well and incubate for 5 minutes.
5. Cells are now free from REAlease Complex and MicroBeads and are ready for analysis or downstream applications.
6. (Optional) For second magnetic labeling with REAlease MicroBeads continue with 2.5.2.

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2.5.2 Second magnetic labeling with REAlease MicroBeads

1. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 40 µL of REAlease Stop Reagent per 10^7 total cells.
3. Mix well.
4. Proceed with steps 4–9 of chapter 2.3 Magnetic labeling.

▲ **Note:** For best recovery and purity of cells, the amount of MACS MicroBeads for the second positive labeling may need optimization as the starting frequency of target cells may be different from a PBMC sample.

2.6 (Optional) Second magnetic labeling with MACS MicroBeads

▲ For second magnetic labeling with MACS Anti-Biotin MicroBeads proceed through all steps of chapter 2.5.

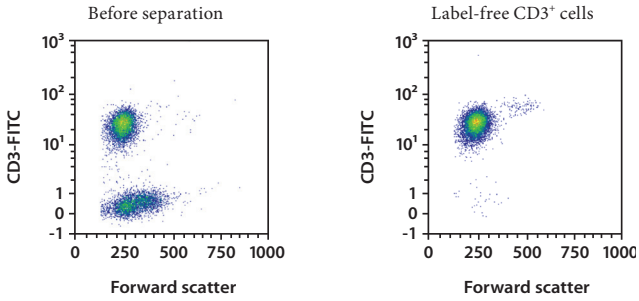
1. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
2. Add the recommended amount of MACS MicroBeads to label the cells magnetically for the second marker. For details refer to the respective MACS MicroBeads data sheet.

▲ **Note:** For best recovery and purity of cells, the amount of MACS MicroBeads for the second positive labeling may need optimization as the starting frequency of target cells may be different from a PBMC sample.

3. Example of a separation using the REAlease CD3 MicroBead Kit

$CD3^+$ cells were isolated from human PBMCs using the REAlease CD3 MicroBead Kit, MS Columns, and a MiniMACS™ Separator. Cells were fluorescently stained with CD3-FITC and analyzed by flow cytometry using the MACSQuant® Analyzer X. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.
The $CD3^+$ T cell content of the isolated fraction is typically 98.4 ± 1.38% (mean ± SD).

A) Cell purity

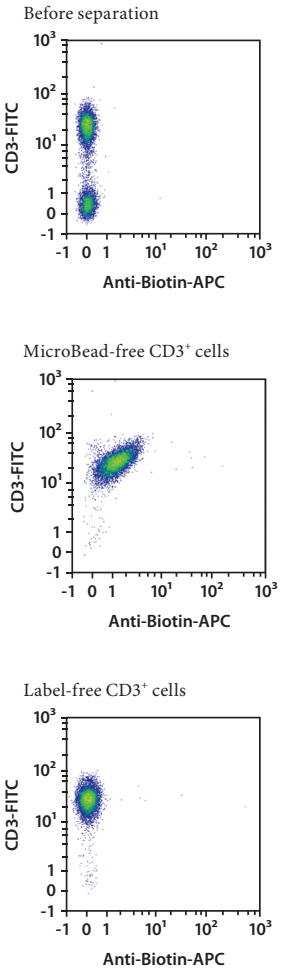


B) Bead-free cells: efficiency of REAlease Anti-Biotin MicroBeads release

Release efficiency was higher than 99% for the REAlease Anti-Biotin MicroBeads (CD3, human). The efficiency was determined by re-applying the isolated cells to a second MACS Column. The ratio between the numbers of cells in the flow-through and the total number of cells applied to the second column allowed us to calculate the efficiency of magnetic labeling removal.

C) Label-free cells: REAlease Biotin Complex release

The efficient removal of all labels was shown by using Anti-Biotin-APC to analyze the cells by flow cytometry for the presence of REAlease Biotin Complex. Directly after isolation, the cells showed staining of biotin ("MicroBead-free $CD3^+$ cells"), whereas the label-free $CD3^+$ cells after the REAlease Biotin Complex release were negative for biotin similar to the non-labeled cells before separation.



Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

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