Miltenyi Biotec

CD154 antibodies
human

Contents

1. Description

Components	1 mL monoclonal CD154 antibodies, human conjugated to various dyes.	
	FITC	130-096-233
	PE	130-092-289
	APC	130-092-290
	VioBlue®	130-096-217
	PE-Vio770™	130-096-793
	APC-Vio770	130-096-603
	Biotin	130-092-690
Clone	5C8 (isotype: mouse IgG2a).	
Capacity	100 tests or up to 10^9 total cells.	
Product format	Antibodies 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.	

Cross-reactivity: The CD154 antibody has been reported to react with rhesus monkey (*Macaca mulatta*) and cynomolgus monkey (*Macaca fascicularis*) cells.

1.1 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. The antibody has been shown to block the activation of antigen-presenting cells by T helper cells *in vitro*. Because of its transient expression within hours after activation, CD154 can be used as a marker for activated antigen-specific CD4$^+$ T cells.[1] The addition of a CD40-blocking antibody during the stimulation of cell suspensions prevents the down-regulation of CD154 expression induced by interaction with CD40 expressed on antigen-presenting cells. For intracellular detection of CD154 expression or if a pure population of enriched T cells is used, blocking of CD40 is not required.

1.2 Applications

- Identification and enumeration of activated CD154$^+$ antigen-specific CD4$^+$ T cells by flow cytometry or fluorescence microscopy.
- Identification and enumeration of antigen-specific CD4$^+$ T cells in combination with a MACS® Cytokine Secretion Assay Kit.
- Evaluation of intracellular cytokine expression in activated antigen-specific CD4$^+$ T cells by using CD154 antibodies in combination with antibodies against human cytokines.

1.3 Recommended antibody dilution

- For intracellular staining the CD154 antibodies should be used at a dilution of 1:10.
  For cell surface staining the CD154 antibodies should be used at a dilution of 1:11.

1.4 Reagent 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).

  ▲ **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.
- Culture medium, e.g., RPMI 1640 (# 130-091-440) containing 5% human serum, for example, autologous AB serum (do not use BSA or FBS because of non-specific stimulation!).
- Reagents for cell culture and stimulation, e.g., CytoStim (# 130-092-172, # 130-092-173), CMV pp65 – Recombinant Protein (# 130-091-823, # 130-091-824), or PepTivator –

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CMV pp65 (# 130-093-435, # 130-093-438) for the stimulation of human T cells. For details refer to the respective data sheet. For more information about antigens refer to www.miltenyibiotec.com.

- (Optional) Anti-Biotin antibodies conjugated to, e.g., PE (# 1130-090-756) as secondary antibody reagent in combination with CD154-Biotin.
- (Optional) Secretion inhibitor, e.g., brefeldin A.
- (Optional) Cytokine Secretion Assay, e.g., IFN-γ Secretion Assay -Detection Kit (PE), human (# 130-054-202).
- (Optional) FcR Blocking Reagent, human (# 130-059-901) to avoid Fc receptor–mediated antibody labeling.
- (Optional) CD40-blocking antibody (clone HB14) (see 2.2.1).
- (Optional) Inside Stain Kit (# 130-090-477) for the fixation and permeabilization of cells.
- (Optional) Fluorochrome-conjugated antibodies for cell surface staining, e.g., CD4-FITC (# 130-080-501), CD4-PE (# 130-091-231), or CD4-APC (# 130-091-232). For more information about antibodies refer to www.miltenyibiotec.com/antibodies.
- (Optional) Fluorochrome-conjugated antibodies for intracellular staining of activation markers, e.g., Anti-IFN-γ-PE (# 130-091-653).
- (Optional) Fixation and Dead Cell Discrimination Kit (# 130-091-163) for cell fixation and flow cytometric exclusion of dead cells.

2. Protocols

2.1 Sample preparation

For activation of T cells, best results are achieved by starting the assay with fresh PBMCs or with other leukocyte-containing single-cell preparations from tissues or cell lines. Alternatively, frozen cell preparations can be used. For details refer to the protocols section at www.miltenyibiotec.com/protocols.

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).

2.2 *In vitro* stimulation of CD4$^+$ T cells

▲ Always include a negative control in the experiment. The sample should be treated in exactly the same manner 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).

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

2.2.1 *In vitro* stimulation for cell surface detection of CD154 expression

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 per mL in culture medium containing 5% human serum. Plate cells in dishes at a density of 5×10^6 cells/cm^2. For details refer to section 5. Appendix: Flask and dish sizes for *in vitro* stimulation of xx cells.
3. Add an antigen or control reagent in the appropriate concentration, for example, CytoStim (20 µL/mL).
4. Add 1 µg/mL of a CD40 antibody (blocking) 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 or by using a cell scraper. Rinse the dish with cold buffer. Check microscopically for any remaining cells, if necessary, rinse the dish again.

2.2.2 *In vitro* stimulation for CD154 staining in combination with intracellular cytokine staining

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/cm^2. For details see 5. Appendix: Flask and dish sizes for stimulation.
3. Add an antigen or control reagent in the appropriate concentration.
4. Incubate cells for 2 hours at 37 °C and 5% CO_2.
5. Add 1 µg/mL brefeldin A and incubate for additional 4 hours at 37 °C and 5% CO_2.
6. Collect cells carefully by pipetting up and down when working with smaller volumes, or by using a cell scraper. Rinse the dish with cold buffer. Check microscopically for any remaining cells. If necessary, rinse the dish again.

2.3 Immunofluorescent staining protocols

2.3.1 Cell surface staining

1. Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant.
2. Resuspend up to 10^7 nucleated cells per 100 µL of buffer.
3. Add 10 µL of the CD154 antibody.
4. (Optional) Add additional staining antibodies, e.g., CD4-FITC (# 130-080-501).
5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2 8 °C).
   ▲ **Note:** Working on ice requires increased incubation times. Higher temperatures

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and/or longer incubation times may lead to non-specific cell labeling.

6. Wash cells by adding 1 2 mL of buffer per 10^7 cells and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
7. (Optional) If CD154-Biotin was used, resuspend the cell pellet in 100 µL of buffer, add 10 µL of anti-biotin antibody (Anti-Biotin-FITC #130-090-857, Anti-Biotin-PE #130-090-756, or Anti-Biotin-APC #130-090-856), and continue as described in steps 4 and 5.
8. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy.

2.3.2 Intracellular staining of cells in suspension

▲ It is recommended to stain 10^6 cells per sample. When working with up to 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 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

1. Wash up to 10^7 cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
2. (Optional) Stain cell surface antigens that are sensitive to fixation with appropriate antibodies according to the manufacturer's recommendations. Then wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend up to 10^7 cells in 500 µL of buffer.
4. Add 500 µL of Inside Fix (Inside Stain Kit). Mix well and incubate for 20 minutes in the dark at room temperature.
5. Centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
6. Wash cells by adding 1 mL of buffer and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.

   ▲ **Note:** Fixed cells may be stored in azide-containing buffer at 2–8 °C for up to 1 week.
7. Wash cells by adding 1 mL of Inside Perm (Inside Stain Kit) and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
8. Resuspend cells in 90 µL of Inside Perm. Add 10 µL of the CD154 antibody.
9. (Optional) Add additional staining antibodies to the solution, for example, for the staining of cytokines.

   ▲ **Note:** For efficient permeabilization upon intracellular staining the volume of Inside Perm should be at least 5× the volume of staining antibodies.
10. Mix well and incubate for 10 minutes in the dark at room temperature.
11. Wash cells by adding 1 mL of Inside Perm and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
12. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy. Store cells at 2–8 °C in the dark until analysis. Mix well before flow cytometric acquisition.

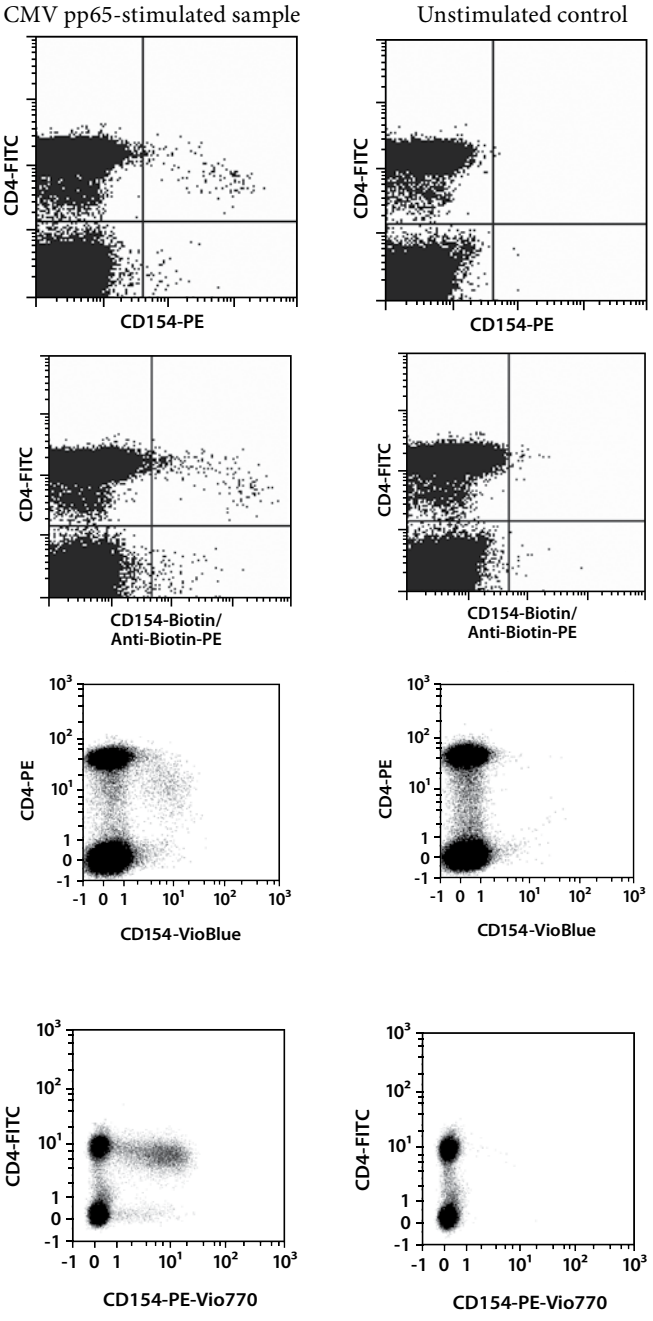
    ▲ **Note:** Samples may be stored at 2–8 °C in the dark for up to 24 hours.

    ▲ **Note:** Do not use propidium iodide (PI) or 7-AAD staining.

3. Examples of immunofluorescent staining with CD154 antibodies

(a) Cell surface staining with CD154 antibodies

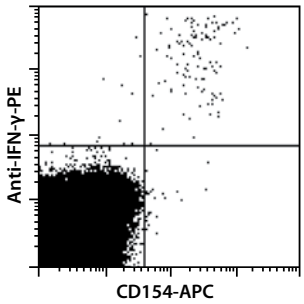
Human PBMCs were restimulated *in vitro* with and without CMV pp65 – Recombinant Protein (# 130-091-823) or CytoStim (# 130-092-172) and CD40 antibody for 16 hours. Cells were harvested, stained with CD4-FITC or CD4-PE and CD154 antibodies conjugated to PE, Biotin, VioBlue, or PE-Vio770 and analyzed by flow cytometry. Cells stained with CD154-VioBlue and CD154-PE-Vio770 were analyzed using the MACSQuant® Analyzer. A lymphocyte gate based on forward and side scatter properties was activated. Cell debris and dead cells were excluded from the analysis according to PI fluorescence.



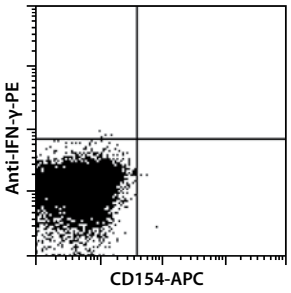
(b) Intracellular staining with CD154 antibodies in combination with Anti-IFN-γ antibodies

Human PBMCs were restimulated *in vitro* with and without CMV pp65 – Recombinant Protein (# 130-091-823) for 6 hours. Brefeldin A was added after 2 hours. The cells were harvested, fixed, stained with CD4-FITC, permeabilized, and intracellularly stained with CD154-APC and Anti-IFN-γ-PE antibodies. Cells were analyzed by flow cytometry. Gating was performed according to CD4 expression and side scatter properties. Cell debris was excluded from the analysis using an FL-2 vs. FL-3 dot plot.

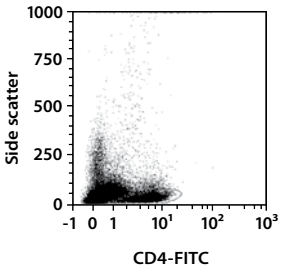
CMV pp65-stimulated sample



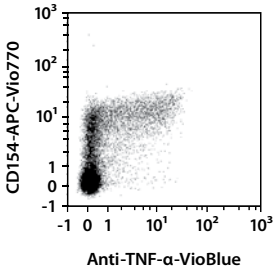
Unstimulated control



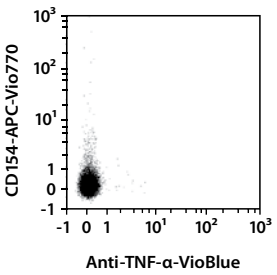
Human PBMCs were restimulated *in vitro* with and without SEB for 16 hours. Brefeldin A was added after 2 hours. The cells were harvested, fixed, stained with CD4-FITC, permeabilized, and intracellularly stained with CD154-APC-Vio770 and Anti-TNF-α-VioBlue. Cells were analyzed by flow cytometry. Gating was performed according to CD4 expression and side scatter properties. Cell debris was excluded from the analysis using an FL-2 vs. FL-3 dot plot.



SEB-stimulated sample



Unstimulated control



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(10): 1118–1124.

5. Appendix: Flask and dish sizes for *in vitro* stimulation of CD154+ cells

For *in vitro* stimulation of CD154+ cells (refer to 2.2) 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.

Warranty

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