Isolation of iNKT cells from mouse spleen using CD1d tetramers

Contents

1. Description

1.1 Principle of the MACS® Separation

Isolation of mouse iNKT cells is performed by using a PE-conjugated, α-GalCer–loaded CD1d tetramer. The tetrameric CD1d/α-GalCer complexes bind to the TCR of iNKT cells. Subsequently, the iNKT cells can be isolated by using Anti-PE MicroBeads.
Because of unspecific binding of the tetramer to B cells, B cells are depleted prior to iNKT cell enrichment by using CD45R (B220) MicroBeads.

1.2 Background information

Natural killer T (NKT) cells represent a distinct lymphocyte population that co-expresses T cell and NK cell surface markers. A subset of mouse NKT cells, referred to as invariant NKT (iNKT) cells, express an invariant TCR α-chain with a restricted set of TCR β-chains (Vα14-Jα18 combined with Vβ2, Vβ7, or Vβ8.2). iNKT cells are implicated in immunoregulatory processes, such as tolerance, host defense, and tumor surveillance. Unlike MHC class I– and II–restricted T cells, they are restricted to antigen presentation by CD1d molecules. A glycosphingolipid, α-Galactosylceramide (α-GalCer), has been identified as a specific and strong ligand for this invariant TCR.

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 (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. Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- MACS Columns and MACS Separators: Depletion of B cells is performed on an LD Column. The subsequent positive selection of iNKT cells is performed on an MS Column. Depletion and positive selection can also be performed by using the autoMACS or the autoMACS Pro Separator.

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

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

- CD45R (B220) MicroBeads, mouse (# 130-049-501).
- PE-conjugated CD1d tetramer (ProImmune Ltd.), loaded with α-GalCer according to the manufacturer´s recommendations.
- Anti-PE MicroBeads (# 130-048-801).
- FcR Blocking Reagent, mouse (# 130-092-575) to avoid Fc receptor–mediated antibody labeling.
- Fluorochrome-conjugated CD3ε antibody for flow cytometric analysis, e.g., CD3ε-FITC (# 130-092-962). For more information about other fluorochrome conjugates see www.miltenyibiotec.com.
- Propidium Iodide Solution (# 130-093-233).

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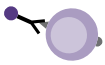
- (Optional) Dead Cell Removal Kit (# 130-090-101) for the depletion of dead cells.
- (Optional) Pre-Separation Filters (# 130-041-407) to remove cell clumps.
- (Optional) PE-conjugated CD1d tetramer, empty, as a negative control.
- (Optional) Anti-NK1.1 antibody and CD45R (B220) antibody for separation control staining, for example, CD45R (B220)-VioBlue (# 130-094-287).

2. Protocol

2.1 Sample preparation

When working with tissues, prepare a single-cell suspension using standard methods. For details see the General Protocols section of the respective separator user manual. The General Protocols are also available at www.miltenyibiotec.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 B 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 separation. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove cell clumps which may clog the column. Wet filter with buffer before use.

▲ The recommended incubation temperature is 2–8 °C. 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×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 90 µL of buffer per 10^7 total cells.
4. Add 10 µL of CD45R (B220) MicroBeads per 10^7 total cells.
5. Mix well and incubate for 15 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 up to 10^8 cells in 500 µL of buffer.
   ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
8. Proceed to magnetic separation (2.3).

2.3 Magnetic separation: Depletion of B cells

Depletion with LD Columns

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details see 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 B cell–depleted fraction. Perform washing steps by adding buffer two times. Only add new buffer when the column reservoir is empty.
5. (Optional) To elute the magnetically labeled $CD45R^+$ cells, remove column from the separator and elute the cells by adding 2×1 mL of buffer.
6. Proceed to 2.4 for the isolation of iNKT cells.

Depletion with the autoMACS™ Separator or the autoMACS™ Pro Separator

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

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

Depletion with the autoMACS™ 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 tube at the uptake port and the fraction collection tubes at port neg1 and port pos1.
3. Choose the following program:
   "Depl025"
   Collect negative fraction from outlet port neg1.
4. Proceed to 2.4 for the isolation of iNKT cells.

Depletion 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 tube in row A of the tube rack and the fraction collection tubes in rows B and C.
3. Choose the following program:
   "Depl025"
   Collect negative fraction in row B of the tube rack.
4. Proceed to 2.4 for the isolation of iNKT cells.

2.4 Labeling of iNKT cells

▲ Volumes given below are for an initial starting cell number of up to 10^7 cells.

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

1. Centrifuge the B cell–depleted fraction at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cells in 45 µL of buffer.
3. Add 5 µL of FcR Blocking Reagent.
4. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).
5. Add 5 µL of the α-GalCer–loaded CD1d tetramer.
6. Mix well and incubate for 20 minutes in the dark in the refrigerator (2–8 °C).
7. Add 5 µL of CD3ε-FITC.

   (Optional) Add other staining antibodies, for example, Anti-NK1.1 according to the manufacturer´s recommendations.
8. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
9. Wash cells by adding 1 mL of buffer per 10^7 cells and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
10. Repeat step 9.
11. Resuspend cell pellet in 80 µL of buffer.
12. Add 20 µL of Anti-PE MicroBeads.
13. Mix well and incubate for 15 minutes in the dark in the refrigerator (2–8 °C).
14. Wash cells by adding 1 mL of buffer per 10^7 cells and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
15. Resuspend cell pellet in 500 µL of buffer.

2.5 Magnetic separation: Positive selection of tetramer$^+$ iNKT cells

Positive selection with two 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 see the MS Column data sheet.
2. Prepare column by rinsing with 500 µL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with 3×500 µL of buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer three times. Only add new buffer 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 purity of iNKT cells, the eluted fraction should 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™ Separator or the autoMACS™ Pro Separator

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

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

Positive selection with the autoMACS™ 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 tube at the uptake port and the fraction collection tubes at port neg1 and port pos1.
3. Choose the following program:

   "Posseld2"
   Collect positive fraction from outlet port pos1.

Positive selection with the autoMACS™ Pro Separator

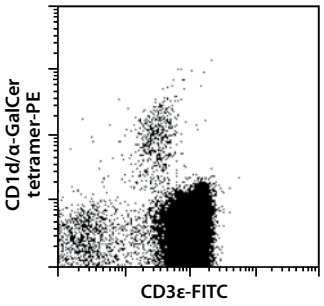
1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.
3. Choose the following program:

   "Posseld2"
   Collect positive fraction in row C of the tube rack.

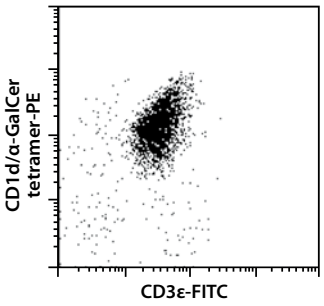
3. Example of an isolation of iNKT cells from mouse spleen using CD1d tetramers

B cells were depleted from mouse splenocytes using CD45R (B220) MicroBeads, an LD Column, and a MidiMACS™ Separator. The subsequent isolation of iNKT cells was performed using a PE-conjugated CD1d tetramer loaded with α-GalCer, Anti-PE MicroBeads, two MS Columns, and a MiniMACS™ Separator. Cells are additionally stained with CD3ε-FITC (# 130-092-962). Cell debris and dead cells are excluded from the analysis based on scatter signals and propidium iodide fluorescence.

Mouse splenocytes before separation



Isolated iNKT cells



4. Reference

1. Godfrey, D. I. *et al.* (2004) NKT cells: what's in a name? Nat. Rev. Immunol. 4: 231–237.

All protocols and data sheets are available at www.miltenyibiotec.com.

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.

Legal Notices

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