



CD154/TNF- α /CD4 Detection Kit human

Order no. 130-092-813

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1. Description

Components	1 mL Anti-TNF-α-PE, human (clone cA2, human IgG1) 1 mL CD154-APC, human (clone 5C8, mouse IgG2a) 1 mL CD4-FITC, human (clone VIT4, mouse IgG2a) 2x25 mL Inside Fix Contains 3.7% formaldehyde (EU Hazard Classification: Xn harmful; R40/20/21/22-43) 30 mL Inside Perm (10x) Contains a detergent 200 μL CytoStim, human 200 μL Brefeldin A (100 μg/mL)
Product format	Antibodies are supplied in a solution containing stabilizer and 0.05% sodium azide. Cytostim is supplied in a solution containing stabilizer. Brefeldin A is supplied in buffer containing 10% DMSO.
Product size	100 tests or up to 10 ⁸ total cells.
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial labels.

1.1 Principle of the CD154/TNF- α /CD4 Detection Kit

The CD154/TNF- α /CD4 Detection Kit was developed for the intracellular detection of TNF- α production in activated CD4⁺ T cells using CD154 as a reliable marker for T cell activation. The kit contains all reagents for the immunofluorescent detection

of TNF- α -producing activated CD4⁺ T cells from whole blood or PBMCs by intracellular staining. In addition, reagents are included for the fixation and permeabilization of cells prior to staining, as well as for the *in vitro* stimulation of T cells as a positive control. Briefly, whole blood cells or PBMCs are cultured with or without antigen for a total of 6 hours during which they begin to secrete cytokines as well as up-regulate activation markers such as CD154¹. After 2 hours of cultivation, Brefeldin A is added to the culture to inhibit transport of proteins to the cellular membrane. As a positive control, cells are stimulated with CytoStim (an antigen-independent stimulant of CD4⁺ and CD8⁺ T cells) and are handled in an identical manner to antigen re-stimulated cells. Cells are fixed and permeabilized before being intracellularly stained with fluorochrome-conjugated CD154, Anti-TNF- α , and CD4 antibodies and analyzed by flow cytometry.

1.2 Background and product applications

TNF- α is a cytokine secreted by activated CD4⁺ T cells, monocytes, macrophages, NK cells, and neutrophils. TNF- α is a mediator of inflammatory immune responses and is selectively toxic for many transformed cells, especially in combination with IFN- γ . CD154 is transiently up-regulated on activated CD4⁺ T cells within hours after activation.¹ By combining the detection of TNF- α production with CD154 and CD4, the sensitive and specific detection of activated antigen-specific CD4⁺ T cells can be reliably performed from whole blood or PBMCs.

Product applications

- Flow cytometric identification and enumeration of TNF- α -producing, CD154⁺CD4⁺ T cells upon *in vitro* stimulation.
- Identification and enumeration of TNF- α -producing, antigen-specific CD154⁺CD4⁺ T cells upon restimulation with the respective antigen.
- Monitoring of specificity of antigen-specific T cell lines.

1.3 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 (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 human serum albumin, human serum, or fetal calf serum. Buffers or media containing Ca²⁺ or Mg²⁺ are not recommended for use.
- Culture medium, e.g., RPMI 1640 (# 130-091-440) containing 5% autologous human serum (do **not** use BSA or FCS due to non-specific stimulation!).
- Dilute Inside Perm 1:10 in distilled water before use.
- (Optional) Red blood cell lysis buffer: Prepare a solution containing 155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA.

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Miltenyi Biotec

www.miltenyibiotec.com



Miltenyi Biotec GmbH
Friedrich-Ebert-Str. 68
51429 Bergisch Gladbach, Germany
Phone +49-2204-8306-0 Fax +49-2204-85197

Miltenyi Biotec Inc.
12740 Earhart Avenue, Auburn CA 95602, USA
Phone 800 FOR MACS, 530 888-8871
Fax 530 888-8925

- (Optional) FcR Blocking Reagent, human (# 130-059-901) to avoid Fc receptor-mediated antibody labeling.

2. Protocols

▲ Always include a **negative control** in the experiment. The sample should be treated exactly the same as the stimulated sample apart from the addition of the stimulus.

▲ A **positive control** is also included in the experiment. Cells are stimulated with CytoStim followed by treatment with Brefeldin A for the inhibition of cytokine secretion.

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

2.1 Sample preparation

The CD154/TNF- α /CD4 Detection Kit can be used with either whole blood or peripheral blood mononuclear cells (PBMCs). PBMCs should be isolated from anti-coagulated peripheral blood by density gradient centrifugation, e.g., using Ficoll-Paque™. For details see section General Protocols in the user manuals or visit www.miltenyibiotec.com/protocols.

▲ **Note:** To remove platelets after density gradient separation, resuspend cell pellet in buffer and centrifuge at 200 \times g for 10–15 minutes at 20 °C. Carefully aspirate supernatant. Repeat washing step.

2.2 Protocol for the *in vitro* stimulation of PBMCs

1. Wash cells by adding medium, centrifuge at 300 \times g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cells at a density of 10⁷ per mL in culture medium containing 5% human serum. For one test, resuspend 10⁶ cells in 100 μ L of medium. Plate cells in dishes at a density of 5 \times 10⁶ cells/cm² (see 5. Appendix: Flask and dish sizes for stimulation).
3. Add 2 μ L of CytoStim per 100 μ L of cell suspension, or add antigen in the appropriate concentration.
4. Mix carefully and incubate cells for 2 hours at 37 °C and 5–7% CO₂.
5. Add 2 μ L Brefeldin A per 100 μ L of cell suspension and incubate for an additional 4 hours at 37 °C and 5–7% CO₂.
6. Collect cells carefully by pipetting up and down 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 Protocol for the *in vitro* stimulation of whole blood

1. Use 250 μ L of whole blood per stimulation/control.
2. Add 2 μ L of CytoStim or antigen in the appropriate concentration.
3. Mix carefully and incubate cells for 2 hours at 37 °C and 5–7% CO₂.
4. Add 2 μ L Brefeldin A and incubate for an additional 4 hours at 37 °C and 5–7% CO₂.
5. Dilute one volume of blood with 5–10 volumes of red blood cell lysis buffer and incubate for 5 minutes at room temperature.
6. Centrifuge at 300 \times g for 10 minutes. Aspirate supernatant completely.
7. Wash cells twice by adding buffer. Centrifuge at 300 \times g for 10 minutes and carefully remove supernatant.
8. For intracellular staining of cells in suspension, resuspend cells in 500 μ L of buffer and proceed to step 3 of section 2.4.

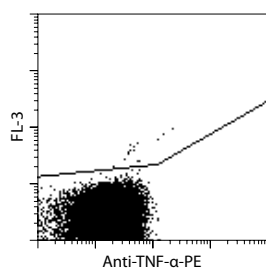
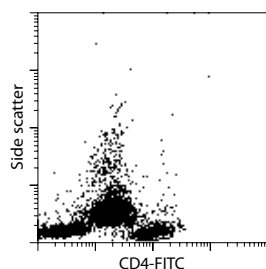
2.4 Intracellular staining of cells in suspension

▲ One test corresponds to staining of 10⁶ cells in a volume of 100 μ L. When working with up to 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 \times 10⁷ nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

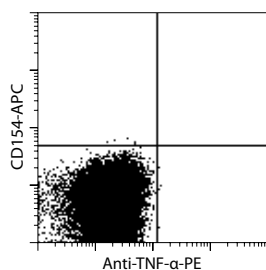
1. Wash cells by adding 1–2 mL of buffer per 10⁷ cells and centrifuge at 300 \times g for 10 minutes. Aspirate supernatant completely.
2. Resuspend 10⁶ cells in 500 μ L of buffer.
3. Add 500 μ L of Inside Fix. Mix well and incubate for 20 minutes at room temperature.
4. Centrifuge at 300 \times g for 5 minutes. Aspirate supernatant carefully.
5. Wash cells by adding 1 mL of buffer and centrifuge at 300 \times g for 5 minutes. Aspirate supernatant carefully.
 - ▲ **Note:** Fixed cells may be stored at 4–8 °C for up to 1 week.
6. Wash cells by adding 1 mL of Inside Perm. Centrifuge at 300 \times g for 5 minutes and remove supernatant carefully.
7. Resuspend cells in 80 μ L of Inside Perm. Add 10 μ L of each of the CD154, Anti-TNF- α , and CD4 antibodies.
8. Mix well and incubate for 10 minutes in the dark at room temperature.
9. Wash cells by adding 1 mL of Inside Perm and centrifuge at 300 \times g for 5 minutes. Aspirate supernatant carefully.
10. 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 analysis. Mix well before flow cytometric data 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 staining with the CD154/TNF- α /CD4 Detection Kit

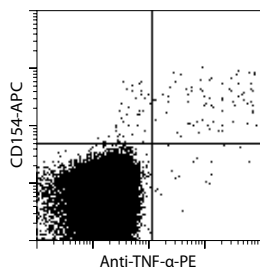
Human PBMCs were incubated with and without CMV pp65 protein (# 130-091-823) for 6 hours. After 2 hours, Brefeldin A was added. Cells were then fixed, permeabilized, and intracellularly stained with CD154-APC, Anti-TNF- α -PE, and CD4-FITC. Cells were analyzed by flow cytometry. A gate on CD4⁺ lymphocytes was activated based on CD4 expression and side scatter properties. Autofluorescent cell debris was excluded in an FL-2 vs. FL-3 dotplot.



Unstimulated control



CMV pp65-stimulated sample



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

For *in vitro* stimulation of PBMCs (see 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
0.5×10^7	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.

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4. Reference

1. Frensch, 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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