Miltenyi Biotec

NK1.1$^+$ iNKT Cell Isolation Kit

mouse

Order no. 130-096-513

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	**2 mL NK1.1$^+$ iNKT Cell Biotin-Antibody Cocktail, mouse:** Cocktail of biotin-conjugated monoclonal anti-mouse antibodies against NKp46, CD45R, CD8a, CD115, and TCRγδ. **1 mL Anti-Biotin MicroBeads:** MicroBeads conjugated to monoclonal anti-biotin antibody (isotype: mouse IgG1). **200 µL Anti-NK1.1-APC, mouse:** Monoclonal anti-mouse NK1.1 antibody conjugated to APC (isotype: mouse IgG2a). **2 mL Anti-APC MicroBeads:** MicroBeads conjugated to monoclonal anti-mouse anti-APC antibody (isotype: mouse IgG1).
Capacity	For 10^9 total cells.
Product format	All components 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 MACS® Separation

The isolation of NK1.1$^+$ iNKT cells is performed in a two-step procedure. First, the non-NK1.1$^+$ iNKT cells are labeled with a cocktail of biotin-conjugated antibodies, Anti-Biotin MicroBeads. The labeled cells are subsequently depleted by separation over a MACS® Column, which is placed in the magnetic field of a MACS Separator.

In the second step, the NK1.1$^+$ iNKT cells are labeled with Anti-NK1.1-APC and Anti-APC MicroBeads and isolated by positive selection from the pre-enriched cell fraction by separation over a MACS Column, which is placed in the magnetic field of a MACS Separator.

After removing the column from the magnetic field, the NK1.1$^+$ iNKT cells can be eluted as the positively selected cell fraction. To increase the purity, the positively selected cell fraction containing the NK1.1$^+$ iNKT cells must be separated over a second column.

Mouse spleen: Depletion of non-NK1.1$^+$ iNKT cells

1. Labeling of non-NK1.1$^+$ iNKT cells with NK1.1$^+$ iNKT Cell Biotin-Antibody Cocktail and Anti-Biotin MicroBeads.
2. Magnetic separation using an LD Column or an autoMACS Column (program "Depl05").

Pre-enriched NK1.1$^+$ iNKT cells (flow-through fraction): Positive selection of NK1.1$^+$ iNKT cells

1. Labeling of NK1.1$^+$ iNKT cells with Anti-NK1.1-APC and Anti-APC MicroBeads.
2. Magnetic separation using two MS Columns or an autoMACS Column (program "Posseld2").

NK1.1$^+$ iNKT cells

1.2 Background information

Natural killer T (NKT) cells are a heterogeneous group of lymphocytes that share properties of both T cells and natural killer (NK) cells. Many of these cells recognize the non-polymorphic CD1d molecule, an antigen-presenting molecule that binds self- and foreign lipids and glycolipids such as the synthetic ligand α-galactosylceramide (α-GalCer).

The term NKT cells was first used in mice to define a subset of T cells that also express the natural killer cell–associated marker NK1.1 (CD161), although NK1.1 is only expressed by the mouse strain C57BL/6. In general, the term iNKT cells refers preferentially to CD1d-restricted T cells, expressing a heavily biased, semi-invariant T cell receptor (TCR Vα14-Jα18, Vß8.2, Vß7, and Vß2) and NK cell markers.

Upon activation, iNKT cells are able to produce large quantities of IFN-γ, IL-4, and GMCSF, as well as multiple other cytokines and chemokines from TH1- and TH2-type. They are an important link between innate and adaptive immune system, promoting or suppressing immune responses.[1–3]

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1.3 Applications

- Isolation of NK1.1-expressing mouse NKT cells after depletion of unwanted cells like NK cells, B cells, macrophages, CD8$^+$, and TCRγδ$^+$ T cells.

1.4 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 (2–8 °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 mouse serum albumin, mouse serum, or fetal bovine serum (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- MACS Columns and MACS Separators: Depletion of non-target cells can be performed on an LD Column. The subsequent positive selection of NK1.1$^+$ iNKT cells can be performed on two MS Columns. Positive selection and depletion can also be performed by using the autoMACS Pro Separator.

Column	Max. number of labeled cells	Max. number of total cells	Separator
Positive selection			
MS	10^7	2×10^8	MiniMACS, OctoMACS, VarioMACS, SuperMACS II
LS	10^8	2×10^9	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II
XS	10^9	2×10^{10}	SuperMACS II
Depletion			
LD	10^8	5×10^8	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II
CS	2×10^8		VarioMACS, SuperMACS II
D	10^9		SuperMACS II
Positive selection or depletion			
autoMACS	2×10^8	4×10^9	autoMACS Pro

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

- (Optional) Fluorochrome-conjugated antibodies for flow cytometric analysis, e.g., CD3ε-VioBlue or Anti-NKp46-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) Dead Cell Removal Kit (# 130-090-101) for the depletion of dead cells.
- (Optional) Pre-Separation Filters (30 µm) (# 130-041-407) to remove cell clumps.

2. Protocol

2.1 Sample preparation

When working with lymphoid organs, non-lymphoid tissues, or peripheral blood, prepare a single-cell suspension using manual methods or the gentleMACS™ Dissociator.

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

▲ Dead cells may bind non-specifically to MACS MicroBeads. To remove dead cells, we recommend using density gradient centrifugation or the Dead Cell Removal Kit (# 130-090-101).

2.2 Magnetic labeling of non-NK1.1$^+$ iNKT 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 10^7 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.

▲ The recommended incubation temperature is 2–8 °C. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

1. Determine cell number.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 80 µL of buffer per 10^7 total cells.
4. Add 20 µL of NK1.1$^+$ iNKT Cell Biotin-Antibody Cocktail per 10^7 total cells.
5. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).
6. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend cell pellet in 90 µL of buffer per 10^7 total cells.
8. Add 10 µL of Anti-Biotin MicroBeads.
9. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
10. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
11. Resuspend up to 10^8 cells in 500 µL of buffer.

    ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.

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2.3 Magnetic separation: Depletion of non-target cells

▲ Choose an appropriate MACS® Column and MACS Separator according to the number of total cells and the number of target cells. For details refer to table in section 1.4.

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

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details refer to LD 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. Collect total effluent; this is the unlabeled pre-enriched NK1.1$^+$ iNKT cell fraction. Perform washing steps by adding buffer two times. Only add new buffer when the column reservoir is empty.
5. Proceed to 2.4 for the labeling of NK1.1$^+$ iNKT cells.

Depletion with the autoMACS® Pro Separator

▲ Refer to the respective user manual for instructions on how to use the autoMACS® Pro Separator.

▲ Buffers used for operating the autoMACS Pro Separator should have a temperature of ≥10 °C.

1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample and collection tubes into the Chill Rack.
3. For a standard separation choose the following program:

   Depletion: Depl05
   Collect negative fraction in row B of the tube rack.
4. Proceed to 2.4 for the labeling of NK1.1$^+$ iNKT cells.

2.4 Magnetic labeling of NK1.1$^+$ cells

▲ Volumes for magnetic labeling given below are for an initial starting cell number of up to 10^7 total cells. For higher initial cell numbers, scale up all volumes accordingly.

1. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 98 µL of buffer.
3. Add 2 µL of Anti-NK1.1-APC.
4. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).
5. Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
6. Resuspend cell pellet in 80 µL of buffer.
7. Add 20 µL of Anti-APC MicroBeads.
8. Mix well and incubate for an additional 15 minutes in the dark in the refrigerator (2–8 °C).
9. Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
10. Resuspend up to 10^8 cells in 500 µL of buffer.

    ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
11. Proceed to magnetic separation (2.5).

2.5 Magnetic separation: Positive selection of NK1.1$^+$ iNKT cells

Positive selection with MS Columns

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

1. Place MS Column in the magnetic field of a suitable MACS Separator. For details refer to MS Column data sheet.
2. Prepare column by rinsing with 500 µL of buffer.
3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
4. Wash column with 3×500 µL of buffer. Collect unlabeled cells that pass through and combine with the effluent from step 3.

   ▲ **Note:** Perform washing steps by adding buffer aliquots only when 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 magnetically labeled cells by firmly pushing the plunger into the column.
7. To increase the purity of NK1.1$^+$ iNKT cells, the eluted fraction must be enriched over a second MS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.

Positive selection with the autoMACS® Pro Separator

1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample and collection tubes into the Chill Rack.
3. For a standard separation choose the following program:

   Positive selection: Posseld2
   Collect positive fraction in row C of the tube rack. This is the enriched target cell fraction.

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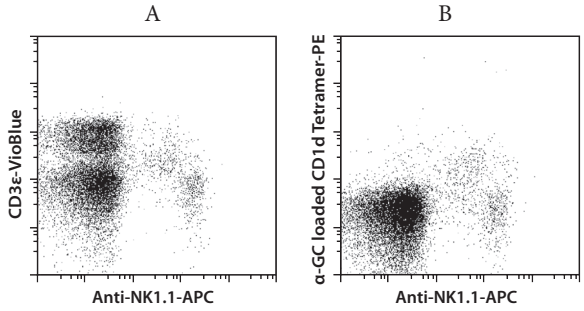
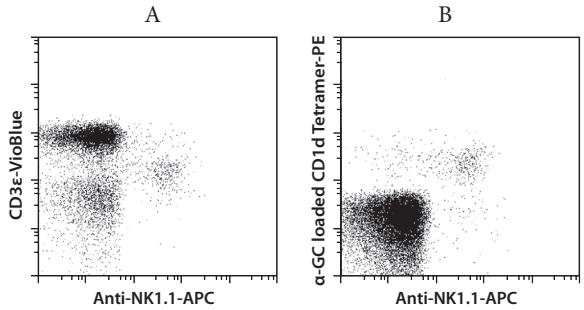
3. Example of a separation using the NK1.1$^+$ iNKT Cell Isolation Kit

Mouse spleen cells from a C57BL/6 mouse were isolated by using the NK1.1$^+$ iNKT Cell Isolation Kit, an LD and two MS Columns, a MidiMACS™ and a MiniMACS™ Separator. The cells were fluorescently stained with Anti-NK1.1-APC, CD3ε-VioBlue®, Anti-NKp46-FITC (A), and α-GC loaded Tetramer-PE (B) and analyzed by flow cytometry using the MACSQuant® Analyzer. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.

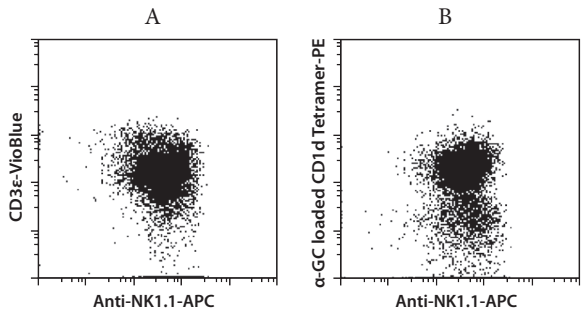
Before separation



After depletion



After enrichment



4. References

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Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com/local to find your nearest Miltenyi Biotec contact.

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