Miltenyi Biotec

Enrichment of CD134 (OX40)$^+$ cells
from activated human PBMCs

Contents

1. Description

1.1 Principle of the MACS® Separation

First, the CD134 (OX40)$^+$ cells are magnetically labeled with CD134 (OX40)-PE and Anti-PE MicroBeads. Then, the cell suspension is loaded onto a MACS® Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled CD134 (OX40)$^+$ cells are retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of CD134 (OX40)$^+$ cells. After removing the column from the magnetic field, the magnetically retained CD134 (OX40)$^+$ cells can be eluted as the positively selected cell fraction.

1.2 Background information

CD134 (OX40) is a member of the tumor necrosis factor/nerve growth factor receptor (TNFR/NGFR) family. CD134 (OX40) is a 50 kDa type I membrane glycoprotein expressed by activated T lymphocytes. The interaction of CD134 (OX40) with OX40L has been implicated in T cell-dependent humoral response, regulation of primary T cell expansion, survival of T cells, size of the memory T cell pool, and regulation of tolerance in the CD4$^+$ T cell compartment.[1]

1.3 Applications

- Enrichment of CD134 (OX40)$^+$ cells from acivated human peripheral blood mononuclear cells (PBMCs).
- Detection of antigen-specific CD4$^+$ T cells.[2]

1.4 Reagent and instrument requirements

- CD134 (OX40)-PE, human (# 130-095-270).
- Anti-PE MicroBeads (# 130-048-801).
- CytoStim, human (# 130-092-172 or # 130-092-173).
- 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 human serum albumin, human 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: CD134 (OX40)$^+$ cells can be enriched by using MS, LS, or XS Columns. Positive selection or depletion can also be performed by using the autoMACS Pro or the autoMACS 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
autoMACS	2×10^8	4×10^9	autoMACS Pro, autoMACS

▲ **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., CD4-FITC (# 130-080-501), CD4 (VIT4)-FITC (# 130-092-358), CD4-APC (# 130-091-232), CD25-APC (# 130-092-858), or CD154-APC (# 130-092-290). For more information about antibodies refer to www.miltenyibiotec.com.
- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD 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 (# 130-041-407) to remove cell clumps.

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, for example, using Ficoll-Paque™.

▲ **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.

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Miltenyi Biotec GmbH
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macs@miltenyibiotec.de
www.miltenyibiotec.com

Miltenyi Biotec Inc.
2303 Lindbergh Street, Auburn, CA 95602, USA
Phone 800 FOR MACS, +1 530 888 8871, Fax +1 530 888 8925
macs@miltenyibiotec.com

For details see the protocols section 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).

2.1.1 Protocol for *in vitro* stimulation for induction of CD134 (OX40) expression

▲ Always include a negative control in the experiment. The sample should be treated exactly the same way as the stimulated sample, except for the addition of the stimulus.

▲ A positive control may also be included in the experiment, such as a sample stimulated with CytoStim (# 130-092-172).

▲ Do not use media containing any non-human proteins, such as BSA or FCS, because of non-specific stimulation.

1. Wash cells by adding cell culture medium, centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cells at a density of 10^7 cells/mL in culture medium containing 5% human serum. Plate cells in dishes at a density of 5×10^6 cells/cm².
3. Add an antigen or control reagent in the appropriate concentration, for example, PepTivator – CMV pp65 (# 130-093-435, # 130-093-438).
4. Incubate cells overnight with antigen and an appropriate control, e.g., CytoStim, at 37 °C and 5% CO_2.
5. Collect cells carefully by pipetting up and down when working with smaller volumes. Rinse the dish with cold buffer. Check microscopically for any remaining cells. If necessary, rinse the dish again.

2.2 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.

▲ 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, # 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 100 µL of buffer per 10^7 total cells.
4. Add 10 µL of CD134 (OX40)-PE 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.
7. Aspirate supernatant completely and add 80 µL of buffer per 10^7 total cells.
8. Add 20 µL of Anti-PE MicroBeads per 10^7 total cells.
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.

    ▲ **Note:** For depletion with LD Columns, resuspend up to 1.25×10^8 cells in 500 µL of buffer.
12. Proceed to magnetic separation (2.3).

2.3 Magnetic separation

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of CD134 $(OX40)^+$ cells. For details refer to the table in section 1.4.

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

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 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 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 only when the column reservoir is empty.
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette the appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

   MS: 1 mL LS: 5 mL
7. (Optional) To increase the purity of CD134 $(OX40)^+$ cells, the eluted fraction can be enriched over a second MS or LS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.

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Magnetic separation with the autoMACS® Pro Separator or the autoMACS® Separator

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

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

▲ Program choice depends on the isolation strategy, the strength of magnetic labeling, and the frequency of magnetically labeled cells. For details refer to the section describing the cell separation programs in the respective user manual.

Magnetic separation 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. For a standard separation choose the following program:

   Positive selection: possel
   Collect positive fraction in row C of the tube rack.

Magnetic separation with the autoMACS® Separator

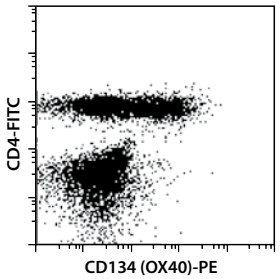
1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube at the uptake port and the fraction collection tubes at port neg1 and port pos1.
3. For a standard separation choose the following program:

   Positive selection: possel
   Collect positive fraction from outlet port pos1.

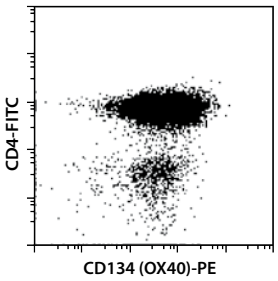
3. Example of a separation using CD134 (OX40)-PE and Anti-PE MicroBeads

CD134 (OX40)$^+$ cells were isolated from activated human PBMCs using CD134 (OX40)-PE, Anti-PE MicroBeads, an MS Column, and a MiniMACS™ Separator. Cells were fluorescently stained with CD134 (OX40)-PE (# 130-095-270) and CD4-FITC (# 130-080-501) 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



Isolated CD134 (OX40)$^+$ cells



4. References

1. Croft, M *et al.* (2009) The significance of OX40 and OX40L to T-cell biology and immune disease. Immunol. Rev. 229: 173–191.
2. Zaunders *et al.* (2009) High Levels of Human Antigen-Specific CD4$^+$ T Cells in Peripheral Blood Revealed by Stimulated Coexpression of CD25 and CD134 (OX40). J. Immunol. 183: 2827–2836.

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.

Warranty

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