



# CD154 Detection Cocktails mouse

CD154 Detection Cocktail (PE) Order no. 130-093-084  
CD154 Detection Cocktail (APC) Order no. 130-093-083

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## 1. Description

<b>Components</b>	<b>0.2 mL CD154 Detection Cocktail (PE), mouse:</b> Cocktail of antibodies containing CD154-PE, CD40 antibody, and CD28 antibody. or <b>0.2 mL CD154 Detection Cocktail (APC), mouse:</b> Cocktail of antibodies containing CD154-APC, CD40 antibody, and CD28 antibody.
<b>Capacity</b>	100 tests or up to 10 <sup>8</sup> total cells.
<b>Product format</b>	The antibody cocktail is supplied in phosphate-buffered saline (PBS) pH 7.2. Low endotoxin.
<b>Storage</b>	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 CD154 Detection Cocktails

The CD154 Detection Cocktails were developed for fast and easy identification and enumeration of antigen-specific CD4<sup>+</sup> T cells. Cells are stimulated with the desired antigen *in vitro* to induce expression of CD154 and are simultaneously incubated with the CD154 Detection Cocktail.

Besides a fluorochrome-conjugated CD154 antibody, which specifically recognizes the mouse CD154 antigen, the CD154 Detection Cocktail contains a CD40 blocking antibody and a CD28 antibody. The CD40 blocking antibody prevents the downregulation of CD154 expression on CD4<sup>+</sup> T cells induced by interaction with CD40 expressed on antigen-presenting cells, thus stabilizing the surface expression of CD154 during stimulation of antigen-specific T cells. The CD28 antibody provides the costimulatory signal for CD4<sup>+</sup> T cells and optimizes the induction of CD154 expression, thus improving the detection of CD154<sup>+</sup> T cells.

The CD154 detection cocktail is optimized to be conveniently added to the cell suspension during stimulation.

### 1.2 Background information

CD154 is a 39 kDa transmembrane glycoprotein, also known as CD40L, gp39, T-BAM, and TRAP. It is transiently up-regulated on activated CD4<sup>+</sup> T cells and to a very low extent on activated CD8<sup>+</sup> 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<sup>+</sup> T cells within hours of activation, CD154 can be used as a marker for activated antigen-specific CD4<sup>+</sup> T helper cells<sup>1-3</sup>.

By combining the stimulation of antigen-specific T cells with the detection of CD154 using the optimized CD154 Detection Cocktail, activated antigen-specific CD4<sup>+</sup> T cells can be reliably detected in single-cell suspensions of mouse lymphoid tissues.

### 1.3 Applications

- Identification and enumeration of viable CD154<sup>+</sup> antigen-specific CD4<sup>+</sup> T cells from single-cell suspensions of lymphoid tissues for phenotypical and functional characterization.
- Identification and enumeration of viable antigen-specific CD4<sup>+</sup> T cells in combination with a MACS<sup>®</sup> Cytokine Secretion Assay Kit.

### 1.4 Reagent requirements

- Buffer: Prepare a solution containing 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<sup>™</sup> 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 mouse serum albumin, mouse serum, or fetal bovine serum. Buffers or media containing Ca<sup>2+</sup> or Mg<sup>2+</sup> are not recommended for use.
- 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 (# 130-091-608) and CD8a-APC (# 130-091-606).

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- (Optional) Cytokine Secretion Assay, for example, IL-2 Secretion Assay – Detection Kit (APC) (# 130-090-987) or IL-2 Secretion Assay – Detection Kit (PE) (# 130-090-491).
- CD4<sup>+</sup>CD62L<sup>+</sup> T Cell Isolation Kit (# 130-091-751).
- (Optional) Propidium iodide (PI) or 7-AAD for flow cytometric exclusion of dead cells without fixation.
- Fixation and Dead Cell Discrimination Kit (# 130-091-163) for cell fixation and flow cytometric exclusion of dead cells.

## 2. Protocol

### 2.1 Sample preparation

Prepare a single-cell suspension from lymphoid organs using a standard preparation method. For details see the General Protocols section of the respective separator user manual. The General Protocols are also available at [www.miltenyibiotec.com/protocols](http://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 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 FCS, because of non-specific stimulation.

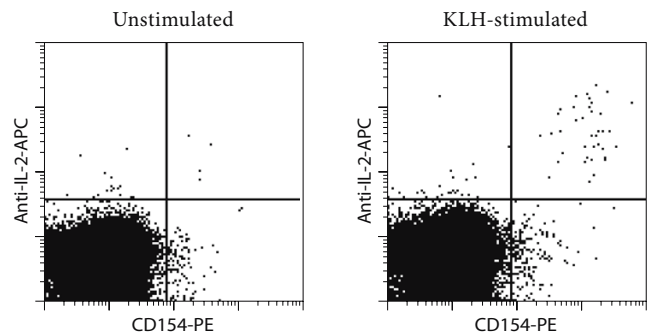
1. Determine cell number.
2. Wash cells by adding cell culture medium, centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cells at a density of 10<sup>7</sup> cells/mL in culture medium, containing 5% mouse serum. For one test resuspend 10<sup>6</sup> cells in 100 μL of medium. Plate cells in dishes at a density of 5×10<sup>6</sup> cells/cm<sup>2</sup> (see 5. Appendix: Flask and dish sizes for stimulation).
4. Add 2 μL of CD154 Detection Cocktail per 100 μL 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<sub>2</sub>, depending on the cell type and the antigen used (see 3.2 and 3.3: Kinetics).
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, centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
9. (Optional) Resuspend up to 10<sup>7</sup> cells in 100 μL of buffer and add additional staining antibodies, e.g., CD4-FITC. Incubate cells for 10 min in the dark in the refrigerator (2–8 °C). Wash cells by adding buffer, centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.

## 3. Examples of detection of CD154 using the CD154 Detection Cocktails (PE) and (APC)

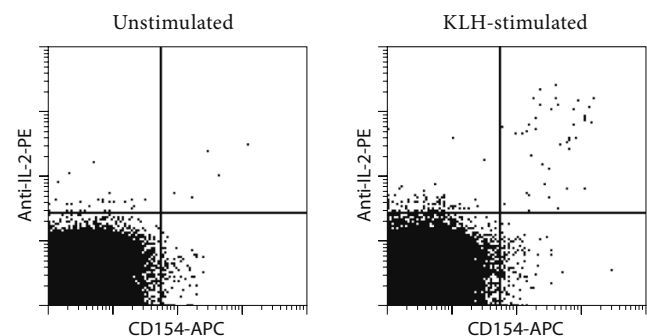
### 3.1 Detection of CD154 with the CD154 Detection Cocktails (PE) or (APC) in combination with the Mouse IL-2 Secretion Assay – Detection Kits (PE) or (APC)

Spleen cells from a BALB/c mouse were activated *in vitro* with 100 μg/mL keyhole limpet hemocyanin (KLH) or were not activated overnight in the presence of the CD154 Detection Cocktail (PE) (a) or the CD154 Detection Cocktail (APC) (b). Then, secreted IL-2 was detected using the Mouse IL-2 Secretion Assay – Detection Kit (APC) (# 130-090-987) or the Mouse IL-2 Secretion Assay – Detection Kit (PE) (# 130-090-491), respectively. For flow cytometric analysis, cells were additionally stained with CD4-FITC and CD45R (B220)-PerCP. Lymphocytes were gated based on scatter properties. Dead cells and B cells were excluded from the analysis according to PI and CD45R (B220)-PerCP fluorescence. Dot plots shown are gated on CD4<sup>+</sup> T cells.

(a) Mouse spleen cells stained with CD154 Detection Cocktail (PE) and the Mouse IL-2 Secretion Assay – Detection Kit (APC).

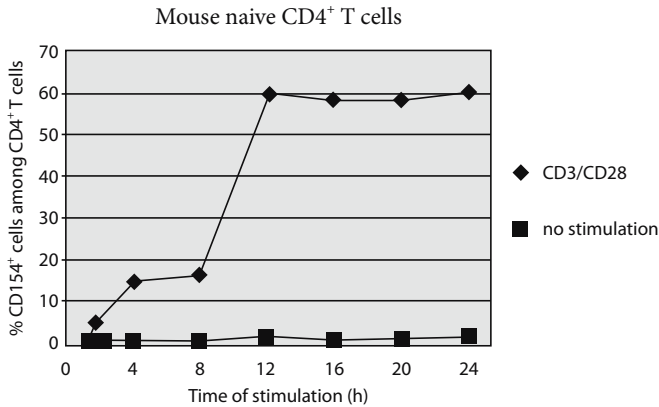


(b) Mouse spleen cells stained with CD154 Detection Cocktail (APC) and the Mouse IL-2 Secretion Assay – Detection Kit (PE).



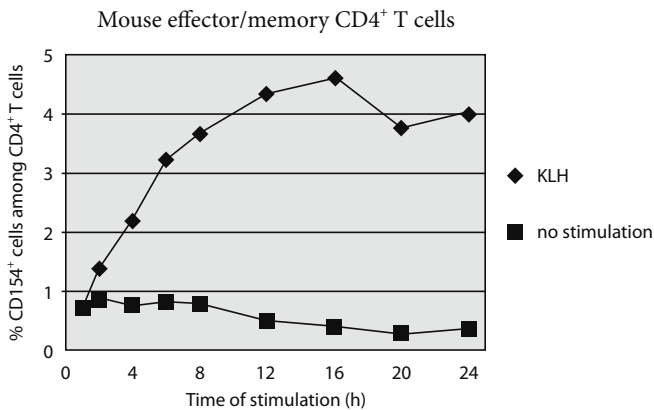
### 3.2 Kinetics of CD154 expression after activation of naive CD4<sup>+</sup> T cells

Naive CD4<sup>+</sup>CD62L<sup>+</sup> T cells were magnetically isolated from spleen cells of BALB/c mice using the CD4<sup>+</sup>CD62L<sup>+</sup> T Cell Isolation Kit (# 130-091-751). CD4<sup>+</sup>CD62L<sup>+</sup> T cells were either stimulated with CD3/CD28 or were not stimulated for 24 hours in the presence of the CD154 Detection Cocktail (PE). CD154 expression was analyzed at different points in time by flow cytometry. The graph displays the percentage of CD154<sup>+</sup> cells among CD4<sup>+</sup> T cells (y-axis) versus stimulation time (x-axis).



### 3.3 Kinetics of CD154 expression after activation of effector/memory CD4<sup>+</sup> T cells

Spleen cells from a BALB/c mouse immunized with KLH were either restimulated *in vitro* with 100 µg/ml KLH or were not stimulated for 24 hours in the presence of the CD154 Detection Cocktail (PE). CD154 expression was analyzed at different points in time by flow cytometry. The graph displays the percentage of CD154<sup>+</sup> cells among CD4<sup>+</sup> T cells (y-axis) versus stimulation time (x-axis).



## 4. References

1. Frensch, M. *et al.* (2005) Direct access to CD4<sup>+</sup> 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<sup>+</sup> 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.* In press.

## 5. Appendix: Flask and dish sizes for *in vitro* stimulation

For *in vitro* stimulation, the cells should be resuspended in culture medium, containing 5% of mouse serum, at a dilution of 10<sup>7</sup> cells/mL. The cells should be plated at a density of 5×10<sup>6</sup> cells/cm<sup>2</sup>. 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 <sup>7</sup>	0.15 mL	96 well	0.64 cm
0.50×10 <sup>7</sup>	0.50 mL	48 well	1.13 cm
1.00×10 <sup>7</sup>	1.00 mL	24 well	1.60 cm
2.00×10 <sup>7</sup>	2.00 mL	12 well	2.26 cm
5.00×10 <sup>7</sup>	5.00 mL	6 well	3.50 cm

Total cell number	Medium volume to add	Culture dish	Dish diameter
4.5×10 <sup>7</sup>	4.5 mL	small	3.5 cm
10.0×10 <sup>7</sup>	10.0 mL	medium	6 cm
25.0×10 <sup>7</sup>	25.0 mL	large	10 cm
50.0×10 <sup>7</sup>	50.0 mL	extra large	15 cm

Total cell number	Medium volume to add	Culture flask	Growth area
12×10 <sup>7</sup>	12 mL	50 mL	25 cm <sup>2</sup>
40×10 <sup>7</sup>	40 mL	250 mL	75 cm <sup>2</sup>
80×10 <sup>7</sup>	80 mL	720 mL	162 cm <sup>2</sup>
120×10 <sup>7</sup>	120 mL	900 mL	225 cm <sup>2</sup>

### 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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