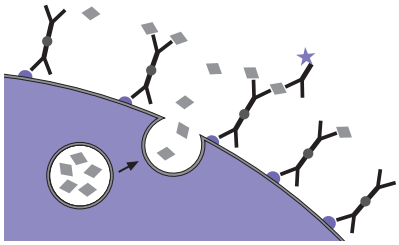
Miltenyi Biotec

IL-12 Secretion Assay – Detection Kit (PE)

human

For 100 tests with 10^6 cells

Order no. 130-092-124



Miltenyi Biotec

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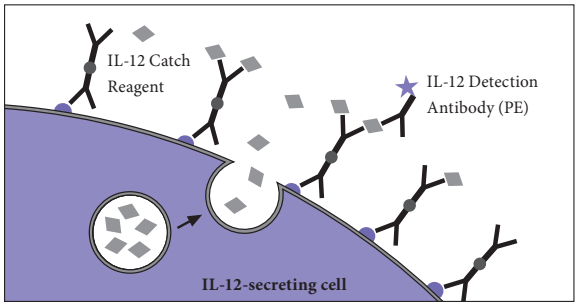
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1. Description

Components	**1 mL IL-12 Catch Reagent, human:** anti-IL-12 monoclonal antibody (mouse IgG1) conjugated to cell surface–specific monoclonal antibody (mouse IgG2a). **1 mL IL-12 Detection Antibody, human:** anti-IL-12 monoclonal antibody (mouse IgG1) conjugated to PE (R-phycoerythrin).
Size	For 100 tests with 10^6 cells.
Product format	All components 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 dates are indicated on the vial labels.

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1.1 Principle of the IL-12 Secretion Assay

IL-12–secreting cells are analyzed using the IL-12 Secretion Assay starting from whole blood, peripheral blood mononuclear cells (PBMCs), or other leukocyte containing single-cell preparations.

The cells are restimulated for a certain period of time with the desired stimulus. Subsequently, an IL-12–specific Catch Reagent is attached to the cell surface of all leukocytes. The cells are then incubated for a short time at 37 °C to allow cytokine secretion. The secreted IL-12 binds to the IL-12 Catch Reagent on the positive, secreting cells. These cells are subsequently labeled with a second IL-12–specific antibody, the IL-12 Detection Antibody conjugated R-phycoerythrin (PE) for sensitive detection by flow cytometry. Since viable cells are analyzed, non-specific background can be minimized by dead cell exclusion. This provides highest sensitivity of analysis.

1.2 Background information

Interleukin 12 (IL-12) consists of a p35 subunit and a p40 subunit that form the biologically active heterodimer IL-12p70. It is produced mainly by monocytes and macrophages, but also by dendritic cells, primarily in response to stimulation with bacterial or viral antigens.

IL-12p70 is produced, e.g., in response to LPS with costimulation of T helper cells through CD40L or IFN-γ.[1] Moreover, monocytes can be induced to synthesize IL-12p70 without costimulation of T cells, through combined stimulation with toll-like receptor (TLR) TLR-4 and TLR-8 ligands.[2] IL-12 displays various effects on both T and NK cells, playing an essential role in the interaction between innate and adaptive immunity.

The IL-12 Cytokine Secretion Assay is designed to detect cells, secreting the active heterodimer IL-12p70.

Quantitative analysis of IL-12–secreting leukocytes can provide important information on the natural course of immune responses.

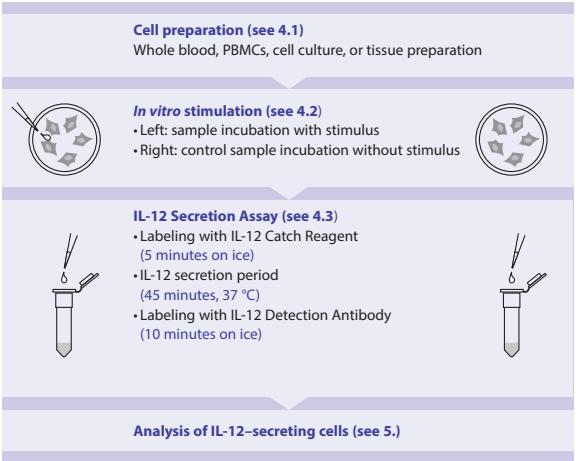
1.3 Applications

- Detection of viable IL-12–secreting leukocytes.
- Detection of IL-12–secreting cells for enumeration and phenotypic analysis.
- Monitoring and analysis of the stimulatory or regulatory functions of cytokine-secreting monocytes, macrophages, and dendritic cells.

1.4 Reagent and instrument 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). Degas buffer before use, as air bubbles could block the column.
- Culture medium, e. g., RPMI, containing 5% human serum, like autologous or AB serum (do not use BSA or FCS because of non-specific stimulation!).
- Propidium Iodide Solution (# 130-093-233) or 7-AAD Staining Solution (# 130-111-568) for flow cytometric exclusion of dead cells.
- Dead Cell Removal Kit (# 130-090-101) for the depletion of dead cells.
- Refrigerated centrifuge (2–8 °C).
- Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753).
- (Optional) Cell stimulation reagents, for example, LPS and R-848 for restimulation of human cells.
- (Optional) Staining reagents such as CD14-FITC, CD14-APC, CD11c-FITC, or CD11c-APC. For more information about fluorochrome-conjugated antibodies see www.miltenyibiotec.com.

2. Protocol overview



3. Experimental setup

3.1 Controls

Negative control
For accurate detection of IL-12–secreting cells, a negative control sample should always be included. This will provide information about IL-12 secretion unrelated to the *in vitro* stimulation, but, for example, due to ongoing *in vivo* immune responses. The control sample should be treated exactly the same as the antigen-stimulated sample except for the addition of stimulus, or by using a control stimulus.

3.2 Counterstaining of cytokine-secreting cells

The IL-12–secreting cells are stained with PE-conjugated IL-12 Detection Antibody. To identify cells of interest, counterstaining for cells with, for example, CD14-FITC, CD14-APC, CD11c-FITC, or CD11c-APC is important.

▲ The samples should be stained with Propidium Iodide Solution (# 130-093-233) or 7-AAD Staining Solution (# 130-111-568) prior to acquisition, to exclude dead cells from analysis. This will reduce non-specific background staining and increase sensitivity.

3.3 Two-color cytokine analysis

IL-12–secreting cells can be analyzed simultaneously for, e.g., IL-10 production by two-color cytokine analysis combining the IL-12 Secretion Assay – Detection Kit (PE) with the IL-10 Secretion Assay – Detection Kit (APC) (# 130-090-761). Detailed protocols are included in the data sheets of the Cytokine Secretion Assay – Detection Kits (APC) and are available at www.miltenyibiotec.com/protocols.

4. Protocol for the IL-12 Secretion Assay

4.1 Cell 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.

▲ **Note:** PBMCs may be stored overnight. The cells should be resuspended and incubated in culture medium as described in 4.2 step 2. However, the stimulus should not be added to the culture until the next day.

▲ **Note:** Remove platelets after density gradient separation. Resuspend cell pellet, fill tube with buffer, and mix. Centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully remove supernatant.

Special protocols for whole blood: You can start the IL-12 Secretion Assay directly from whole blood. For details on the procedure refer to www.miltenyibiotec.com/protocols.

4.2 *In vitro* stimulation

▲ Always include a negative control in the experiment (see 3.1). A positive control may also be included.

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



Protocol for *in vitro* stimulation

1. Wash cells by adding medium, centrifuge at 300×g for 10 minutes.
2. Resuspend cells in culture medium, containing 5% human serum, adjust to 10^6 cells/mL and 5×10^5 cells/cm² (see 7. Appendix A: Flask and dish sizes for *in vitro* stimulation).
3. Add stimulus or control reagent, for example:

   LPS (TLR-4 agonist) and R-848 (TLR-7/-8 agonist): LPS (e.g. 1 µg/mL) and R-848 (e.g. 2.5 µg/mL) for 4 hours at 37 °C, 5–7% CO_2.
   For comparison of different experiments, the stimulation time should always be the same.
4. Collect cells carefully by using a cell scraper. Rinse the dish with cold buffer. Check microscopically for any remaining cells, if necessary, rinse the dish again.

4.3 Cytokine Secretion Assay

General considerations

▲ The assay is optimized for cell samples containing <5% of total IL-12–secreting cells. If ≥5% of IL-12–secreting cells are expected, it is necessary to dilute the cells further during the cytokine secretion period, and therefore a larger test tube will be needed (see table below). The dilution prevents non-specific staining of cells not secreting IL-12 during this period.

▲ For each test with 10^6 total cells, prepare:
50 mL of cold buffer (2–8 °C)
100 µL of cold medium (2–8 °C)
2 mL (or 10 mL; see table below) of warm medium (37 °C).

▲ 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 (exception: warm medium during secretion period).

▲ Volumes given below are for up to 10^6 total cells. When working with fewer than 10^6 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 2×10^6 total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ Do not remove supernatant by decanting. This will lead to cell loss and incorrect incubation volumes. Aspirate supernatant.

▲ Dead cells may bind non-specifically to antibodies. Therefore, when working with cell preparations containing large amounts of dead cells, they should be removed before starting the IL-12 Secretion Assay, e.g., by densitiy gradient centrifugation or by using the Dead Cell Removal Kit (# 130-090-101).



Labeling cells with IL-12 Catch Reagent

1. Use 10^6 total cells in a 15 mL closable tube per sample.
2. Wash cells by adding 10 mL of cold buffer, centrifuge at 300×g for 10 minutes at 2–8 °C, aspirate supernatant completely.
3. Resuspend cell pellet in 90 µL of cold medium per 10^6 total cells.
4. Add 10 µL of IL-12 Catch Reagent per 10^6 total cells, mix well, and incubate for 5 minutes on ice.

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IL-12 secretion period

1. Add warm (37 °C) medium to dilute the cells according to the following table:

Expected number of IL-12–secreting cells	Dilution	Amount of medium to add per 10^6 total cells
<5%	5×10^5 cells/mL	2 mL
≥5%	$\leq10^5$ cells/mL	10 mL

▲ **Note:** For frequencies of cytokine-secreting cells >20% the cells need to be further diluted, e.g., by a factor of 5.

▲ **Note:** Choose an appropriate tube to allow for addition of cold medium to stop the secretion period.

2. Incubate cells in closed tube for 45 minutes at 37 °C under slow continuous rotation using the MACSmix™ Tube Rotator (# 130-090-753), or turn tube every 5 minutes to resuspend settled cells.

▲ **Note:** During this step it is crucial to prevent contact of cells to avoid cross contamination with cytokines.



Labeling cells with IL-12 Detection Antibody

1. Put the tube on ice.
2. Wash the cells by filling up the tube with cold buffer and centrifuge at 300×g for 10 minutes at 2–8 °C. Aspirate supernatant completely.

   ▲ **Note:** If the volume of the cell suspension was higher than the volume of the added buffer, then repeat the wash step.
3. Resuspend cell pellet in 90 µL of cold buffer per 10^6 total cells.
4. Add 10 µL of IL-12 Detection Antibody per 10^6 total cells.
5. (Optional) Add staining antibodies, e.g., CD14-FITC, CD14-APC, CD11c-FITC, or CD11c-APC according to manufacturer's recommendation.
6. Mix well and incubate for 10 minutes on ice.
7. Add 10 mL of cold buffer and centrifuge at 300×g for 10 minutes at 2–8 °C. Aspirate supernatant completely.

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5. Detection and analysis of IL-12–secreting cells

▲ Add Propidium Iodide Solution (# 130-093-233) or 7-AAD to a final concentration of 0.5 µg/mL just prior to acquisition to exclude dead cells from flow cytometric analysis. Incubating with propidium iodide for longer periods will affect the viability of the cells.
Do not fix the cells when using Propidium Iodide Solution or 7-AAD.

▲ For optimized sensitivity, an appropriate number of viable cells has to be acquired from the stimulated sample as well as from the control sample.

- Acquire 2×10^5 viable cells from each sample.

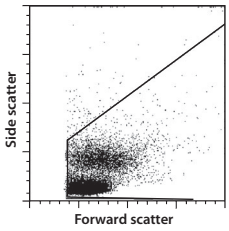
To illustrate the analysis, we describe the detection of IL-12–secreting cells using the IL-12 Secretion Assay. The detailed description, including how to set gates, should serve as a model for the analysis of your own sample.

1. 10^6 human PBMCs have been stimulated for 4 hours with LPS (1 µg/mL) and R-848 (2.5 µg/mL) or have been left untreated.
2. The IL-12 Secretion Assay was performed on the stimulated and the unstimulated sample.
3. Counterstaining of cells was performed using CD11c-APC.
4. Dead cells were stained with Propidium Iodide Solution, which was added just prior to flow cytometric analysis to a final concentration of 0.5 µg/mL.

5. 200,000 viable cells were acquired by flow cytometry, from the stimulated as well as from the unstimulated sample.
6. A lymphocyte gate based on forward and side scatter properties (FSC/SSC) was activated prior to further gating to exclude debris (A).
7. Dead cells were excluded according to PI-staining in a fluorescence 2 (PE) versus fluorescence 3 (PI) plot (B).

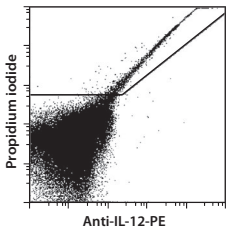
   The dead cell exclusion is crucial for the analysis of rare cells, as dead cells may bind non-specifically to antibodies or MicroBeads. This could lead to false positive events.
8. Analysis of secreted IL-12 (PE) versus CD11c-APC staining of viable leukocytes is displayed (C).

A. Leukocyte gate using FSC versus SSC



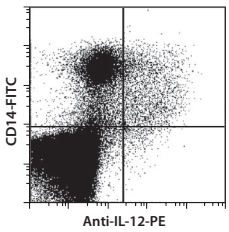
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B. Dead cell exclusion

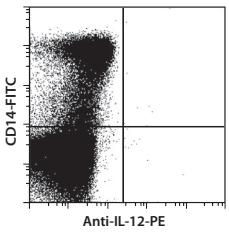


C. Counterstaining of IL-12-secreting cells using CD14-FITC

Sample stimulated with LPS and R-848



Unstimulated control sample



6. References

1. Krug, A. *et al.* (2001) Toll-like receptor reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. Eur. J. Immunol. 31: 3026–3037. [1215]
2. Bekeredjian-Ding, I. *et al.* (2006) T Cell-Independent, TLR-Induced IL-12p70 Production in Primary Human Monocytes. J. Immunol. 176: 7438–7446. [8765]

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

For *in vitro* stimulation (see 4.2) the cells should be resuspended in culture medium, containing 5% of human serum, at a dilution of 10^6 cells/mL. The cells should be plated at a density of 5×10^5 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
0.15×10^6	0.15 mL	96 well	0.64 cm
0.50×10^6	0.50 mL	48 well	1.13 cm
1.00×10^6	1.00 mL	24 well	1.60 cm
2.00×10^6	2.00 mL	12 well	2.26 cm
5.00×10^6	5.00 mL	6 well	3.50 cm
Total cell number	**Medium volume to add**	**Culture dish**	**Dish diameter**
4.5×10^6	4.5 mL	small	3.5 cm
10.0×10^6	10.0 mL	medium	6 cm
25.0×10^6	25.0 mL	large	10 cm
50.0×10^6	50.0 mL	extra large	15 cm
Total cell number	**Medium volume to add**	**Culture flask**	**Growth area**
12×10^6	12 mL	50 mL	25 cm^2
40×10^6	40 mL	250 mL	75 cm^2
80×10^6	80 mL	720 mL	162 cm^2
120×10^6	120 mL	900 mL	225 cm^2

Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

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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