Miltenyi Biotec

CD154 MicroBead Kit
human

Order no. 130-092-658

Contents

1. Description
   1.1 Principle of the MACS® Separation
   1.2 Background information
   1.3 Applications
   1.4 Reagent and instrument requirements
2. Protocols
   2.1 Sample preparation
      2.1.1 *In vitro* stimulation for induction of CD154 expression
   2.2 Magnetic labeling
   2.3 Magnetic separation
3. Example of a separation using the CD154 MicroBead Kit
4. Reference
5. Appendix: Flask and dish sizes for *in vitro* stimulation of T cells

1. Description

Components **1 mL CD154-Biotin, human:** Monoclonal anti-human CD154 antibody conjugated to biotin (clone: 5C8, isotype: mouse IgG2a).

2 mL Anti-Biotin MicroBeads: MicroBeads conjugated to monoclonal anti-biotin antibody (isotype: mouse IgG1).

Capacity For 10^9 total cells, up to 100 separations.

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.

The CD154 antibody has been tested to cross-react with rhesus monkey (*Macaca mulatta*) and cynomolgus monkey (*Macaca fascicularis*).

1.1 Principle of the MACS® Separation

First, the CD154$^+$ cells are magnetically labeled with CD154-Biotin antibody and Anti-Biotin 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 CD154$^+$ cells are retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of CD154$^+$ cells. After removing the column from the magnetic field, the magnetically retained CD154$^+$ cells can be eluted as the positively selected cell fraction. To increase the purity, the positively selected cell fraction containing the CD154$^+$ cells is separated over a second column.

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1.2 Background information

The antibody specifically recognizes the human CD154 antigen, a 39 kDa transmembrane glycoprotein also known as CD40L, gp39, T-BAM, TRAP, or Ly-62. CD154 is transiently up-regulated on activated CD4$^+$ T cells and plays an important role as a costimulatory molecule in T cell/antigen-presenting cell interactions through ligation of CD40. Clone 5C8 has been shown to block the activation of antigen-presenting cells by T helper cells *in vitro*. Due to its transient expression within hours after activation, CD154 can be used as a marker for activated antigen-specific CD4$^+$ T cells.[1] Adding a CD40-blocking antibody during the stimulation of cell suspensions prevents down-regulation of CD154 expression induced by interaction with CD40 expressed on antigen-presenting cells. Blocking of CD40 is not required if a pure population of enriched T cells is used.

1.3 Applications

- Positive selection of activated CD154$^+$ antigen-specific CD4$^+$ T cells.
- Isolation of CD154$^+$ cells for phenotypical and 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 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: CD154$^+$ cells can be enriched by using MS, or LS Columns (positive selection). Positive selection 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
LS	10^8	2×10^9	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
autoMACS	2×10^8	4×10^9	autoMACS Pro, 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.

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- (Optional) Fluorochrome-conjugated antibodies for flow cytometric analysis, e.g., CD4-FITC (# 130-080-501) and Anti-Biotin-PE (# 130-090-756). For more information about fluorochrome-conjugated antibodies see 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.
- (Optional) CMV pp65 – Recombinant Protein, human (# 130-091-823) for stimulation of T cells.
- (Optional) CytoStim (# 130-092-172, # 130-092-172).
- (Optional) CD40 pure – functional grade (# 130-094-133) antibody to block down-regulation of CD154-expression (see 2.1.1).

2. Protocols

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.

When working with tissues or lysed blood, prepare a single-cell suspension using standard methods.

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).

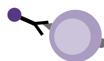
2.1.1 *In vitro* stimulation for induction of CD154 expression

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

▲ A positive control should also be included in the experiment, for example, a sample stimulated with CytoStim (# 130-092-172,# 130-092-173).

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

1. Wash cells by adding medium and centrifuge at 300×g for 10 minutes. Aspirate supernatant.
2. Resuspend cells at a density of 10^7 cells per mL in culture medium containing 5% human serum. Plate cells in dishes at a density of 5×10^6 cells/cm². For details see 5. Appendix: Flask and dish sizes for in vitro stimulation of T cells.
3. Add an antigen or control reagent in the appropriate concentration.
4. (Optional) Add 1 µg/mL of CD40 pure – functional grade (# 130-094-133) to the cell suspension.
   ▲ **Note:** The addition of a CD40-blocking antibody prevents the down-regulation of CD154 expression on T cells induced by interaction with CD40 on antigen-presenting cells.
5. Incubate cells for 4–16 hours at 37 °C and 5% CO_2.
   ▲ **Note:** CD154 is transiently expressed on activated $CD4^+$ T cells. The highest levels are detected 4–16 hours after *in vitro* stimulation. Therefore, staining with CD154 antibodies should be performed immediately after stimulation.
6. 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. 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 40 µL of buffer per 10^7 total cells.
4. Add 10 µL of CD154-Biotin per 10^7 total cells.
5. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
6. Wash cells by adding 0.5–1 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend cell pellet in 80 µL of buffer per 10^7 total cells.
8. Add 20 µL of Anti-Biotin 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.
12. Proceed to magnetic separation (2.3).

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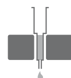

2.3 Magnetic separation

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of $CD154^+$ cells. For details see 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 see 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 effluent 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.
   ▲ **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 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, enrich the eluted fraction 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.

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

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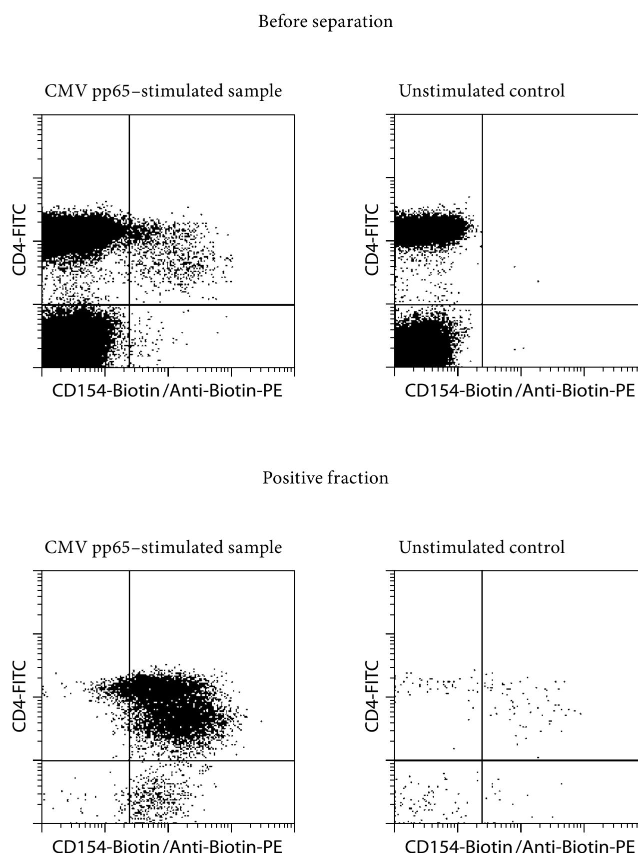
Magnetic separation with the autoMACS® Separator

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 one of the following programs:
   Positive selection: "Posseld2"
   Collect positive fraction from outlet port pos2.

3. Example of a separation using the CD154 MicroBead Kit

$CD154^+$ T cells were isolated from human PBMCs of a CMV^+ donor using the CD154 MicroBead Kit, two MS Columns, and a MiniMACS™ Separator.
PBMCs were stimulated for 16 hours with CMV pp65 – Recombinant Protein (# 130-091-823), a CD40 blocking-antibody was added during the stimulation to prevent down-regulation of CD154. Subsequently, $CD154^+$ cells were separated using the CD154 MicroBead Kit. Cells were fluorescently stained with Anti-Biotin-PE (# 130-090-756) and CD4-FITC (# 130-080-501) to detect $CD154^+$ cells. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.



4. Reference

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.

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5. Appendix: Flask and dish sizes for *in vitro* stimulation of T cells

For *in vitro* stimulation of T cells, the cells should be resuspended in culture medium, containing 5% of human serum, at a dilution of 10^7 cells/mL. The cells should be plated at a density of 5×10^6 cells/cm². Both the dilution and the cell density are important to assure optimum stimulation.

The following table lists culture plate, dish and flask sizes suitable for different cell numbers. It also indicates the appropriate amount of medium to add.

Total cell number	Medium volume to add	Culture plate	Well diameter
0.15×10^7	0.15 mL	96 well	0.64 cm
0.50×10^7	0.50 mL	48 well	1.13 cm
1.00×10^7	1.00 mL	24 well	1.60 cm
2.00×10^7	2.00 mL	12 well	2.26 cm
5.00×10^7	5.00 mL	6 well	3.50 cm
Total cell number	**Medium volume to add**	**Culture dish**	**Dish diameter**
4.5×10^7	4.5 mL	small	3.5 cm
10.0×10^7	10.0 mL	medium	6 cm
25.0×10^7	25.0 mL	large	10 cm
50.0×10^7	50.0 mL	extra large	15 cm
Total cell number	**Medium volume to add**	**Culture flask**	**Growth area**
12×10^7	12 mL	50 mL	25 cm²
40×10^7	40 mL	250 mL	75 cm²
80×10^7	80 mL	720 mL	162 cm²
120×10^7	120 mL	900 mL	225 cm²

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.

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