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1. Description

Components	1 mL Anti-IL-12 (p40/p70) antibodies, human conjugated to various dyes.
	PE 130-092-774
	APC 130-092-775
	or
	0.5 mL Anti-IL-12 (p40/p70) antibodies, human
	pure – functional grade 130-095-755
Clone	C8.6 (isotype: mouse 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. <i>The functional grade product contains no preservative and is sterile filtered; always handle under aseptic conditions.</i>
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-12 (p40/p70) antibody has been tested to react with rhesus monkey (*Macaca mulatta*) and cynomolgus monkey (*Macaca fascicularis*) cells.

1.1 Background information

Interleukin-12 (IL-12) is a 70–75 kDa heterodimeric cytokine composed of a 40 kDa heavy chain and a 35 kDa light chain. It is produced mainly by monocytes and macrophages as well as dendritic cells primarily in response to stimulation with bacterial or viral antigens. IL-12 has also been described to be produced by non-hematopoietic cells such as keratinocytes and epidermoid carcinomas.

IL-12 displays various effects on both NK and T cells, playing an essential role in the interaction between innate and adaptive immunity: IL-12 induces the production of IFN-γ alone or in synergy with IL-2, is a co-stimulator of both NK and T cell proliferation, and enhances cytolytic activity of both cell types. Moreover, IL-12 promotes the development of T helper 1 (Th1)-specific immune responses by promoting the differentiation of CD4⁺ cells towards a Th1 phenotype while inhibiting the production of Th2 cells.

The antibody clone C8.6 reacts with the p40 kDa chain and the p70 kDa heterodimer of human, rhesus monkey, and cynomolgus monkey IL-12.

1.2 Applications

- Identification and enumeration of IL-12–producing activated dendritic cells, monocytes, and macrophages by flow cytometry or fluorescence microscopy.
- Analysis of cytokine production of rare cells by solid-phase staining technology, in combination with magnetic enrichment of cells. For details refer to protocol in section 2.3.2.
- The Anti-IL pure – functional grade antibody is suited for functional assays, for example, neutralization of IL-12 p40/p70 activity.

1.3 Recommended antibody dilution

- Anti-IL-12 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 Ca²⁺ or Mg²⁺ are not recommended for use.
- Culture medium, for example, RPMI 1640 (# 130-091-440) containing 5% autologous human serum (do not use BSA or FBS due to non-specific stimulation!).

- Reagents for lymphocyte stimulation, such as lipopolysaccharide (LPS) and R-848.
- Secretion inhibitor, e.g. brefeldin A.
- (Optional) FcR Blocking Reagent, human (# 130-059-901) to avoid Fc receptor-mediated antibody labeling.
- (Optional) Inside Stain Kit (# 130-090-477) for the fixation and permeabilization of cells.
- (Optional) CD14-FITC (# 130-080-701). 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

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 exactly the same as the stimulated sample, except for the addition of the stimulus.

▲ A positive control may also be included in the experiment, for example, a sample stimulated with lipopolysaccharide (LPS) and R-848.

▲ 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 10⁷ per mL in culture medium containing 5% human serum. Plate cells in dishes at a density of 5×10⁶ cells/cm². For details refer to 4. Appendix: Flask and dish sizes for *in vitro* stimulation.
3. Add 10 ng/mL of LPS.
4. Incubate cells for 1 hour at 37 °C and 5–7% CO₂.
5. Add 2.5 µg/mL of R-848 and incubate for 1 hour at 37 °C and 5–7% CO₂.

6. Add 2 µg/mL of brefeldin A and incubate for an additional 4 hours at 37 °C and 5–7% CO₂.
7. 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 10⁶ cells per sample. 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 2×10⁷ nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

1. Wash up to 10⁷ 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 10⁷ 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 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. 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-12 (p40/p70) antibody.
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 CD69.
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 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 given below are for up to 10^7 nucleated 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 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

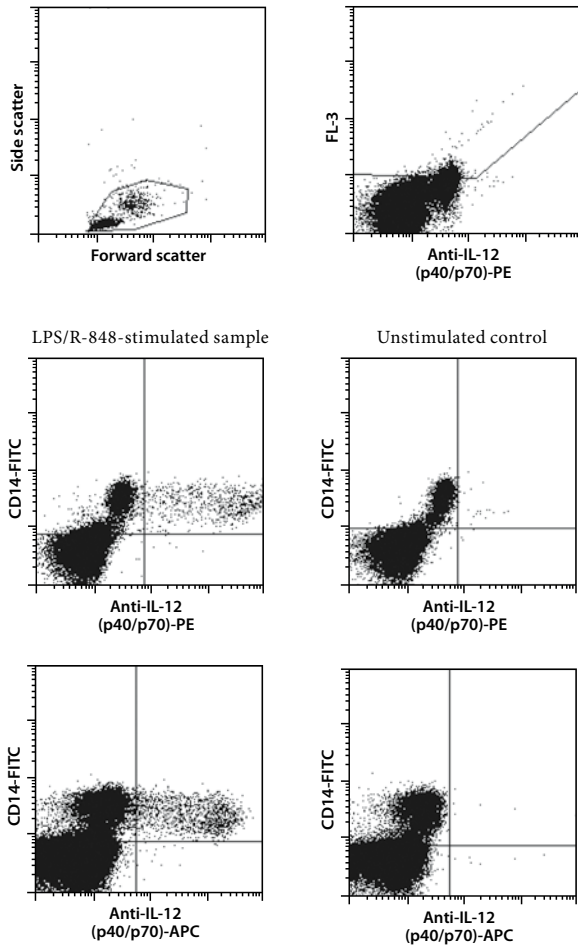
▲ For optimal performance it is important to obtain a single-cell suspension before magnetic separation. Pass cells through 30 μ m nylon mesh (Pre-Separation Filters, 30 μ m, # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

1. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge cell suspension at 300 \times g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 80 μ L of buffer per 10^7 total cells.
3. Add 20 μ L of MACS MicroBeads 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).
▲ Note: Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.
5. (Optional) Stain cell surface antigens with antibodies that are sensitive to fixation according to the manufacturer's recommendations. Then wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300 \times g for 10 minutes. Aspirate supernatant completely.
6. Resuspend cell pellet in 500 μ L 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 μ L of buffer.
9. Apply cell suspension onto the column.
10. Collect unlabeled cells that pass through and wash column with 3 \times 500 μ L of buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty.

11. Remove column from the separator and place it on a suitable collection tube.
12. Pipette 500 μ L of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
13. Add 500 μ L 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 μ L of buffer.
15. Apply the fixed cell suspension onto the column.
16. Wash cells by rinsing the column with 1 \times 500 μ L of buffer, followed by 2 \times 500 μ L of Inside Perm.
17. Prepare a solution of 10 μ L of Anti-IL-12 (p40/p70) antibodies and 90 μ L 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 or for the staining of antigens accumulating in the cell, such as CD69.
▲ Note: Do not exceed the total solution volume of 150 μ L.
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 \times 500 μ L of Inside Perm followed by 1 \times 500 μ L of buffer.
21. Remove column from the separator and place it on a suitable collection tube.
22. Pipette 500 μ L 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 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.

3. Examples of immunofluorescent staining with Anti-IL-12 (p40/p70) antibodies

Human peripheral blood mononuclear cells (PBMCs) were incubated with and without LPS and R-848 for 6 hours. After 2 hours, brefeldin A was added. Cells were then fixed, permeabilized, and intracellularly stained with Anti-IL-12 (p40/p70) antibodies conjugated to PE or APC. Cell surface staining was performed with CD14-FITC (# 130-080-701). Cells were analyzed by flow cytometry. A lymphocyte gate was activated based on the scatter properties of the cells. Autofluorescent cell debris was excluded in an FL-2 versus FL-3 dot plot.



4. References

1. Trinchieri, G. (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3: 133–146.
2. D’Andrea, A. *et al.* (1992) Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J. Exp. Med.* 176: 1387–1398.

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

For *in vitro* stimulation (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/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
0.50×10^7	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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