Miltenyi Biotec

CD154 MicroBead Kit
human

Order no. 130-092-658

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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	**1 mL CD154-Biotin, human:** Monoclonal anti-human CD154 antibody conjugated to biotin (clone: 5C8, isotype: mouse IgG2a). **2 mL Anti-Biotin MicroBeads UltraPure:** UltraPure MicroBeads conjugated to monoclonal anti-biotin antibody (isotype: mouse IgG1).
Capacity	For 10^9 total cells, up to 100 separations.
Product format	All reagents 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 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$^+$ 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.

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 can also be performed by using the autoMACS Pro or the MultiMACS™ Cell24 Separator.

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140-001-861.06

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
Multi-24	10^8	10^9	MultiMACS Cell24

▲ **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) and Anti-Biotin-PE (# 130-090-756). For more information about fluorochrome-conjugated antibodies refer to ww.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.
- (Optional) PepTivator® CMV pp65 – premium grade # 130-093-438) 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. 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.

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

For details refer to 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 FBS, 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.
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, 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 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 UltraPure 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).

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 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 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 MultiMACS™ Cell24 Separator Plus

Refer to the the MultiMACS™ Cell Separator Plus user manual for instructions on how to use the instrument

Magnetic separation 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.

▲ 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.

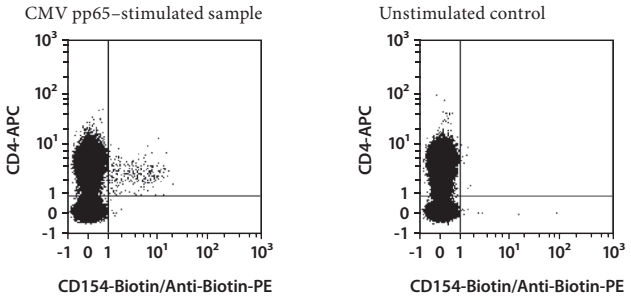
1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.
3. For a standard separation choose the following program:

   Positive selection: Posseld2
   Collect positive fraction in row C of the tube rack

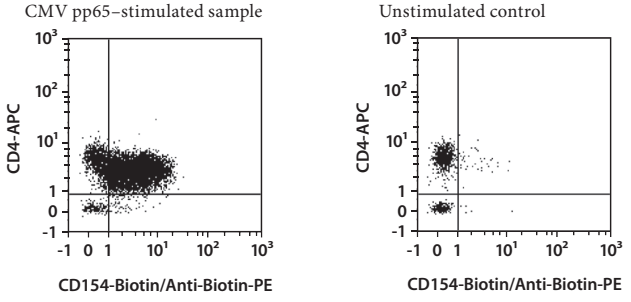
3. Example of a separation using the CD154 MicroBead Kit

$CD154^+$ 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 PepTivator CMV pp65 (# 130-093-438), 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 (VIT4)-APC (# 130-092-374) to detect $CD154^+$ cells. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.

A) Before separation



B) After separation



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.

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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