Miltenyi Biotec

CD154 Enrichment and Detection Kit (PE)

mouse

Order no. 130-093-129

Contents

1. **Description**
   - 1.1 **Principle of the MACS® Separation**
   - 1.2 **Background information**
   - 1.3 **Applications**
   - 1.4 **Reagent and instrument requirements**
2. **Protocol**
   - 2.1 **Sample preparation**
   - 2.2 ***In vitro* stimulation of antigen-specific T cells and cell surface staining**
   - 2.3 **Magnetic labeling**
   - 2.4 **Magnetic separation**
3. **Example of a separation using the CD154 Enrichment and Detection Kit (PE)**
4. **References**

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 CD154 Detection Cocktail (PE), mouse:** Cocktail of antibodies containing CD154-PE, CD40 antibody, and CD28 antibody. **2 mL Anti-PE MicroBeads UltraPure:** UltraPure MicroBeads conjugated to monoclonal anti-PE antibodies (isotype: mouse IgG1).
Capacity	For 10^9 total cells, up to 100 separations.
Product format	The antibody cocktail is supplied in phosphate-buffered saline (PBS) pH 7.2. Low endotoxin. Anti-PE MicroBeads UltraPure 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 CD154 Enrichment and Detection Kit (PE) is designed for easy enrichment and enumeration of antigen-specific T cells. Detection and stimulation is conveniently done in one step. Using the CD154 Enrichment and Detection Kit (PE), mouse antigen-specific $CD4^+$ T cells are indirectly magnetically labeled with a PE-conjugated CD154 antibody as primary labeling reagent, and an anti-PE monoclonal antibody conjugated to MicroBeads, as secondary labeling reagent. To optimize the detection of CD154 on activated antigen-specific $CD4^+$ T cells, cells are stimulated with the desired antigen *in vitro* to induce the expression of CD154. Simultaneously, the cells are incubated with the CD154 Detection Cocktail (PE). Besides a PE-conjugated CD154 antibody, the detection cocktail contains a CD40 blocking antibody to prevent the down-regulation of CD154 surface expression, and a CD28 antibody to provide a costimulatory signal. After stimulation, cells are magnetically labeled with Anti-PE MicroBeads UltraPure. Then, the cell suspension is loaded onto a MACS® Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled $CD154^+$ T cells are retained within the column while the unlabeled cells run through. After removing the column from the magnetic field, the magnetically retained $CD154^+$ T cells can be eluted as the positively selected cell fraction.

1.2 Background information

The CD154 antibody used in the detection cocktail specifically recognizes the mouse CD154 antigen. CD154 is a 39 kDa transmembrane glycoprotein, also known as CD40L, gp39, T-BAM, and TRAP. It is transiently up-regulated on activated $CD4^+$ T cells and to a very low extent on activated $CD8^+$ T cells. CD154 plays an important role as a costimulatory molecule in the interaction between T cells and antigen-presenting cells through ligation of CD40. Because of its transient expression on $CD4^+$ T cells within hours of activation, CD154 can be used as a marker for activated antigen-specific $CD4^+$ T helper cells[1-3].

To stabilize the surface expression of CD154 during stimulation of antigen-specific T cells, the CD154 Detection Cocktail (PE) contains a CD40 blocking antibody that prevents the down-regulation of CD154 expression induced by interaction with CD40 expressed on antigen-presenting cells. The CD28 antibody included in the cocktail provides the costimulatory signal for $CD4^+$ T cells and optimizes the induction of CD154 expression, thus improving the enrichment and detection of $CD154^+$ T cells. The detection cocktail is optimized to be conveniently added to the cell suspension during stimulation.

1.3 Applications

- Enrichment of stimulated $CD154^+$ antigen-specific $CD4^+$ T cells from single-cell suspensions of lymphoid tissues.
- Detection and enrichment of $CD154^+$ antigen-specific $CD4^+$ T cells for enumeration, expansion, and phenotypic as well as functional characterization.

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

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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: $CD154^+$ T cells can be enriched by using MS or LS Columns (positive selection). Positive selection can also be performed by using the autoMACS Pro Separator 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.

- Cell culture medium, e.g., RPMI 1640 (# 130-091-440) supplemented with 5% mouse serum.
- (Optional) Fluorochrome-conjugated CD4 and CD8a antibodies for flow cytometric analysis, e.g., CD4-FITC (# 130-102-541), CD8a-APC (# 130-102-490), and CD45R (B220)-APC (# 130-102-228). For more information about antibodies refer to www.miltenyibiotec.com/antibodies.
- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD for flow cytometric exclusion of dead cells.
- (Optional) Pre-Separation Filters, 30 µm (# 130-041-407) to remove cell clumps.

2. Protocol

2.1 Sample preparation

Prepare a single-cell suspension from lymphoid organs using a standard preparation method.

For details refer to the protocols section at www.miltenyibiotec.com/protocols.

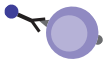
2.2 Protocol for *in vitro* stimulation of antigen-specific T cells and cell surface staining

▲ Always include a negative control in the experiment. The sample for the negative control 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, for example, a sample stimulated with *Staphylococcus* enterotoxin B (SEB).

▲ Do not use media containing any non-mouse proteins, for example, BSA or FBS, because of non-specific stimulation.

1. Determine cell number.
2. Wash cells by adding cell culture medium, centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cells at a density of 10^7 cells/mL in culture medium, containing 5% mouse serum. Plate cells at a density of 5×10^6 cells/cm².
4. Add 20 µL of CD154 Detection Cocktail (PE) per 1 mL of cell suspension.
5. Add an antigen or control reagent at the appropriate concentration.
6. Incubate cells for 6–14 hours at 37 °C and 5% CO_2.
7. Collect cells carefully by pipetting up and down. Rinse the dish with cold buffer. Check microscopically for any remaining cells, if necessary rinse the dish again.
8. Wash cells by adding buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
9. Proceed to magnetic labeling (2.3).



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, 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. Resuspend cell pellet in 80 µL of buffer per 10^7 total cells.
2. Add 20 µL of Anti-PE MicroBeads UltraPure per 10^7 total cells.
3. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
4. (Optional) Add staining antibodies, e.g., 10 µL of CD4-FITC (# 130-102-541), and incubate for 5 minutes in the dark in the refrigerator (2–8 °C).
5. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.

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6. Resuspend up to 10^8 cells in 500 µL of buffer.

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

7. (Optional) Take an aliquot of the labeled cells for flow cytometric analysis and cell count before enrichment.

8. Proceed to magnetic separation (2.4).

2.4 Magnetic separation

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of $CD154^+$ T 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

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

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 as soon as 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. To increase purity of $CD154^+$ 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.

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.

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: Posseld2
   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 pos2.

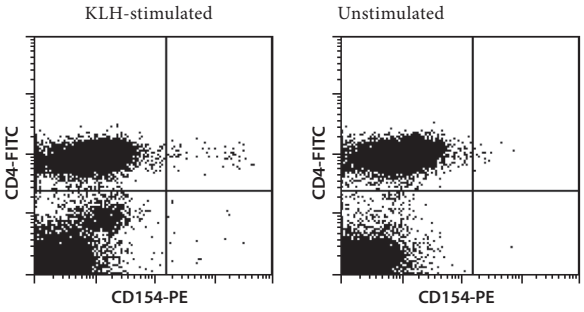
3. For a standard separation choose the following program:

   Positive selection: Posseld2
   Collect positive fraction from port pos2.

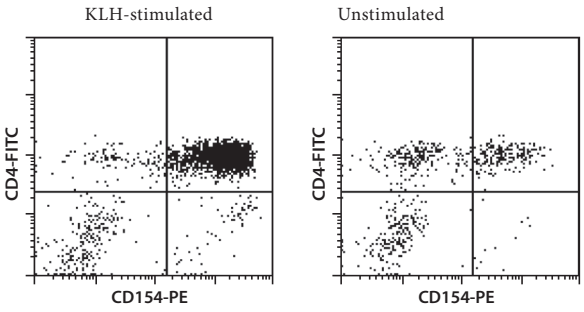
3. Example of a separation using the CD154 Enrichment and Detection Kit (PE)

Mouse spleen cells have been separated using the CD154 Enrichment and Detection Kit (PE), two MS Columns, and a MiniMACS™ Separator. Spleen cells from a BALB/c mouse immunized with keyhole limpet hemocyanin (KLH) were either restimulated *in vitro* with 100 µg/mL KLH or were cultured without an antigen in the presence of the CD154 Detection Cocktail (PE) overnight.
Afterwards, $CD154^+$ T cells were magnetically isolated using Anti-PE MicroBeads UltraPure. Cells were fluorescently stained with CD4-FITC (# 130-102-541) and CD45R (B220)-PerCP (# 130-102-213). Dead cells and B cells were excluded from the analysis according to propidium iodide and CD45R (B220)-PerCP fluorescence.

Before separation



After separation



4. References

1. Frentsch, M. *et al.* (2005) Direct access to $CD4^+$ T cells specific for defined antigens according to CD154 expression. Nat. Med. 11: 1118–1124.
2. Chattopadhyay, P. K. *et al.* (2005) A live-cell assay to detect antigen-specific $CD4^+$ T cells with diverse cytokine profiles. Nat. Med. 11: 1113–1117.
3. Kirchhoff, D. *et al.* (2007) Identification and isolation of murine antigen-reactive T cells according to CD154 expression. Eur. J. Immunol. 37: 2370–2377.

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