Miltenyi Biotec

# REAlease® CD4 (TIL) MicroBead Kit
human

Order no. 130-121-559

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## 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 CD4 (TIL)-Biotin, human** |
| | **2.5 mL REAlease Anti-Biotin MicroBeads (CD4 (TIL), human)** |
| | **4 mL REAlease Bead Release Reagent (50×)** |
| | **4 mL REAlease Release Reagent** |
| | **4 mL REAlease Stop Reagent** |
| **Capacity** | For $5\times10^8$ total cells, up to 50 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 CD4 (TIL)-Biotin, human) 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 from single-cell suspensions of solid human tumors as well as xenotransplanted tumors of mice with a humanized immune system are labeled with REAlease CD4 (TIL)-Biotin, human (REAlease Biotin Complex). Subsequently, REAlease Anti-Biotin MicroBeads (CD4 (TIL), human) bind to the 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 $CD4^+$ 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 cells. 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 CD4 (TIL) MicroBead Kit, human has been developed for the isolation of $CD4^+$ tumor-infiltrating leukocytes (TILs) from single-cell suspensions of solid human tumors as well as xenotransplanted tumors of mice with a humanized immune system. CD4 is expressed on T helper cells, at a lower level on monocytes and dendritic cells, and at a very low level on hematopoietic progenitor cells.
The REAlease CD4 (TIL) MicroBead Kit, human is an indirect magnetic labeling system that allows to obtain cells free of MicroBeads and the REAlease Biotin Complex.

### 1.3 Applications

- Positive selection of $CD4^+$ T cells from solid human tumors.
- Isolation of specific $CD4^+$ tumor-infiltrating T cell subsets. The selected $CD4^+$ T helper cell population can be sorted according to a second marker of interest, for example, CD25, CD137 (4-1BB), CD223 (LAG-3), CD279 (PD1), or CD366 (TIM-3).
- Isolation of $CD4^+$ tumor-infiltrating T cells which need to be label-free.



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### 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: $CD4^+$ cells can be enriched by using MS or LS Columns.

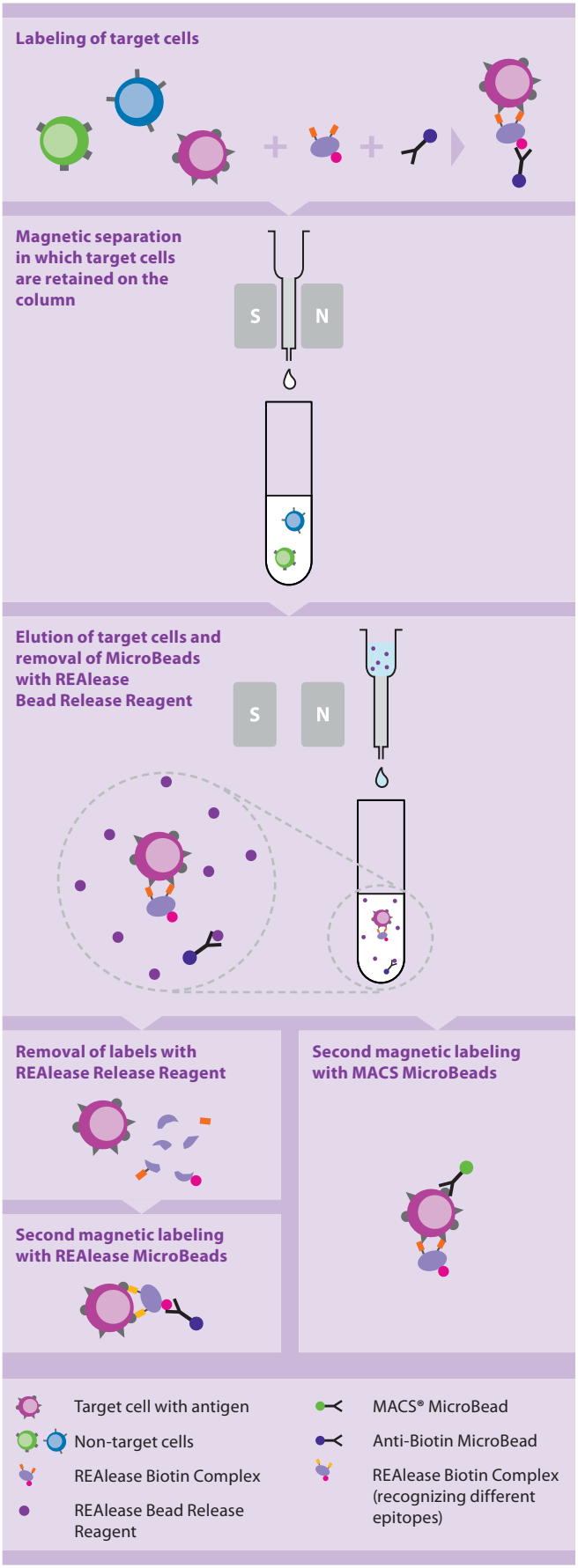
| Column | Max. number of labeled cells | Max. number of total cells | Separator |
|---|---|---|---|
| **Positive selection** | | | |
| MS | $10^7$ | $2×10^7$ | MiniMACS, OctoMACS, SuperMACS II |
| LS | $4×10^7$ | $5×10^7$ | MidiMACS, QuadroMACS, SuperMACS II |

  ▲ **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 CD4 antibodies for flow cytometric analysis, e.g., CD4-VioGreen™. 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) or Viobility™ Fixable Dyes (# 130-109-812, # 130-109-814, # 130-109-816) for flow cytometric exclusion of dead cells.
- (Optional) Pre-Separation Filters (30 µm) (# 130-041-407) to remove cell clumps.
- (Optional) MACS SmartStrainers (30 µm) (# 130-098-458) to remove cell clumps.

## 2. Protocol

### 2.1 Protocol overview



### 2.2 Sample preparation

For preparation of a single-cell suspension from solid human or xenotransplanted tumors use the Tumor Dissociation Kit, human (# 130-095-929) in combination with the gentleMACS™ Dissociators.

For details refer to www.gentlemacs.com/protocols.



### 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 $2\times10^7$ 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 (MACS SmartStrainer (30 µm), # 130-098-458) 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 30 µL of separation buffer per $10^7$ total cells.
4. Add 20 µL of REAlease CD4 (TIL)-Biotin per $10^7$ total cells.
5. Mix well and incubate for 5 minutes.
6. Add 50 µL of REAlease Anti-Biotin MicroBeads (CD4 (TIL), human) per $10^7$ total cells.
7. Mix well and incubate for 5 minutes.
8. (Optional) Add staining antibodies, e.g., CD4-VioGreen™, 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 $5\times10^7$ 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 $CD4^+$ cells. For details refer to the table in section 1.4.

▲ 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 (30 µm), # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

▲ 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. (Optional) If removal of MicroBeads is not required, pipette appropriate amount of separation buffer. Immediately flush out target cells by firmly pushing the plunger into the column. Eluted cells are ready for downstream applications, e.g. flow cytometry analysis.
   MS: 1 mL LS: 5 mL
7. For removal of MicroBeads proceed with step 8.
8. 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
9. Mix well and incubate for 10 minutes.
10. Cells are now free from MicroBeads and ready for analysis and downstream applications.
11. (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 to remove the REAlease Biotin Complex.

#### 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.

### 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. For a second magnetic labeling follow the labeling protocol in the respective REAlease MicroBead Kit 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 single-cell suspension of solid human tumors as well as xenotransplanted tumors of mice with a humanized immune system.

### 2.6 (Optional) Second magnetic labeling with MACS MicroBeads

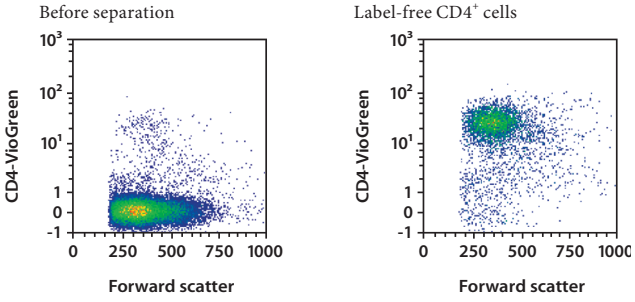
▲ For second magnetic labeling with MACS Anti-Biotin MicroBeads proceed through all steps of chapter 2.5 to remove the REAlease Biotin Complex.

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 single-cell suspension of solid human tumors as well as xenotransplanted tumors of mice with a humanized immune system.

## 3. Example of a separation using the REAlease CD4 (TIL) MicroBead Kit

A human ovarian carcinoma sample was dissociated using the gentleMACS™ Octo Dissociator with Heaters in combination with the Tumor Dissociation Kit, human (# 130-095-929). $CD4^+$ TILs were isolated from the single-cell suspension using the REAlease CD4 (TIL) MicroBead Kit, LS Columns, and a MidiMACS™ Separator. Cells were fluorescently stained with CD4-VioGreen™ 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 7-AAD fluorescence.

### A) Cell purity

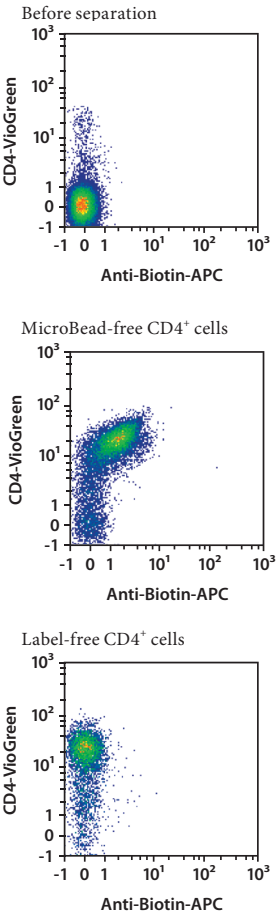



### B) Bead-free cells: efficiency of REAlease Anti-Biotin MicroBeads release

Release efficiency was higher than 99% for the REAlease Anti-Biotin MicroBeads (CD4 (TIL)). 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 $CD4^+$ cells"), whereas the label-free $CD4^+$ 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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