



# CD56<sup>+</sup>CD8<sup>+</sup>/CD8<sup>-</sup> NK Cell Isolation Kit

## human

Order no. 130-092-659

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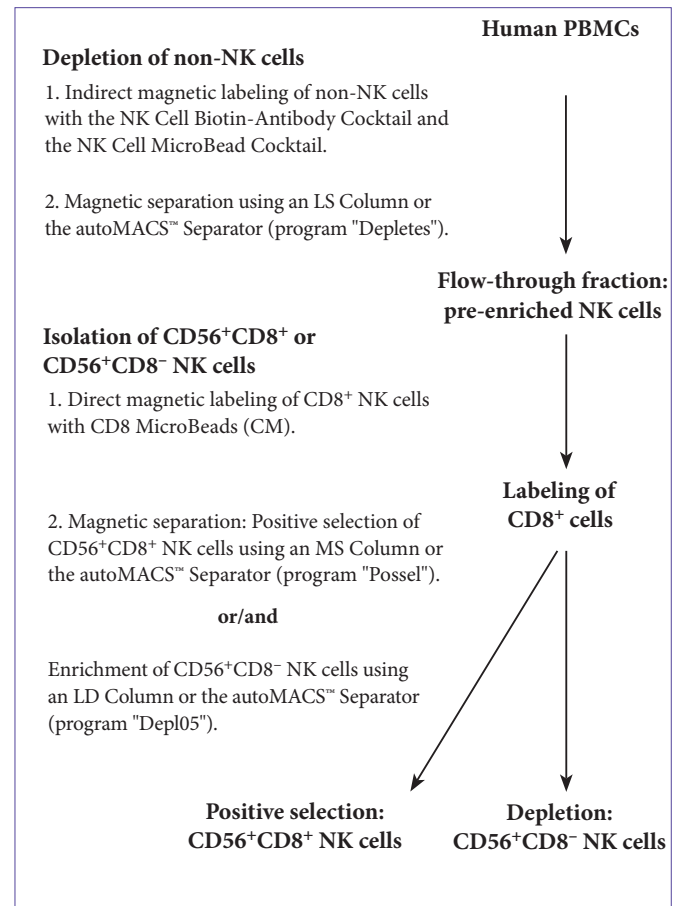
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### 1. Description

<b>Components</b>	<b>2 mL NK Cell Biotin-Antibody Cocktail, human:</b> Cocktail of biotin-conjugated monoclonal anti-human antibodies against antigens not expressed by NK cells. <b>2 × 2 mL NK Cell MicroBead Cocktail, human:</b> Cocktail of MicroBeads conjugated to monoclonal antibodies. <b>2 mL CD8 MicroBeads (CM), human:</b> MicroBeads conjugated to a monoclonal CD8 antibody (isotype: mouse IgG2a).
<b>Size</b>	For 2 × 10 <sup>9</sup> total cells, up to 20 separations.
<b>Product format</b>	All components are supplied in buffer containing stabilizer and 0.05% sodium azide.
<b>Storage</b>	Store protected from light at 4–8 °C. Do not freeze. The expiration date is indicated on the vial label.

### 1.1 Principle of MACS<sup>®</sup> Separation

The isolation of both CD56<sup>+</sup>CD8<sup>+</sup> and/or CD56<sup>+</sup>CD8<sup>-</sup> NK cell subsets is performed in a two-step procedure. In both cases, non-NK cells, i.e. T cells, B cells, dendritic cells, stem cells, monocytes, granulocytes, and erythroid cells are first indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies against lineage-specific antigens and a cocktail of MicroBeads. Upon subsequent magnetic separation of the cells over a MACS<sup>®</sup> Column that is placed in a magnetic field of a MACS Separator, the magnetically labeled non-NK cells are retained within the column, while the unlabeled NK cells run through. In the second step, the pre-enriched NK cells are directly labeled with CD8 MicroBeads (CM). Upon subsequent magnetic separation, either the non-labeled CD56<sup>+</sup>CD8<sup>-</sup> NK cells are retrieved from the flow-through fraction, or the labeled CD56<sup>+</sup>CD8<sup>+</sup> NK cells are eluted after removing the column from the magnetic field.



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## 1.2 Background and product applications

NK cells are not a homogeneous cell population, but can be subdivided into several subsets according to functional and phenotypic differences. The CD56<sup>+</sup>CD8<sup>+</sup>/CD8<sup>-</sup> NK Isolation Kit was developed to enrich two NK cell populations: CD3<sup>-</sup>CD56<sup>+</sup>CD8<sup>+</sup> and CD3<sup>-</sup>CD56<sup>+</sup>CD8<sup>-</sup> NK cells. CD3<sup>-</sup>CD56<sup>+</sup>CD8<sup>+</sup> NK cells represent approx. 3% and CD3<sup>-</sup>CD56<sup>+</sup>CD8<sup>-</sup> NK cells about 4% of all PBMCs in healthy donors.

Both NK cell populations differ in the presence of homodimeric CD8 formed by two  $\alpha$ -chains. Recently, the expression of CD8 $\alpha$  on CD3<sup>-</sup> NK cells revealed to be of fundamental significance, as the CD3<sup>-</sup>CD56<sup>+</sup>CD8<sup>+</sup> subset was shown to have a stronger cytotoxic function compared to its CD8<sup>-</sup> counterpart.<sup>1</sup>

### Example applications

- Specific isolation of CD56<sup>+</sup>CD8<sup>+</sup> or CD56<sup>+</sup>CD8<sup>-</sup> NK cells from peripheral blood for phenotypical and functional characterization.
- Characterization of NK subset-specific receptor expression or cytokine secretion patterns.
- Analysis of cell-cell or cytokine-mediated interactions with other cells of the innate and adaptive immunity.
- Studies on cell-mediated cytotoxicity and the involved signal transduction pathways.
- Analysis of NK cell differentiation and maturation.
- Studies on function and benefits of using a distinct NK cell subset for anti-cancer treatment.
- Studies on the function of NK cell subsets in distinct diseases, e.g. sepsis, septic shock, multiple-organ dysfunction, HIV, and Hepatitis B infection, as well as during pregnancy.

## 1.3 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 (4–8 °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 gelatine, human serum, or fetal calf serum. Buffers or media containing Ca<sup>2+</sup> or Mg<sup>2+</sup> are not recommended for use.

- MACS Columns and MACS Separators: Depletion of non-NK cells is performed on an LS Column. The subsequent positive selection of CD56<sup>+</sup>CD8<sup>+</sup> NK cells is performed on an MS Column. If CD56<sup>+</sup>CD8<sup>-</sup> NK cells are to be enriched, then an LD Column is used for the second cell separation step. If both NK cell populations are needed, first isolate the CD56<sup>+</sup>CD8<sup>+</sup> NK cells over an MS Column. The flow-through fraction of this separation can then be passed over an LD Column to additionally enrich the CD56<sup>+</sup>CD8<sup>-</sup> NK cell population. 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
<b>Depletion</b>			
LS	10 <sup>8</sup>	2×10 <sup>9</sup>	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
<b>Positive selection</b>			
MS	10 <sup>7</sup>	2×10 <sup>8</sup>	MiniMACS, OctoMACS, VarioMACS, SuperMACS
<b>Depletion</b>			
LD	10 <sup>8</sup>	5×10 <sup>8</sup>	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
<b>Depletion and positive selection</b>			
autoMACS	2×10 <sup>8</sup>	4×10 <sup>9</sup>	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 antibody for flow cytometric analysis, e.g. CD3-FITC (# 130-080-401), CD3-PE (# 130-091-374), CD3-APC (# 130-091-373), CD56-PE (# 130-090-755) or CD56-APC (# 130-090-843) and Anti-Biotin-FITC (# 130-090-857), Anti-Biotin-PE (# 130-090-756), or Anti-Biotin-APC (# 130-090-856).
- ▲ **Note:** For staining of CD8, we recommend using antibodies that recognize an other epitope than clone BW135/80.
- (Optional) Propidium iodide (PI) or 7-AAD for flow cytometric exclusion of dead cells without cell fixation.
- (Optional) Pre-Separation Filters (# 130-041-407) to remove cell clumps.
- (Optional) Dead Cell Removal Kit (# 130-090-101) for the labeling and discrimination of dead cells by flow cytometry.

## 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, e.g. Ficoll-Paque™. For details see section General Protocols in the User Manuals or visit [www.miltenyibiotec.com](http://www.miltenyibiotec.com).

▲ **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.



### 2.2 Magnetic labeling of non-NK 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<sup>8</sup> cells. When working with fewer than 10<sup>8</sup> 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<sup>8</sup> 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.

▲ 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  $300\times g$  for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 400  $\mu\text{L}$  of buffer per  $10^8$  cells.
4. Add 100  $\mu\text{L}$  of **NK Cell Biotin-Antibody Cocktail** per  $10^8$  cells.
5. Mix well and refrigerate for 10 minutes (4–8 °C).
6. Add additional 300  $\mu\text{L}$  of buffer per  $10^8$  cells.
7. Add 200  $\mu\text{L}$  of **NK Cell MicroBead Cocktail** per  $10^8$  cells.
8. Mix well and refrigerate for additional 15 minutes (4–8 °C).
9. Wash cells by adding 10–20 mL of buffer and centrifuge at  $300\times g$  for 10 minutes at 4–8 °C. Aspirate supernatant completely.
10. Resuspend up to  $10^8$  cells in 500  $\mu\text{L}$  of buffer.  
▲ **Note:** For larger cell numbers, scale up buffer volume accordingly.
11. Proceed to magnetic separation (2.3).



### 2.3 Magnetic separation: Depletion of non-NK cells

#### Depletion with an LS Column

1. Place LS Column in the magnetic field of a suitable MACS Separator. For details see respective MACS 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  $3\times 3$  mL of buffer. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty. Collect total effluent; this contains the pre-enriched NK cell fraction.
5. Proceed to 2.4 for the isolation of  $\text{CD}56^+\text{CD}8^+$  or  $\text{CD}56^+\text{CD}8^-$  NK 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 "Depletes".
3. Collect unlabeled fraction from outlet port neg1. This is the pre-enriched NK cell fraction.
4. Proceed to 2.4 for the isolation of  $\text{CD}56^+\text{CD}8^+$  or  $\text{CD}56^+\text{CD}8^-$  NK cells.



### 2.4 Magnetic labeling of $\text{CD}56^+\text{CD}8^+$ NK cells

▲ Volumes for magnetic labeling given below are for an initial starting cell number of up to  $10^8$  cells. For larger initial cell numbers, scale up volumes accordingly.

1. Centrifuge cells at  $300\times g$  for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 400  $\mu\text{L}$  of buffer.
3. Add 100  $\mu\text{L}$  of **CD8 MicroBeads (CM)**.
4. Mix well and refrigerate for 15 minutes (4–8 °C).  
▲ **Note:** Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.
5. Wash cells by adding 5–10 mL of buffer and centrifuge at  $300\times g$  for 10 minutes. Aspirate supernatant completely.
6. Resuspend cells in 500  $\mu\text{L}$  of buffer.
7. Proceed to magnetic separation (2.5, 2.6, or 2.7).



### 2.5 Magnetic separation: Positive selection of $\text{CD}56^+\text{CD}8^+$ NK cells

#### Positive selection with MS Column

1. Place MS Column in the magnetic field of a suitable MACS Separator. For details see respective MACS Column data sheet.
2. Prepare column by rinsing with 500  $\mu\text{L}$  of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with  $3\times 500$   $\mu\text{L}$  of buffer. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty.
5. (Optional) For isolation of  $\text{CD}56^+\text{CD}8^-$  NK cells, keep flow-through and wash fractions (2.7).
6. Remove column from the separator and place it on a suitable collection tube.
7. Pipette 1 mL of buffer onto the column. Immediately flush out the fraction with magnetically labeled cells ( $\text{CD}56^+\text{CD}8^+$  NK cells) by firmly pushing the plunger into the column.

▲ **Note:** To increase the purity of the magnetically labeled fraction pass the cells over a new, freshly prepared column.

#### Positive selection 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 "Possel".
3. Collect positive fraction from outlet port pos1. This is the enriched  $\text{CD}56^+\text{CD}8^+$  NK cell fraction.
4. (Optional) Collect negative fraction from outlet port neg 1. This fraction can be used for isolation of  $\text{CD}56^+\text{CD}8^-$  NK cells (2.7).



## 2.6 Magnetic separation: Enrichment of CD56<sup>+</sup>CD8<sup>-</sup> NK cells

### Depletion with an LD Column

1. Place an LD Column in the magnetic field of a suitable MACS Separator. For details see respective MACS Column data sheet.
2. Prepare column by rinsing with 2 mL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with 2×1 mL of buffer. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty. Collect total effluent; this contains the enriched CD56<sup>+</sup>CD8<sup>-</sup> NK cell fraction.

### 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 "Depl05".
3. Collect unlabeled fraction from outlet port neg1. This is the enriched CD56<sup>+</sup>CD8<sup>-</sup> NK cell fraction.



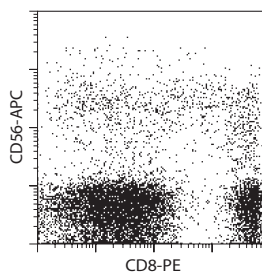
## 2.7 Magnetic separation: Isolation of CD56<sup>+</sup>CD8<sup>+</sup> and CD56<sup>+</sup>CD8<sup>-</sup> NK cells

▲ If both NK cell populations – CD56<sup>+</sup>CD8<sup>+</sup> and CD56<sup>+</sup>CD8<sup>-</sup> – are needed, first isolate the CD56<sup>+</sup>CD8<sup>+</sup> NK cells over an MS Column or using the autoMACS Separator, program "Possel" (see section 2.5). The flow-through fraction of this separation can then be passed over an LD Column or using the autoMACS Separator, program "Depl05" (see section 2.6) to additionally enrich the CD56<sup>+</sup>CD8<sup>-</sup> NK cell population.

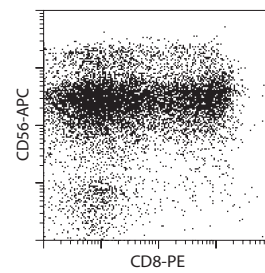
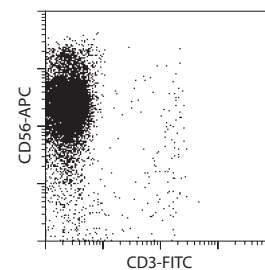
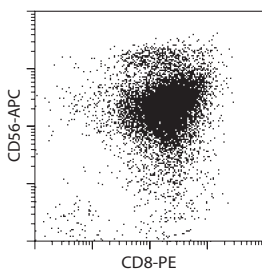
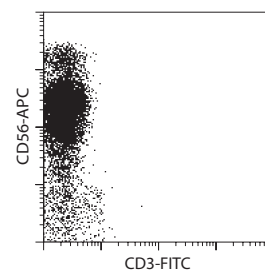
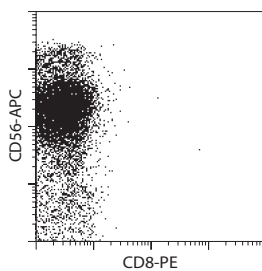
### 3. Examples of a separation using the CD56<sup>+</sup>CD8<sup>+</sup>/ CD8<sup>-</sup> NK Cell Isolation Kit

Both the CD56<sup>+</sup>CD8<sup>+</sup> and CD56<sup>+</sup>CD8<sup>-</sup> NK cell subsets were isolated from human PBMCs using the CD56<sup>+</sup>CD8<sup>+</sup>/CD8<sup>-</sup> NK Cell Isolation Kit. Non-NK cells were depleted by separation using an LS Column. The enriched NK cells were then magnetically labeled with CD8 MicroBeads (CM), and CD56<sup>+</sup>CD8<sup>+</sup> NK cells were isolated by positive selection using an MS Column. For isolation of the CD56<sup>+</sup>CD8<sup>-</sup> NK cells the wash and flow-through of the MS Column were collected and separated using an LD Column. The CD56<sup>+</sup>CD8<sup>-</sup> NK cells are finally collected in the flow-through of the LD Column. Cells are fluorescently stained with CD8-PE, CD56-APC, and CD3-FITC. Cell debris and dead cells are excluded from the analysis based on scatter signals and PI fluorescence.

PBMCs before separation



Pre-enriched NK cells after depletion of non-NK cells

Isolated CD56<sup>+</sup>CD8<sup>+</sup> NK cellsIsolated CD56<sup>+</sup>CD8<sup>-</sup> NK cells

## 4. Reference

1. Addison, E. G. *et al.* (2005) Ligation of CD8α on human natural killer cells prevents activation-induced apoptosis and enhances cytolytic activity. *Immunology* 116: 254–361.

### Warning

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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