Anti-IL-10 antibodies
human

1. Description

Components	**1 mL monoclonal Anti-IL-10 antibodies, human** conjugated to	
	PE	130-096-043
	APC	130-096-042
	or	
	0.5 mL monoclonal Anti-IL-10 antibodies, human	
	pure – functional grade	130-096-041
Clone	JES3-9D7 (isotype: rat IgG1).	
Capacity	100 tests or up to 10^9 total cells.	
	The functional grade antibody is supplied at a concentration of 1 mg/mL.	
Product format	Antibodies are supplied in buffer containing stabilizer and 0.05% sodium azide.	
	Functional grade antibodies are supplied in phosphate-buffered saline (PBS), pH 7.2. Endotoxin levels have been tested and do not exceed 0.01 ng/µg of protein.	
	The functional grade product contains no preservative and is sterile filtered; always handle under aseptic conditions.	
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.	

Cross-reactivity: The Anti-IL-10 antibody has been reported to react with IL-10 produced by cells from rhesus monkey (*Macaca mulatta*).

1.1 Background information

Interleukin 10 (IL-10) is a cytokine predominantly secreted by $CD4^+$ memory and effector T cells and antigen-presenting cells, for example, monocytes/macrophages. It is believed to have important suppressive functions on immune responses and may also be involved in the maintenance of tolerance.

Anti-IL-10 antibodies are designed for intracellular staining of IL-10–producing cells. Cells can be stimulated for IL-10 production, for example, by polyclonal stimulation with mitogens. For induction of IL-10 production by antigen-specific T cells, cells are restimulated with respective antigen. IL-10 can be accumulated in the cells by addition of secretion inhibitors like brefeldin A. After fixation and permeabilization of the cell sample, IL-10–producing cells can be stained intracellularly with Anti-IL-10 antibodies. Staining of surface markers allows simultaneous flow cytometric analysis of subsets and activation status of the IL-10–producing cells.
Magnetically enriched cells can be stained intracellularly for IL-10 production directly on the MACS® Column. This procedure ensures higher sensitivity of detection and minimizes loss of cells during washing procedures. The protocol is useful for cytokine analysis of rare cells, for example, $CD4^+$ T cells in HIV patients, or other cell sources than PBMCs, e.g., bronchoalveolar lavages, or synovial fluids. For details refer to section 2.3.2.

1.2 Applications

- Flow cytometric identification and enumeration of IL-10–producing cells upon polyclonal stimulation with mitogens.
- Identification and enumeration of IL-10–producing antigen-specific T cells upon restimulation with the respective antigen.
- Monitoring of specificity of antigen-specific T cell lines.
- Analysis of cytokine production of rare cells by solid-phase staining technology, in combination with magnetic enrichment of cells. For details refer to the protocol in section 2.3.2.
- The Anti-IL-10 pure – functional grade antibody is suited for functional assays, for example, neutralization of IL-10 activity.

1.3 Recommended antibody dilution

- Anti-IL-10 antibodies should be used at a dilution of 1:10.

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.

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- Culture medium, for example, RPMI 1640 (# 130-091-440) containing 5% human serum, e.g., autologous or AB serum (do not use BSA or FBS because of non-specific stimulation!).
- Reagents for T cell stimulation, such as phorbol myristate acetate (PMA)/ionomycin, CytoStim (# 130-092-172), staphylococcal enterotoxin B (SEB), antigenic peptide or protein, e.g., peptide pools (PepTivators), or CMV pp65 – Recombinant Protein (# 130-091-824). For details refer to the respective data sheet. For more information about antigens refer to www.miltenyibiotec.com.
- Secretion inhibitor, e.g., brefeldin A.
- Inside Stain Kit (# 130-090-477) for fixation and permeabilization of cells.
- (Optional) Fluorochrome-conjugated antibodies for surface staining, for example, CD4-FITC (# 130-080-501) and/or CD154-PE (# 130-092-289) or CD154-APC (# 130-092-290). For more information about antibodies refer to www.miltenyibiotec.com/antibodies.
- (Optional) FcR Blocking Reagent, human (# 130-059-901) to avoid Fc receptor–mediated antibody labeling.

Additional requirements for intracellular cytokine staining in combination with magnetic cell separation (refer to protocol 2.3.2)

- MACS MicroBeads, e.g., CD4 MicroBeads (# 130-045-101).
- MS Columns and suitable MACS Separator (MiniMACS™, OctoMACS™, VarioMACS™, or SuperMACS™ II Separator).

  ▲ **Note:** Column adapters are required to insert certain columns into the VarioMACS or SuperMACS II Separators. For details refer to the respective MACS Separator data sheet.
- (Optional) Pre-Separation Filters, 30 µm (# 130-041-407) to remove cell clumps.

2. Protocols

2.1 Sample preparation

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.

2.2 *In vitro* stimulation of PBMCs

▲ 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 PMA/ionomycin as high control, CytoStim (# 130-092-172) or staphylococcal enterotoxin B (SEB) as medium control for IL-10–producing cells.

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

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/cm². For details refer to section 4. Appendix: Flask and dish sizes for *in vitro* stimulation of T cells.
3. Add antigen or control reagent:
   1–10 µg/mL peptide
   10–100 µg/mL protein
   20 µL/mL CytoStim
   1 µg/mL SEB
   20 ng/mL PMA and 1 µg/mL ionomycin
4. Incubate cells for 2 hours at 37 °C and 5–7% CO_2.
5. Add 1 µg/mL brefeldin A and incubate for an additional 4 hours at 37 °C and 5–7% 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 Intracellular immunofluorescent cytokine staining

2.3.1 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 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 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. (Optional) Stain cell surface antigens that are sensitive to permeabilization 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.
8. Wash cells by adding 1 mL of Inside Perm (Inside Stain Kit) and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
9. Resuspend cells in 90 µL of Inside Perm. Add 10 µL of the Anti-IL-10 antibody.

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10. (Optional) Add additional staining antibodies to the solution, for example, for the staining of cell surface antigens internalized upon cell activation, such as CD3 and TCR α/β, or for the staining of antigens accumulating in the cell, such as CD154.

    ▲ **Note:** For efficient permeabilization upon intracellular staining the volume of Inside Perm should be at least 5× the volume of staining antibodies.

11. Mix well and incubate for 10 minutes in the dark at room temperature.

12. Wash cells by adding 1 mL of Inside Perm and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.

13. 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.

2.3.2 Intracellular staining in combination with magnetic cell separation (solid phase intracellular staining)

▲ Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ Volumes for magnetic labeling given below are for **up to 10^7** total cells. When working with fewer than 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 total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic separation. Pass cells through a 30 µm nylon mesh (Pre-Separation Filters, 30 µm, # 130-041-407) to remove cell clumps which may clog the column. Wet filter with buffer before use.

▲ The recommended incubation temperature is 2–8 °C. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

▲ Always wait until the column reservoir is empty before proceeding to the next step.

1. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.

2. Resuspend cell pellet in 80 µL of buffer per 10^7 total cells.

3. Add 20 µL of MACS MicroBeads, for example, CD4 MicroBeads (# 130-045-101) per 10^7 total cells.

    ▲ **Note:** For details on the procedure refer to the respective MACS MicroBead data sheet.

4. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).

5. (Optional) Stain cell surface antigens with antibodies that are sensitive to fixation according to the manufacturer's recommendations, for example, add 10 µL of CD4-FITC (# 130-080-501) or CD4-APC (# 130-091-232), mix well, and incubate for an additional 5 minutes at 2–8 °C. Then wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.

6. Resuspend cells in 500 µL of buffer.

7. Place MS Column in the magnetic field of a suitable MACS Separator. For details refer to the MS Column data sheet.

8. Prepare column by rinsing with 500 µL of buffer.

9. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.

10. Wash column with 3×500 µL of buffer. Collect unlabeled cells that pass through and combine with effluent from step 9.

11. Remove column from the separator and place it on a suitable collection tube.

12. Pipette 500 µL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

13. Add 500 µL of Inside Fix to the eluted cell fraction and incubate for 20 minutes at room temperature.

14. Place a second MS Column in the magnetic field of a suitable MACS Separator and prepare column by rinsing with 500 µL of buffer.

15. Apply the fixed cell suspension onto the column.

16. Wash cells by rinsing the column with 1×500 µL of buffer. Then permeabilize cells by rinsing the column with 2×500 µL of Inside Perm.

17. Prepare a solution of 10 µL of Anti-IL-10 antibody and 90 µL of Inside Perm.

18. (Optional) Add additional staining antibodies to the solution, for example, for the staining of cell surface antigens internalized upon cell activation, such as CD3 and TCR α/β, or for the staining of antigens accumulating in the cell, such as CD154.

    ▲ **Note:** Do not exceed the total solution volume of 150 µL.

19. Apply the solution onto the column and incubate for 10 minutes at room temperature.

    ▲ **Note:** The MACS Column has a flow-stop mechanism that will retain the solution in the column.

20. Wash cells by rinsing the column with 2×500 µL of Inside Perm followed by 1×500 µL of buffer.

21. Remove column from the separator and place it on a suitable collection tube.

22. Pipette 500 µL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

23. Cells are now ready for analysis. 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.

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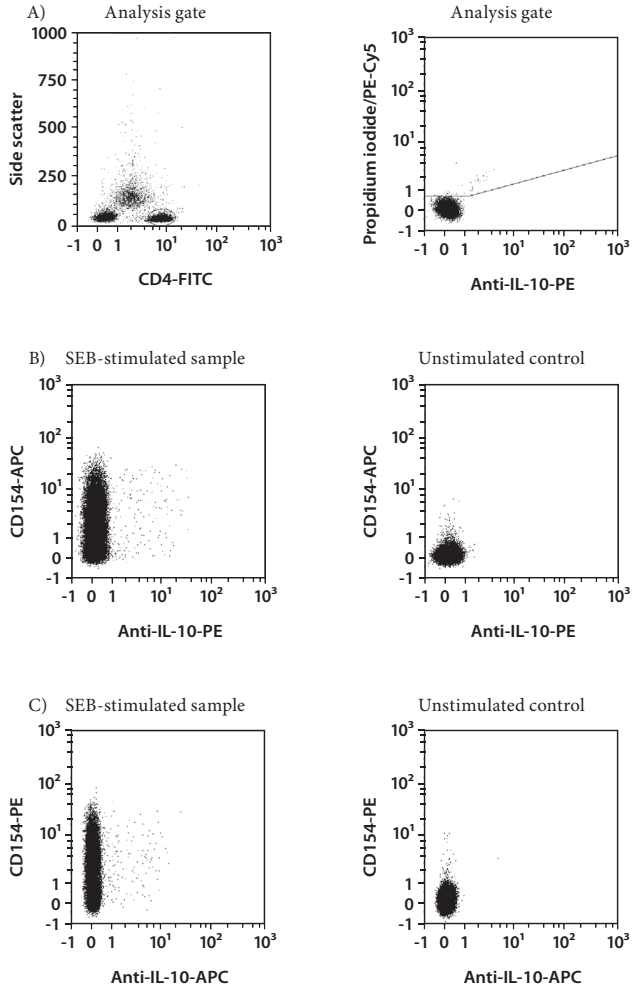
3. Examples of immunofluorescent staining with Anti-IL-10 antibodies

Human PBMCs were incubated with and without CytoStim for 6 hours. After 2 hours, brefeldin A was added. The cells were fixed, permeabilized, and intracellularly stained with Anti-IL-10 antibodies conjugated to PE (B) or APC (C) and analyzed by flow cytometry using the MACSQuant® Analyzer. Cell surface staining was performed with CD4-FITC (# 130-080-501) and CD154-PE (# 130-092-289) or CD154-APC (# 130-092-290). Gating was performed according to the CD4 expression and side scatter properties of the cells. Autofluorescent cell debris was excluded in an FL-3 versus FL-4 dot plot.



4. Appendix: Flask and dish sizes for *in vitro* stimulation of T cells

For *in vitro* stimulation of T 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/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
0.50×10^7	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 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

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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