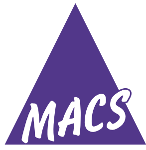


CD154/IL-2/CD4 Detection Kit
human

Order no. 130-092-818

Index

1. Description
    - 1.1 Principle of the Detection Kit
    - 1.2 Background and product applications
    - 1.3 Reagent requirements
2. Protocols
    - 2.1 Sample preparation
    - 2.2 *In vitro* stimulation of PBMCs
    - 2.3 *In vitro* stimulation of whole blood
    - 2.4 Intracellular staining of cells in suspension
3. Examples of immunofluorescent staining with the CD154/IL-2/CD4 Detection Kit
4. Reference
5. Appendix: Flask and dish sizes for *in vitro* stimulation of PBMCs

1. Description

Components

1 mL Anti-IL-2-PE, human (clone N7.48A, mouse IgG2a)

1 mL CD154-APC, human (clone 5C8, mouse IgG2a)

1 mL CD4-FITC, human (clone VIT4, mouse IgG2a)

2×25 mL Inside Fix
Contains 3.7% formaldehyde (EU Hazard Classification: Xn harmful; R40/20/21/22-43)

30 mL Inside Perm (10×)
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/IL-2/CD4 Detection Kit

The CD154/IL-2/CD4 Detection Kit was developed for the intracellular detection of IL-2 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 IL-2-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 CD154[1]. 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-IL-2, and CD4 antibodies and analyzed by flow cytometry.

1.2 Background and product applications

IL-2 is rapidly secreted by naive T helper cells and by certain subsets of memory T cells upon activation. It promotes growth and differentiation of T cells and has pleiotropic effects on many other leukocytes. The quantitative analysis of IL-2 producing cells can provide important information on the course of an immune response.

CD154 is transiently up-regulated on activated $CD4^+$ T cells within hours after activation.[1] By combining the detection of IL-2 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 IL-2-producing $CD154^+CD4^+$ T cells upon *in vitro* stimulation.
- Identification and enumeration of IL-2-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 Ca^{2+} or Mg^{2+} 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 NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA.

140-001-898.02

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/IL-2/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×g for 10–15 minutes at 20 °C. Carefully aspirate supernatant. Repeat washing step.

2.2 *In vitro* stimulation of PBMCs

1. Wash cells by adding medium, centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cells at a density of 10^7 per mL in culture medium containing 5% human serum. For one test, resuspend 10^6 cells in 100 µL of medium. Plate cells in dishes at a density of 5×10^6 cells/cm^2 (see 5. Appendix: Flask and dish sizes for stimulation).
3. Add 2 µL of CytoStim per 100 µL 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% CO_2.
5. Add 2 µL Brefeldin A per 100 µL of cell suspension and incubate for an additional 4 hours at 37 °C and 5–7% CO_2.
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 *In vitro* stimulation of whole blood

1. Use 250 µL of whole blood per stimulation/control.
2. Add 2 µL of CytoStim or antigen in the appropriate concentration.
3. Mix carefully and incubate cells for 2 hours at 37 °C and 5–7% CO_2.
4. Add 2 µl Brefeldin A and incubate for an additional 4 hours at 37 °C and 5–7% CO_2.
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×g for 10 minutes. Aspirate supernatant completely.
7. Wash cells twice by adding buffer. Centrifuge at 300×g for 10 minutes and carefully remove supernatant.
8. For intracellular staining of cells in suspension, resuspend cells in 500 µL 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 10^6 cells in a volume of 100 µL. 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 cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend 10^6 cells in 500 µL of buffer.
3. Add 500 µL of Inside Fix. Mix well and incubate for 20 minutes at room temperature.
4. Centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
5. 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 at 4–8 °C for up to 1 week.
6. Wash cells by adding 1 mL of Inside Perm. Centrifuge at 300×g for 5 minutes and remove supernatant carefully.
7. Resuspend cells in 80 µL of Inside Perm. Add 10 µL of each of the CD154, Anti-IL-2, 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×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.

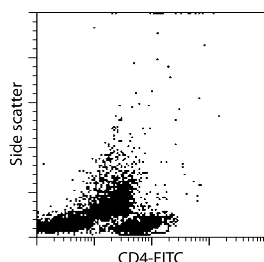
▲ **Note:** Samples may be stored at 4–8 °C in the dark for up to 24 hours.
▲ **Note:** Do not use propidium iodide (PI) or 7-AAD staining.

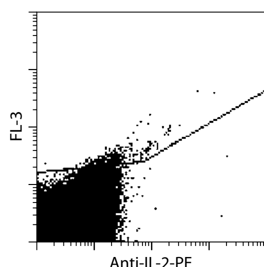
140-001-898.02

Unless otherwise specifically indicated, all Miltenyi Biotec products and services are for research use only and not for diagnostic or therapeutic use.

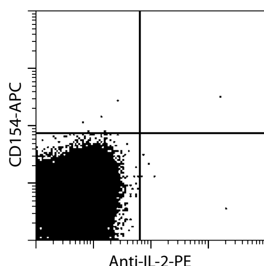
3. Examples of immunofluorescent staining with the CD154/IL-2/CD4 Detection Kit

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-IL-2-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.

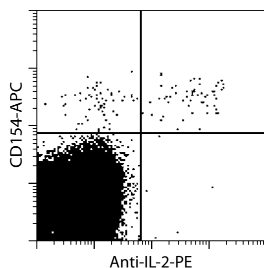




Unstimulated control



CMV pp65-stimulated sample



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.

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/cm^2. 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.

Warranty

The products sold hereunder are warranted only to be free from defects in workmanship and material at the time of delivery to the customer. Miltenyi Biotec GmbH makes no warranty or representation, either expressed or implied, with respect to the fitness of a product for a particular purpose. There are no warranties, expressed or implied, which extend beyond the technical specifications of the products. Miltenyi Biotec GmbH's liability is limited to either replacement of the products or refund of the purchase price. Miltenyi Biotec GmbH is not liable for any property damage, personal injury or economic loss caused by the product.

MACS is a registered trademark of Miltenyi Biotec GmbH.
autoMACS is a trademark of Miltenyi Biotec GmbH.

Ficoll-Paque is a trademark of GE Healthcare companies.

© 2007 Miltenyi Biotec GmbH. Printed in Germany.

140-001-898.02

Unless otherwise specifically indicated, all Miltenyi Biotec products and services are for research use only and not for diagnostic or therapeutic use.