Miltenyi Biotec

StraightFrom® Leukopak® REAlease® CD56 MicroBead Kit
human

Order no. 130-128-759

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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

2 mL StraightFrom Leukopak® REAlease CD56-Biotin, human

5 mL StraightFrom Leukopak® REAlease Anti-Biotin MicroBeads (CD56), human

4 mL REAlease Bead Release Reagent (50×)

4 mL REAlease Release Reagent

4 mL REAlease Stop Reagent

1× Multi-24 Column Block
1× Multi-24 Column Block and 1× 24-well Deep Well Plate, sterile packed.

Capacity For one ½ Leukopak®.

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. Store Multi-24 Column Block dry at 10–35 °C and protected from light. The expiration date is indicated on the vial or box 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 CD56-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.
Sample preparation in form of an initial wash is required. No density gradient centrifugation or erythrocyte lysis is needed. $CD56^+$ cells are labeled with StraightFrom Leukopak® REAlease CD56-Biotin (REAlease Biotin Complex). Subsequently, StraightFrom Leukopak® REAlease Anti-Biotin MicroBeads (CD56) bind to the REAlease Biotin Complex. Then, the cell suspension is loaded onto a Multi-24 Column Block, which is placed in the magnetic field of a MultiMACS Cell24 Separator Plus. The magnetically labeled cells are retained within the column. The unlabeled non-target cells flow through; this cell fraction is thus depleted of $CD56^+$ NK and NKT cells. After removing the column from the magnetic field, the target cells are eluted using 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.
In addition, $CD56^+CD3^+$ cells can be depleted in a second magnetic separation using CD3 MicroBeads.

1.2 Technical specifications of the Multi-24 Column Block

One Multi-24 Column Block is a unit of 24 columns, enabling up to 24 separations in parallel.

- Column capacity: 1×10^8 magnetically labeled cells from up to 1×10^9 total cells per single column.
- Columns are “flow stop” and do not run dry.
- Void volume: 250 µL. Reservoir volume: 5 mL.
  ▲ **Note:** If sample volume exceeds 5 mL per column, apply sample in aliquots.
- Multi-24 Column Blocks are for single use only.

1.3 Background information

During leukapheresis white blood cells are separated from whole blood and collected as highly concentrated leukocytes in Leukopaks®. Thus, Leukopaks® are ideal for the isolation of large numbers of various leukocyte subsets.
In blood, CD56 is expressed on human NK cells, NKT cells, and also on a small subpopulation of T cells. The StraightFrom Leukopak®

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REAlease CD56 MicroBead Kit, human has been developed for the positive selection of CD56$^+$ cells directly from a Leukopak® by using the MultiMACS Cell24 Separator Plus.
The StraightFrom Leukopak® REAlease CD56 MicroBead Kit is an indirect magnetic labeling system that allows to obtain cells free of MicroBeads and REAlease Biotin Complex.
In addition, CD56$^+$CD3$^+$ cells can be depleted in a second magnetic separation using CD3 MicroBeads in combination with LD Columns.

1.4 Applications

- Isolation of CD56$^+$ cells from Leukopak®. The purified CD56$^+$ cells are well suited for further magnetic separation, i.e., for the depletion of CD56$^+$CD3$^+$ cells with CD3 MicroBeads. The cells are also suited for further flow cytometric analyses, molecular biology applications, and functional studies.
- Isolation of CD56$^+$ cell subset that needs to be label-free.

1.5 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). Keep buffer cold (2–8 °C). Alternatively, use autoMACS Running Buffer. 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 (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 50 mL add 1 mL of REAlease Bead Release Reagent to 49 mL of separation buffer.

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

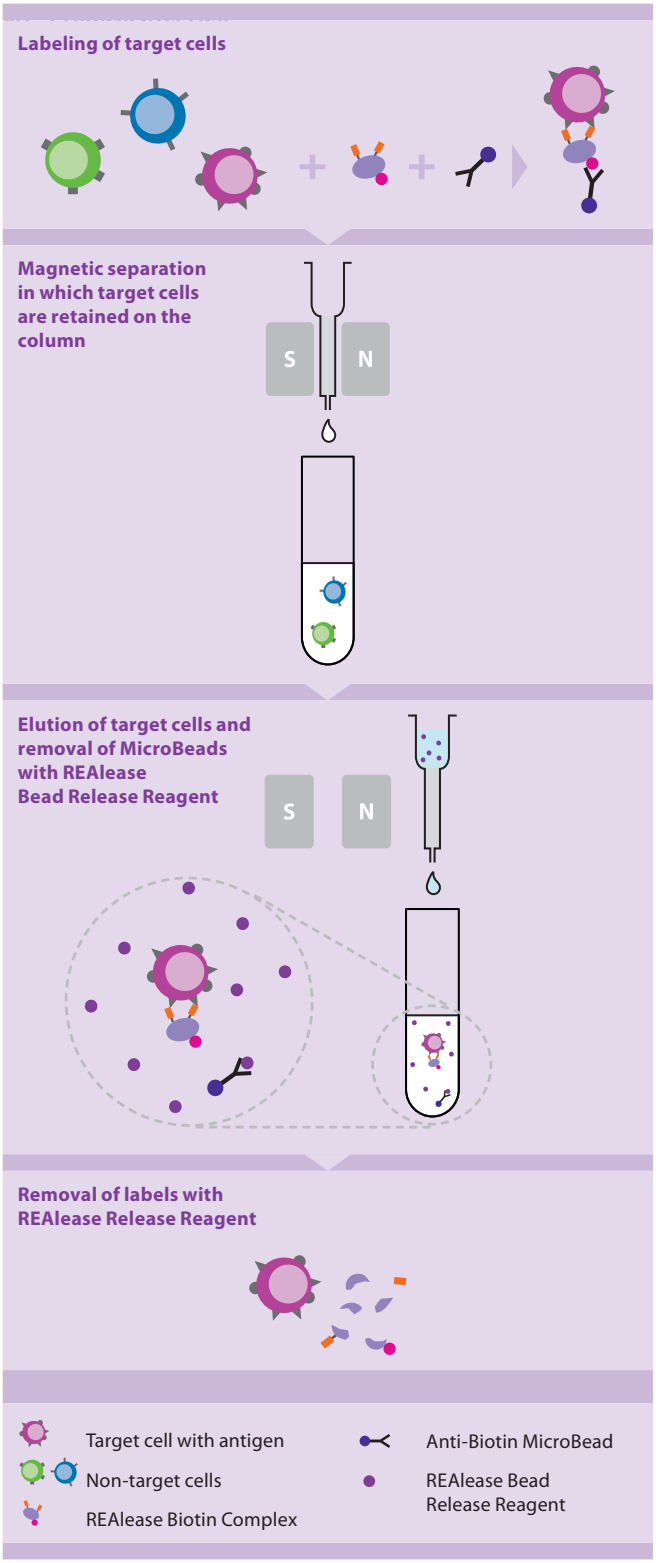
- Semi-automated separation:
  - MultiMACS Cell24 Separator Plus (# 130-098-637)
  - 24-well Deep Well Plates (# 130-110-500) or Single-well Deep Well Plates (# 130-114-966)
- (Optional) Fluorochrome-conjugated REAfinity™ Antibodies for flow cytometric analysis, e.g.,
  - CD3 Antibody, anti-human, FITC, REAfinity,
  - CD56 Antibody, anti-human, APC, REAfinity, or
  - CD45 Antibody, anti-human, VioGreen™, REAfinity.

  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) MACS SmartStrainers (30 µm) (# 130-098-458) to remove cell clumps.
- (Optional) Red Blood Cell Lysis Solution (10×) (# 130-094-183)
- (Optional) For additional depletion of CD3$^+$CD56$^+$ cells:
  - CD3 MicroBeads, human (# 130-050-101)
  - LD Columns (# 130-042-901)

  Semi-automated separation:
  - MultiMACS Cell24 Separator Plus (# 130-098-637)
  - MultiMACS Single-Column Adapter (# 130-098-637)

  Manual separation:
  - MidiMACS™ Separator (# 130-042-302) or QuadroMACS™ Separator (# 130-090-976)
  - MACS Acrylic Tube Rack (# 130-041-406) or MACS 15 mL Tube Rack (# 130-091-052)

2. Protocol



2.2 Preparation of Leukopak®

1. Divide ½ Leukopak® equally into eight collection tubes (max. 25 mL per tube). If the volume per tube is less than 25 mL, fill up to 25 mL with separation buffer.
2. Centrifuge cell suspension at 200×g for 15 minutes. Aspirate supernatant completely.
3. Resuspend each of the eight cell pellets in separation buffer, pool all resuspended samples, and fill up to a final volume of 50 mL with separation buffer.
4. Proceed to magnetic labeling (2.3).



2.3 Magnetic labeling

▲ 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.

▲ The StraightFrom Leukopak® CD56 MicroBead Kit has been developed for positive selection of target cells from one ½ Leukopak® with up to 200 mL (5×10^9–1×10^{10} cells). When working with smaller volumes, scale down all reagents and total volumes accordingly (e.g. for ¼ Leukopak®, fill up to 25 mL after centrifugation and use 1/2 vial of each reagent and buffer as well as half of the Multi-24 Column Block). When working with higher volumes per ½ Leukopak®, please contact the Technical Support team.

▲ Perform labeling and separation steps for each tube one after the other.

▲ The recommended incubation temperature is room temperature (19–25 °C). Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

1. Add 2 mL of StraightFrom Leukopak® REAlease CD56-Biotin to the tube containing 50 mL sample.
2. Mix well by inverting the tube and incubate for 5 minutes at room temperature (19–25 °C).
3. Add 45 mL of separation buffer.
4. Add 5 mL of StraightFrom Leukopak® REAlease Anti-Biotin MicroBeads (CD56).
5. Mix well by inverting the tube and incubate for 10 minutes at room temperature (19–25 °C).
6. Wash cells 10 min 300×g and resuspend in 100 mL of separation buffer.
7. Proceed to magnetic separation (2.4).



2.4 Magnetic separation and removal of magnetic labeling

2.4.1 Semi-automated magnetic separation with the MultiMACS Cell24 Separator Plus

▲ The MultiMACS Cell24 Separator Plus, including the MACS Elution Station, has to be used with a Multi-24 Column Block and two Single-well Deep Well Plates for magnetic separation with StraightFrom Leukopak® REAlease MicroBeads.

▲ **Note:** To reach maximum cell recovery, rinse the Deep Well Plate after removal of the positive fraction and combine with the positive fraction. For the collection of the negative fraction and the wash fractions, use one Deep Well Plate.

▲ Buffer volumes are as follows:

Equilibration: 2 mL (separation buffer)
Wash: 3×1 mL (separation buffer)
Elution: 2 mL (1:50 REAlease Bead Release Reagent)

▲ If equilibration solution and negative fraction should be collected in the same Deep Well Plate as waste, select the program **POSSEL** and follow the on-screen instructions of the MultiMACS Cell24 Separator Plus.

▲ If equilibration solution and negative fraction should be collected in separate Deep Well Plates, select the program **POSSEL2** and follow the on-screen instructions of the MultiMACS Cell24 Separator Plus.

▲ For more detailed instructions on how to use the MultiMACS Cell24 Separator Plus, please refer to the user manual.

1. Equilibrate using 2 mL of separation buffer per column.
2. Divide the sample equally between the 24 columns of the Multi-24 Column Block, e.g., when starting volume is 100 mL, add 4 mL onto each column.
3. Wash using 3×1 mL of separation buffer per column.
4. Elute using 2 mL of REAlease Bead Release Reagent (1:50) per column.
5. After the separation, mix well and incubate for 10 minutes. Cells are now free from MicroBeads and ready for analysis or downstream applications.
6. (Optional) Remaining erythrocytes can be lysed using Red Blood Cell Lysis Solution (10×).
7. (Optional) Proceed to 2.5 Removal of REAlease Biotin Complex.

▲ **Note:** To reach optimal NK cell purity, it is recommended to perform a second magnetic separation using CD3 MicroBeads for depletion of $CD56^+CD3^+$ cells.

2.5 (Optional) Removal of the REAlease Biotin Complex

▲ The recommended incubation temperature is room temperature (19–25 °C).

1. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in appropriate amount of separation buffer. For ½ Leukopak®: 10 mL.
3. Add an appropriate amount of REAlease Release Reagent. For ½ Leukopak®: 200 µL.
4. Mix well and incubate for 5 minutes.
5. Cells are now free from REAlease Biotin Complex and MicroBeads and are ready for analysis or downstream applications.
6. (Optional) For second magnetic labeling, centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely. Resuspend cell pellet in 4 mL of REAlease Stop Reagent and mix well. Then, proceed with 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.

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2.6 (Optional) Depletion of CD3$^+$CD56$^+$ cells

▲ To reach optimal NK cell purity, it is recommended to perform a second magnetic separation using CD3 MicroBeads for depletion of CD56$^+$CD3$^+$ cells. Please note that CD3 MicroBeads are not contained in this kit and have to be ordered separately.

▲ Before second magnetic labeling with MACS MicroBeads proceed through all steps of chapter 2.5.

▲ Buffer volumes are as follows:

Equilibration: 2 mL (separation buffer)
Wash: 2×1 mL (separation buffer)

▲ Equilibration solution should be collected in the Deep Well Plate as waste, select the program **DEPLETE** and follow the on-screen instructions of the MultiMACS Cell24 Separator Plus.

The program **DEPLETE** enables only collection of negative fraction.

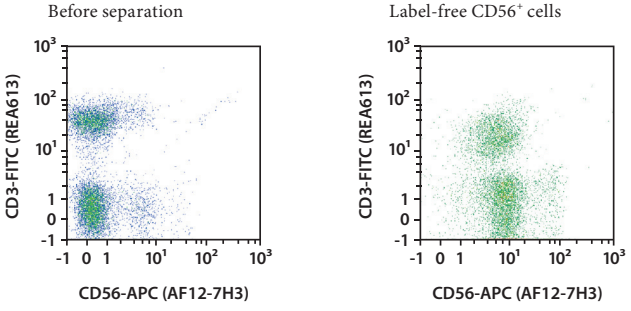
▲ For more detailed instructions on how to use the MultiMACS Cell24 Separator Plus, please refer to the user manual.

1. Add 1 mL CD3 MicroBeads.
2. Mix well by inverting the tube and incubate for 15 minutes in the refrigerator (2–8 °C).
3. Equilibrate 5 LD Columns using 2 mL of separation buffer per column.
4. After incubation with CD3 MicroBeads, add 1 mL of the sample onto each of the 5 LD Columns placed in a MultiMACS Single-Column Adapter.
5. Wash LD Columns using 2×1 mL of separation buffer per column.

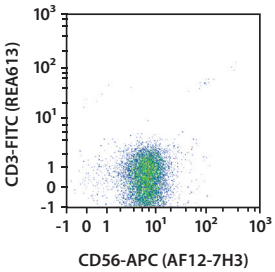
3. Example of a separation using the StraightFrom Leukopak® REAlease CD56 MicroBead Kit and CD3 MicroBeads

CD56$^+$ cells were isolated from ½ Leukopak® using the StraightFrom Leukopak® REAlease CD56 MicroBead Kit, CD3 MicroBeads, Multi-24 Column Block, LD Columns for CD3 depletion, and a MultiMACS Cell24 Separator Plus. Cells were fluorescently stained with REAfinity Antibodies CD56-APC, CD3-FITC, and Biotin Antibody-PE 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 CD56$^+$ NK cell content of the isolated fraction is typically 98.5 ± 0.4% (mean ± SD).

A) Cell purity



Cells after CD3 depletion

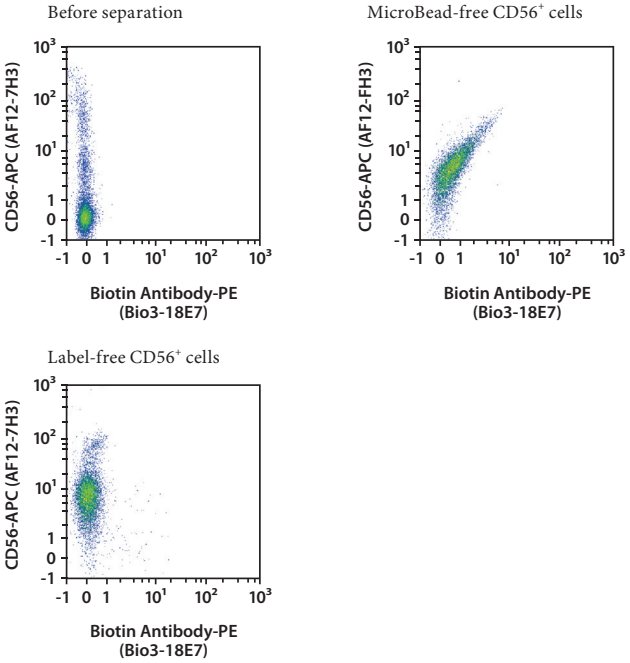


B) Bead-free cells: release efficiency of StraightFrom Leukopak® REAlease Anti-Biotin MicroBeads

Release efficiency of the REAlease Anti-Biotin MicroBeads was higher than 95% for the StraightFrom Leukopak® REAlease Anti-Biotin MicroBeads (CD56). 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: release of REAlease Biotin Complex

The efficient removal of all labels was shown by using Biotin Antibody-PE 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 CD56$^+$ cells"), whereas the label-free CD56$^+$ 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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