

CD154 antibodies

mouse

CD154-PE	130-092-106
CD154-APC	130-092-105
CD154-Biotin	130-092-104

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1. Description

Clone	MR1 (isotype: hamster IgG3).
Product format	1 mL CD154 antibodies, mouse: monoclonal CD154 antibodies conjugated to R-phycoerythrin (PE), allophycocyanin (APC) or biotin (Biotin). Antibodies are supplied in a solution containing stabilizer and 0.05% sodium azide.
Product size	100 tests (for up to 10 ⁹ nucleated cells).
Storage	Store protected from light at 4–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Background and product applications

The antibody specifically recognizes the mouse 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. The addition of 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. For intracellular detection of CD154 expression or if a pure population of enriched T cells is used, blocking of CD40 is not required.

Product applications

- Identification and enumeration of activated 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[®] Mouse Cytokine Secretion Assay–Detection Kit (PE).
- Evaluation of intracellular cytokine expression in activated antigen-specific CD4⁺ T cells by using CD154 antibodies in combination with antibodies against mouse cytokines.

1.2 Examples of staining concentrations for mouse cells.

CD154 conjugate	PE	APC	Biotin
Recommended antibody dilution			
Flow cytometry^a			
- in general	1:11	n. r.	1:11
- formaldehyde-fixed cells	1:11	n. r.	1:11
- formaldehyde-fixed and permeabilized cells	1:11	1:11	n. r.
Immunohistochemistry^b			
a) Given antibody dilutions are for a cell concentration of up to 1×10 ⁷ cells/mL buffer. b) For immunohistochemical staining, the optimal antibody dilution has to be tested. n. r. The antibody is not recommended for the indicated application.			

1.3 Reagent requirements

- Buffer: Prepare a solution containing PBS (phosphate buffered saline) pH 7.2, 0.5% BSA (bovine serum albumin) and 2 mM EDTA, e.g. by diluting MACS[®] BSA Stock Solution (# 130-091-376) 1:20 in autoMACS[™] Rinsing Solution (# 130-091-222). Keep buffer cold (4–8 °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 mouse serum albumin, mouse serum or fetal calf serum. Buffers or media containing Ca²⁺ or Mg²⁺ are not recommended for use.
- Cell culture medium, e.g. RPMI 1640 (#130-091-440) with 5% mouse serum.
- (Optional) CD40 blocking antibody for cell surface detection of CD154.
- (Optional) Brefeldin A.
- (Optional) CD4-FITC (# 130-091-608), CD4-PE (# 130-091-607), or CD4-APC (# 130-091-611).
- (Optional) Anti-Biotin-PE (# 130-090-756), or Anti-Biotin-APC (# 130-090-856) as secondary antibody reagent in combination with CD154-Biotin (# 130-092-104).
- (Optional) Inside Stain Kit (# 130-090-477).
- (Optional) Intracellular cytokine staining antibodies, e.g. Anti-IFN-γ-PE (# 130-092-346).
- (Optional) Mouse Cytokine Secretion Assay, e.g. Mouse IL-2 Cytokine Secretion Assay–Detection Kit (PE) (# 130-090-491).
- (Optional) PI (propidium iodide) or 7-AAD for flow cytometric exclusion of dead cells. For cell fixation and flow cytometric exclusion of dead cells, the Fixation and Dead Cell Discrimination Kit (# 130-091-163) is recommended.

2. Protocols

2.1 Sample preparation

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

2.2 Protocols for *in vitro* stimulation of antigen-specific CD4⁺ T cells

▲ 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, like a sample stimulated with SEB.

▲ Do not use media containing any non-murine proteins, like BSA or FCS, because of non-specific stimulation.

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

1. Wash cells by adding cell culture medium, centrifuge at 300×g for 10 minutes. Pipette off supernatant.
2. Resuspend cells at a density of 10⁷ cells/mL in culture medium, containing 5% mouse serum. Plate cells in dishes at a density of 5×10⁶ cells/cm² (see Appendix).
3. Add an antigen or control reagent in the appropriate concentration.
4. Add 10 µg/mL CD40 antibody (blocking) to the cell suspension.
▲ **Note:** Addition of CD40-blocking antibody prevents down-regulation of CD154 expression on T cells induced by interaction with CD40 expressed on antigen-presenting cells.
5. Incubate cells for 6–8 hours at 37 °C and 5% CO₂.
▲ **Note:** CD154 is transiently expressed on activated CD4⁺ T cells. The highest levels are detected 6–8 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.

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

1. Wash cells by adding medium, centrifuge at 300×g for 10 minutes. Pipette off supernatant.
2. Resuspend cells at a density of 10⁷ cells/mL in culture medium, containing 5% mouse serum. Plate cells in dishes at a density of 5×10⁶ cells/cm² (see Appendix).
3. Add an antigen or control reagent in the appropriate concentration.
4. Incubate cells for 2 hours at 37 °C and 5% CO₂.
5. Add 1 µg/mL Brefeldin A and incubate for additional 4 hours at 37 °C and 5% CO₂.
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.

2.3 Protocols for immunofluorescent staining

2.3.1 Protocol for cell surface staining

▲ Volumes for fluorescent labeling given below are for up to 10⁷ total cells. When working with fewer than 10⁷ 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 2×10⁷ total cells, use twice the volume of all indicated reagent volumes and total volumes).

1. Wash cells by adding 1–2 mL of buffer, centrifuge at 300×g for 10 minutes. Pipette off supernatant completely.
2. Resuspend up to 10⁷ nucleated cells per 100 µL of buffer.
3. Add 10 µL of CD154 antibody.
4. Mix well and incubate for 10 minutes in the dark at 4–8 °C.
▲ **Note:** Working on ice requires increased incubation time. Higher temperatures and/or longer incubation times lead to non-specific cell labeling.
5. Wash cells by adding 1–2 mL of buffer per 10⁷ cells and centrifuge at 300×g for 5 minutes. Pipette off supernatant completely.
▲ **Note:** If CD154-Biotin is used for staining, repeat steps 2–5 for staining with a secondary antibody reagent (e.g. Anti-Biotin-APC).
6. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy.

2.3.2 Protocol for intracellular staining

▲ Volumes for fluorescent labeling given below are for up to 10⁷ total cells. When working with fewer than 10⁷ 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 2×10⁷ total cells, use twice the volume of all indicated reagent volumes and total volumes).

1. Wash cells by adding 1–2 mL of buffer, centrifuge at 300×g for 10 minutes. Pipette off supernatant completely.
2. (Optional) Stain for cell surface antigens, which are sensitive to fixation, according to the manufacturer's recommendations. Subsequently, wash cells by adding 1–2 mL of buffer. Centrifuge at 300×g for 10 minutes. Pipette off supernatant completely.
3. Resuspend up to 10⁷ nucleated cells per 500 µL of buffer.
4. Add 500 µL of Inside Fix. Mix well and incubate for 20 minutes at room temperature.
5. Centrifuge for 5 minutes at 300×g. Remove supernatant carefully.
6. Wash cells by adding 1 mL of buffer. Centrifuge for 5 minutes at 300×g and remove supernatant carefully.
▲ **Note:** Fixed cells may be stored at 4–8 °C for up to 1 week.
7. Wash cells by adding 1 mL of Inside Perm. Centrifuge for 5 minutes at 300×g and remove supernatant carefully.
8. Resuspend cells in 100 µL of Inside Perm. Add 10 µL of CD154 antibody.
9. (Optional) Add additional staining antibodies to the solution, e.g. 10 µL of CD4-FITC (# 130-091-608) and 10 µL of Anti-IL-2-PE (# 130-092-302).
10. Mix well and incubate for 10 minutes at room temperature.
11. Wash cells by adding 1 mL of Inside Perm. Centrifuge at 300×g for 5 minutes and pipette off supernatant carefully.

- Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy. Store cells at 4–8 °C in the dark until analyzed. Mix well before flow cytometric acquisition.

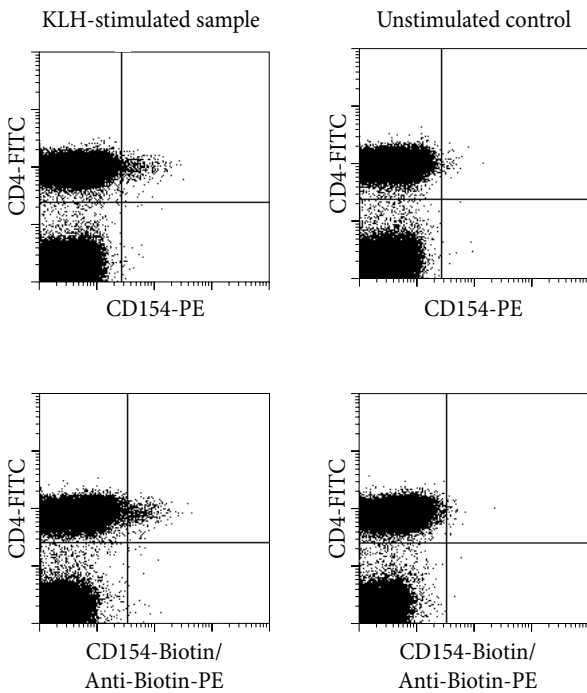
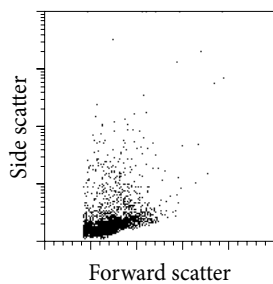
▲ **Note:** Samples may be stored at 4–8 °C in the dark for up to 24 hours.

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

3. Examples of immunofluorescent stainings with CD154 antibodies

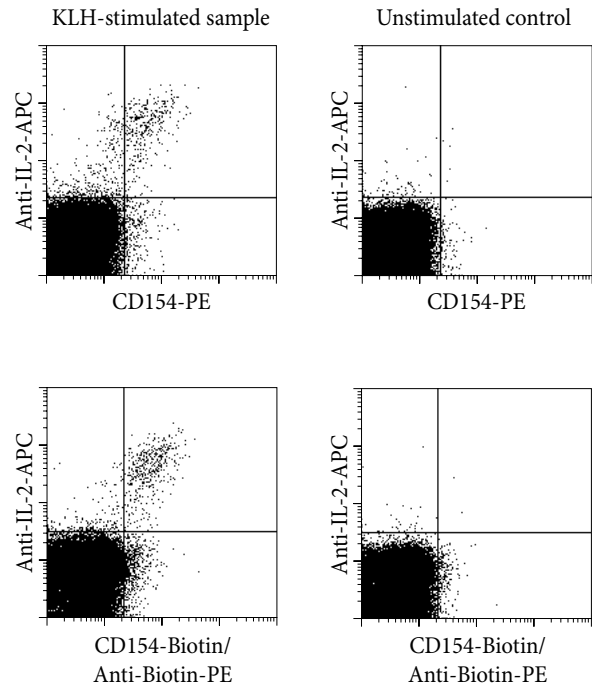
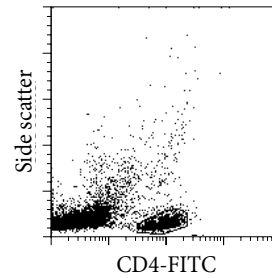
(a) Cell surface staining with CD154 antibodies

Spleen cells from a KLH-immunized mouse were restimulated *in vitro* with or without KLH and with CD28 and CD40 antibodies for 6 hours. The cells were harvested, stained with CD4-FITC and CD154-PE or CD154-Biotin and Anti-Biotin-PE and analyzed by flow cytometry. A lymphocyte gate based on forward and side scatter properties was activated. Dead cells and B cells were excluded according to PI- and CD45R/B220-PerCP™-staining in a fluorescence 2 versus fluorescence 3 dot plot.



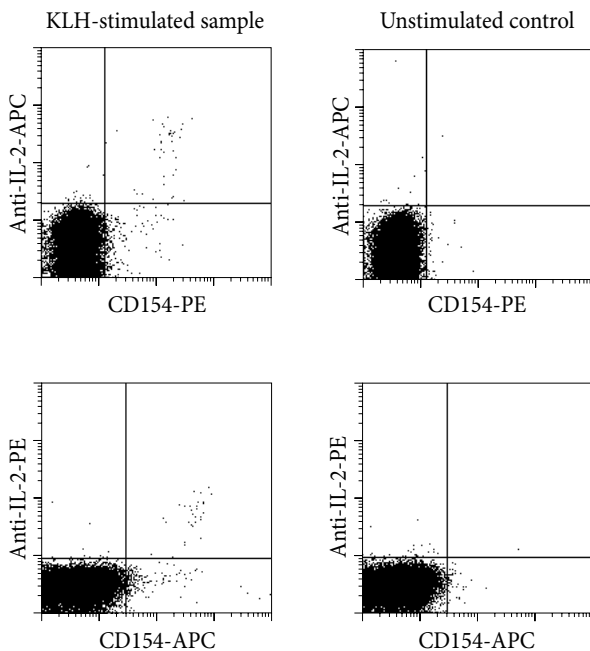
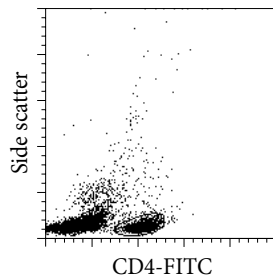
(b) Cell surface staining with CD154 antibodies in combination with the Mouse IL-2 Cytokine Secretion Assay–Detection Kit (APC)

Spleen cells from a KLH-immunized mouse were restimulated *in vitro* with or without KLH and with CD28 and CD40 antibodies for 6 hours. The cells were harvested and the Mouse IL-2 Cytokine Secretion Assay–Detection Kit (APC) was performed. Subsequently, the cells were counterstained with CD4-FITC, CD154-PE or CD154-Biotin and Anti-Biotin-PE and analyzed by flow cytometry. Gating was performed according to CD4 expression and side scatter properties. Dead cells and B cells were excluded according to PI- and CD45R/B220-PerCP™-staining in a fluorescence 2 versus fluorescence 3 dot plot.



(c) Intracellular staining with CD154 antibodies in combination with Anti-IL-2 antibodies

Spleen cells from a KLH-immunized mouse were restimulated *in vitro* with or without KLH 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 and Anti-IL-2 antibodies. Cells were analyzed by flow cytometry. Gating was performed according to CD4 expression and side scatter properties. Cell debris and dead cells were excluded from the analysis in a fluorescence 2 versus fluorescence 3 dot plot.



4. Appendix

Flask and dish sizes for stimulation

For *in vitro* stimulation the cells should be resuspended in culture medium, containing 5% mouse serum, at 10^7 cells/mL and 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 T 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
0.5×10^7	0.5 mL	48 well	1.13 cm
1×10^7	1 mL	24 well	1.6 cm
2×10^7	2 mL	12 well	2.26 cm
5×10^7	5 mL	6 well	3.5 cm

total cell number	medium volume to add	culture dish	dish diameter
4.5×10^7	4.5 mL	small	3.5 cm
10×10^7	10 mL	medium	6 cm
25×10^7	25 mL	large	10 cm
50×10^7	50 mL	extra large	15 cm

total cell number	medium volume to add	culture flask	growth area
12×10^7	12 mL	50 mL	25 cm ²
40×10^7	40 mL	250 mL	75 cm ²
80×10^7	80 mL	720 mL	162 cm ²
120×10^7	120 mL	900 mL	225 cm ²

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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