Miltenyi Biotec

Anti-IL-2 antibodies
human

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1. Description

Components	1 mL monoclonal Anti-IL-2 antibodies, human conjugated to various dyes. PE 130-091-646 APC 130-091-644 APC-Vio770™ 130-097-011
Clone	N7.48A (isotype: mouse IgG2a).
Capacity	100 tests or up to 10^9 total cells.
Product format	Antibodies are supplied in buffer containing stabilizer and 0.05% sodium azide.
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-2 antibody has been reported to react with IL-2 produced by a subset of peripheral blood mononuclear cells (PBMCs) from rhesus monkey (*Macaca mulatta*) cells.

1.1 Background information

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. Quantitative analysis of IL-2–producing cells can provide important information on the course of immune responses.
Anti-IL-2 antibodies are designed for intracellular staining of IL-2–producing cells. Cells can be stimulated for IL-2 production, for example, by polyclonal stimulation with mitogens. For induction of IL-2 production by antigen-specific T cells, cells are restimulated with respective antigen. IL-2 can be accumulated in the cells by addition of secretion inhibitors like brefeldin A. After fixation and permeabilization of the cell sample, IL-2–producing cells can be stained intracellularly with Anti-IL-2 antibodies. Staining of surface markers allows simultaneous flow cytometric analysis of subsets and activation status of the IL-2–producing cells.
Magnetically enriched cells can be stained intracellularly for IL-2 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, for example, bronchoalveolar lavages, or synovial fluids. For details refer to section 2.3.2.

1.2 Applications

- Flow cytometric identification and enumeration of IL-2–producing cells upon polyclonal stimulation with mitogens.
- Identification and enumeration of IL-2–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.

1.3 Recommended antibody dilution

- Anti-IL-2 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.
- Culture medium, for example, RPMI 1640 (# 130-091-440) containing 5% human serum, for example, 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, staphylococcal enterotoxin B (SEB), antigenic peptite or protein, e.g., peptide pools (PepTivator® Peptide Pools), or CMV pp65 – Recombinant Protein (# 130-091-824). For details refer to the respective

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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 the fixation and permeabilization of cells.
- Fluorochrome-conjugated antibodies for surface staining, e.g., CD4-PE (# 130-091-231), and/or CD8-FITC (# 130-080-601) or CD69-FITC (# 130-092-166). For more information about antibodies refer to 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.
- 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

To detect and isolate cytokine-secreting cells, best results are achieved by starting the assay with fresh PBMCs or with other leukocyte-containing single-cell preparations from tissues or cell lines. Alternatively, frozen cell preparations can be used. For details refer to the protocols section at www.miltenyibiotec.com/protocols.

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.

2.2 Protocol for *in vitro* stimulation of PBMCs

▲ Always include a negative control in the experiment. The sample 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, such as a sample stimulated with PMA/ionomycin as high control or SEB as medium control for IL-2–producing cells.

▲ Do not use media containing any 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 4. Appendix: Flask and dish sizes for *in vitro* T cell stimulation.
3. Add antigen or control reagent:

   1–10 µg/mL peptide
   10–100 µg/mL protein
   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 in the dark 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 at 2–8 °C for up to 1 week.
7. (Optional) Stain cell surface antigens with antibodies that are sensitive to permeabilization according to the manufacturer's recommendations, for example, resuspend 10^6 cells in 100 µL of buffer, add 10 µL of CD4-PE (# 130-091-231), mix well, and incubate for 10 minutes at 2–8 °C. 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). Centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.

10. Resuspend cells in 90 µL of Inside Perm. Add 10 µL of the Anti-IL-2 antibody.
11. (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 CD69.
12. Mix well and incubate for 10 minutes in the dark at room temperature.
13. Wash cells by adding 1 mL of Inside Perm and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
14. 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

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cytometric data 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 labeling. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, 30 µm # 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.

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 of choice, e.g., 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) Counterstain cell surface antigens with antibodies that are sensitive to fixation according to the manufacturer's recommendations.
6. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend cells in 500 µL of buffer.
8. Place MS Column in the magnetic field of a suitable MACS Separator.
9. Prepare column by rinsing with 500 µL of buffer.
10. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
11. Wash column with 3×500 µL of buffer. Collect unlabeled cells that pass through and combine with effluent from step 10.
12. Remove column from the separator and place it on a suitable collection tube.
13. Pipette 500 µL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
14. Add 500 µL of Inside Fix to the eluted cell fraction and incubate for 20 minutes at room temperature.
15. Place a second MS Column in the magnetic field of a suitable MACS Separator and prepare column by rinsing with 500 µL of buffer.
16. Apply the fixed cell suspension onto the column.
17. Wash cells by rinsing the column with 1×500 µL of buffer, followed by 2×500 µL of Inside Perm.
18. Prepare a solution of 10 µL of Anti-IL-2 antibodies and 90 µL of Inside Perm.
19. (Optional) Add additional staining antibodies to the solution, e.g., for the staining of cell surface antigens internalized upon cell activation or antigens which accumulate in the cell.

    ▲ **Note:** Do not exceed the total solution volume of 150 µL.
20. Apply the solution onto the column and incubate for 10 minutes at room temperature.

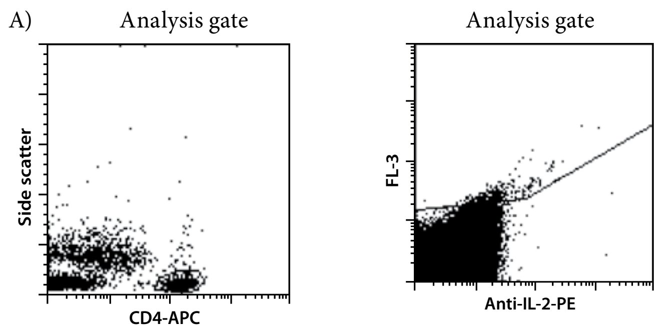
    ▲ **Note:** The MACS Column has a flow-stop mechanism that will retain the solution in the column.
21. Wash cells by rinsing the column with 2×500 µL of Inside Perm followed by 1×500 µL of buffer.
22. Remove column from the separator and place it on a suitable collection tube.
23. Pipette 500 µL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
24. 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-2 antibodies

Human PBMCs were incubated with and without SEB for 6 hours. After 2 hours, brefeldin A was added.
Cells were then fixed, permeabilized, and intracellularly stained with Anti-IL-2 antibodies conjugated to PE (B), APC (C), or APC-Vio770 (D) as described. Cell surface staining was performed with CD4-PE (# 130-091-231) or CD4-APC (# 130-091-232) and CD69-FITC (# 130-092-166). Cells were analyzed by flow cytometry. Gating was performed according to the CD4-expression and side scatter properties of the cells. Autofluorescent cell debris was excluded in an fluorescence 2 versus fluorescence 3 dot plot (A).



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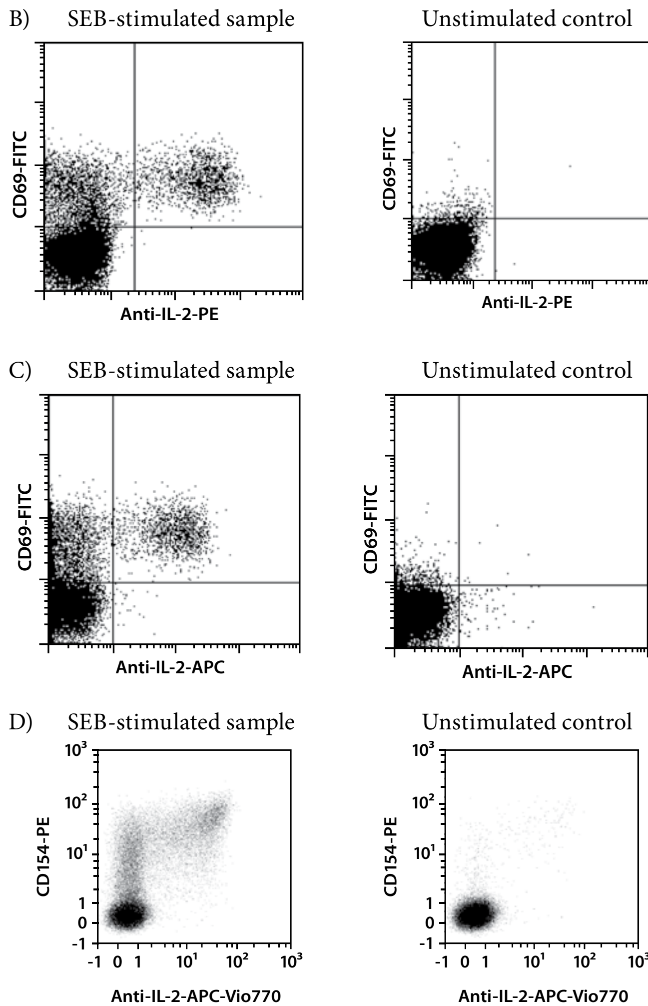

4. Appendix: Flask and dish sizes for *in vitro* T cell stimulation

For *in vitro* T cell stimulation (refer to 2.2) the cells should be resuspended in culture medium, containing 5% of human serum, at 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.

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