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## 1. Description

<b>Components</b>	<p><b>1 mL monoclonal Anti-IL-4 antibodies, human conjugated to PE</b> 130-091-647</p> <p>or</p> <p><b>0.5 mL monoclonal Anti-IL-4 antibodies, human pure – functional grade</b> 130-095-753</p>
<b>Clone</b>	7A3-3 (isotype: mouse IgG1).
<b>Capacity</b>	100 tests or up to 10 <sup>9</sup> total cells.
<b>Product format</b>	<p>Antibodies are supplied in buffer containing stabilizer and 0.05% sodium azide.</p> <p>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.</p> <p><i>The functional grade product contains no preservative and is sterile filtered; always handle under aseptic conditions.</i></p>
<b>Storage</b>	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-4 antibody has been tested to react with IL-4 produced by a subset of peripheral blood mononuclear cells (PBMCs) from rhesus monkey (*Macaca mulatta*) and cynomolgus monkey (*Macaca fascicularis*) cells.

### 1.1 Background information

Interleukin-4 (IL-4) is a cytokine predominantly secreted by CD4<sup>+</sup> effector and memory T cells, basophils, and mast cells. IL-4 especially induces and supports humoral responses, e.g., by its effects on activation, proliferation, and antibody production of B cells.

The Anti-IL-4 antibody is designed for intracellular staining of IL-4-producing cells. Cells can be stimulated for IL-4-production, e.g., by polyclonal stimulation with mitogens. For induction of IL-4 production by antigen-specific T cells, cells are restimulated with respective antigen. IL-4 can be accumulated in the cells by addition of secretion inhibitors like brefeldin A. After fixation and permeabilization of the cell sample, IL-4-producing cells can be stained intracellularly with the Anti-IL-4 antibody. Staining of surface markers allows simultaneous flow cytometric analysis of subsets and activation status of the IL-4-producing cells. Magnetically enriched cells can be stained intracellularly for IL-4 production directly on the MACS® Column. This procedure ensures higher sensitivity of detection and minimizes loss of cells during washing procedures. The protocol is very useful for cytokine analysis of rare cells, e.g., CD4<sup>+</sup> T cells in HIV patients, or other cell sources than PBMCs, e.g., bronchoalveolar lavages, or synovial fluids (refer to protocol 2.3.2).

### 1.2 Applications

- Identification and enumeration of IL-4-producing cells upon polyclonal stimulation with mitogens by flow cytometry.
- Identification and enumeration of IL-4-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 section 2.3.2.
- The Anti-IL-4 pure – functional grade antibody is suited for functional assays, for example, neutralization of IL-4 activity.

### 1.3 Recommended antibody dilution

- Anti-IL-4 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  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  are not recommended for use.

- Culture medium, e.g., RPMI 1640 (# 130-091-440) containing 5% human serum, for example, autologous 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), staphyl-ococcal enterotoxin B (SEB), antigenic peptide or protein, e.g., 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](http://www.miltenyibiotec.com).
- Secretion inhibitor, e.g., brefeldin A.
- Inside Stain Kit (# 130-090-477) for the fixation and permeabilization of cells.
- Fluorochrome-conjugated antibodies for cell surface staining, e.g., CD4-APC (# 130-091-232), and/or CD8-FITC (# 130-080-601). For more information about antibodies refer to [www.miltenyibiotec.com/antibodies](http://www.miltenyibiotec.com/antibodies).

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

- MACS MicroBeads of choice, 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  $\mu\text{m}$  (# 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  $200\times g$  for 10–15 minutes at 20 °C. Carefully aspirate supernatant. Repeat washing step.

When working with tissues, prepare a single-cell suspension using standard methods.

For details refer to the protocols section at [www.miltenyibiotec.com/protocols](http://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 SEB as medium control for IL-4-producing cells.

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

1. Wash cells by adding medium and centrifuge at  $300\times g$  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  $5\times 10^6$  cells/cm<sup>2</sup>. For details refer to section 4. Appendix: Flask and dish sizes for *in vitro* stimulation of T cells.
3. Add an antigen or control reagent:  
1–10  $\mu\text{g/mL}$  peptide  
10–100  $\mu\text{g/mL}$  protein  
20  $\mu\text{L/mL}$  CytoStim  
1  $\mu\text{g/mL}$  SEB  
20 ng/mL PMA and 1  $\mu\text{g/mL}$  ionomycin
4. Incubate cells for 2 hours at 37 °C and 5–7%  $\text{CO}_2$ .
5. Add 1  $\mu\text{g/mL}$  brefeldin A and incubate for an additional 4 hours at 37 °C and 5–7%  $\text{CO}_2$ .
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 protocols

#### 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  $2\times 10^7$  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  $300\times g$  for 10 minutes. Aspirate supernatant completely.
2. (Optional) Stain cell surface antigens that are sensitive to fixation with appropriate antibodies according to the manufacturer's recommendations. Then wash cells by adding 1–2 mL of buffer and centrifuge at  $300\times g$  for 10 minutes. Aspirate supernatant completely.
3. Resuspend up to  $10^7$  cells in 500  $\mu\text{L}$  of buffer.
4. Add 500  $\mu\text{L}$  of Inside Fix (Inside Stain Kit). Mix well and incubate for 20 minutes in the dark at room temperature.
5. Centrifuge at  $300\times g$  for 5 minutes. Aspirate supernatant carefully.
6. 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 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  $300\times g$  for 10 minutes. Aspirate supernatant completely.
8. Wash cells by adding 1 mL of Inside Perm (Inside Stain Kit) and centrifuge at  $300\times g$  for 5 minutes. Aspirate supernatant carefully.
9. Resuspend cells in 90  $\mu\text{L}$  of Inside Perm. Add 10  $\mu\text{L}$  of the Anti-IL-4 antibody.

- (Optional) Add additional staining antibodies to the solution, for example, for antigens which are internalized upon cell activation such as CD3 and TCR  $\alpha/\beta$ , or which are accumulating in the cell like CD69.

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

- Mix well and incubate for 10 minutes in the dark at room temperature.
- Wash cells by adding 1 mL of Inside Perm and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
- 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.

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic labeling. Pass cells through 30  $\mu\text{m}$  nylon mesh (Pre-Separation Filters, 30  $\mu\text{m}$  # 130-041-407) to remove cell clumps which may clog the column. Moisten 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.

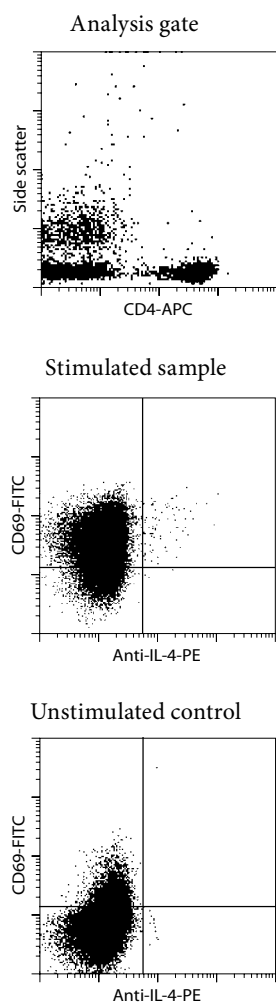
▲ Always wait until the column reservoir is empty before proceeding to the next step.

- 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.
- Resuspend cell pellet in 80  $\mu\text{L}$  of buffer per  $10^7$  total cells.
- Add 20  $\mu\text{L}$  of MACS MicroBeads, e.g., CD4 MicroBeads (# 130-045-101), per  $10^7$  total cells.  
▲ **Note:** For details on the procedure refer to the respective MACS MicroBeads data sheet.
- Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
- (Optional) Add surface staining antibodies, e.g., add 10  $\mu\text{L}$  of CD4-APC (# 130-091-232), and incubate for an additional 5 minutes at 2–8 °C.
- Wash cells by adding 1–2 mL of buffer per  $10^7$  cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.

- Resuspend cells in 500  $\mu\text{L}$  of buffer.
- Place MS Column in the magnetic field of a suitable MACS Separator.
- Prepare column by rinsing with 500  $\mu\text{L}$  of buffer.
- Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
- Wash column with 3×500  $\mu\text{L}$  of buffer. Collect unlabeled cells that pass through and combine with effluent from step 10.
- Remove column from the separator and place it on a suitable collection tube.
- Pipette 500  $\mu\text{L}$  of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
- Add 500  $\mu\text{L}$  of Inside Fix to the eluted cell fraction and incubate for 20 minutes at room temperature.
- Place a second MS Column in the magnetic field of a suitable MACS Separator and prepare column by rinsing with 500  $\mu\text{L}$  of buffer.
- Apply the fixed cell suspension onto the column.
- Wash cells by rinsing the column with 1×500  $\mu\text{L}$  of buffer, followed by 2×500  $\mu\text{L}$  of Inside Perm.
- Prepare a solution of 10  $\mu\text{L}$  of Anti-IL-4 antibodies and 90  $\mu\text{L}$  of Inside Perm.
- (Optional) Add additional staining antibodies to the solution, e.g., for antigens which are internalized upon cell activation such as CD3 and TCR  $\alpha/\beta$ , or which are accumulating in the cell like CD69.  
▲ **Note:** Do not exceed the total solution volume of 150  $\mu\text{L}$ .
- 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.
- Wash cells by rinsing the column with 2×500  $\mu\text{L}$  of Inside Perm followed by 1×500  $\mu\text{L}$  of buffer.
- Remove column from the separator and place it on a suitable collection tube.
- Pipette 500  $\mu\text{L}$  of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
- 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.

### 3. Examples of immunofluorescent staining with Anti-IL-4 antibodies

Human PBMCs were incubated with or without CytoStim for 6 hours. After 2 hours, brefeldin A was added. Cells were then fixed, permeabilized, and intracellularly stained with Anti-IL-4-PE and analyzed by flow cytometry. Cell surface staining was performed with CD4-APC (# 130-091-232) and CD69-FITC. Gating was performed according to CD4 expression and side scatter properties of the cells.



### 5. 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  $5 \times 10^6$  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
$0.15 \times 10^7$	0.15 mL	96 well	0.64 cm
$0.50 \times 10^7$	0.50 mL	48 well	1.13 cm
$1.00 \times 10^7$	1.00 mL	24 well	1.60 cm
$2.00 \times 10^7$	2.00 mL	12 well	2.26 cm
$5.00 \times 10^7$	5.00 mL	6 well	3.50 cm

Total cell number	Medium volume to add	Culture dish	Dish diameter
$4.5 \times 10^7$	4.5 mL	small	3.5 cm
$10.0 \times 10^7$	10.0 mL	medium	6 cm
$25.0 \times 10^7$	25.0 mL	large	10 cm
$50.0 \times 10^7$	50.0 mL	extra large	15 cm

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

All protocols and data sheets are available at [www.miltenyibiotec.com](http://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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