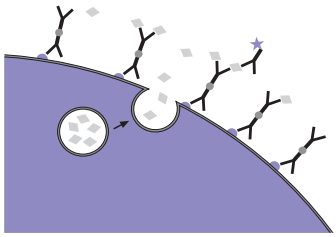
Miltenyi Biotec

Mouse IL-4 Secretion Assay
Detection Kit (PE)

For 100 tests with 10^6 cells Order no. 130-090-479



Miltenyi Biotec

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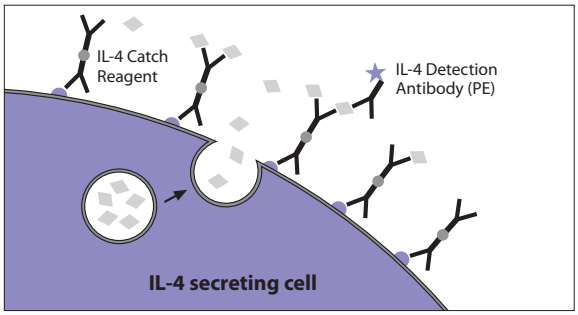
1. Description

Components	1 ml **Mouse IL-4 Catch Reagent**: anti-IL-4 monoclonal antibody (rat IgG1) conjugated to cell surface specific monoclonal antibody (rat IgG2b). 1 ml **Mouse IL-4 Detection Antibody**: anti-IL-4 monoclonal antibody (rat IgG2b) conjugated to PE* (phycoerythrin).
Size	For 100 tests with 10^6 cells
Product format	All components are supplied as a suspension containing 0.1% gelatine and 0.05% sodium azide.
Storage	Store protected from light at 4°C. Do not freeze. The expiration dates are indicated on the vial labels.

1.1 Principle of the Mouse IL-4 Secretion Assay

For analysis of murine antigen-specific T cells using the Mouse IL-4 Secretion Assay, mouse spleen cells or other leukocyte containing single cell preparations are restimulated for a short period of time with specific peptide, protein or other antigen preparations.

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Subsequently, an IL-4-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-4 binds to the IL-4 Catch Reagent on the positive, secreting cells. These cells can subsequently be labeled with a second IL-4-specific antibody, the **Mouse IL-4 Detection Antibody** conjugated to 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 and product applications

The Mouse IL-4 Secretion Assay is designed for the detection and analysis of viable IL-4 secreting murine leukocytes. It is specially developed for the detection of antigen-specific T cells after in vitro restimulation with specific antigen to induce secretion of IL-4.

IL-4 is predominantly secreted by $CD4^+$ memory and effector 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 by B cells.

Quantitative analysis of antigen-specific T cell populations can provide important information on the natural course of immune responses.

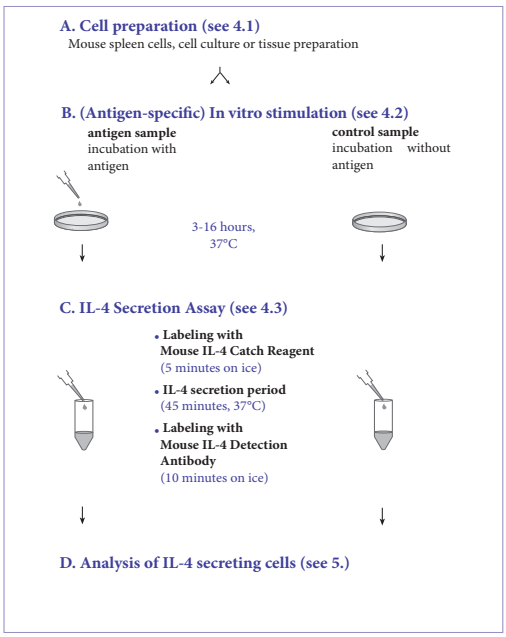
Examples of applications

- Detection of viable IL-4 secreting mouse leukocytes for phenotypic and functional characterisation.
- Detection of IL-4 secreting antigen-specific T cells for enumeration and phenotypic characterization.

1.3 Reagent and instrument requirements

- **Buffer** (degassed): phosphate buffered saline pH 7.2, containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA (e.g. 4 ml of a 0.5 M EDTA stock solution per 1 liter of buffer).
- (Optional) 0.5 M EDTA stock solution: dissolve 56 g sodium hydroxide (NaOH) in 900 ml dd H_2O. Add 146.2 g ethylene-diamine-tetraacetic acid, adjust pH to 7.5, fill up to 1000 ml with dd H_2O.
- **Culture medium**, e.g. RPMI 1640 containing 5% murine serum (do **not** use **BSA** or **FCS** because of non-specific stimulation!).
- **Propidium iodide (PI)** or **7-AAD** to exclude dead cells from the analysis.
- (Optional) Staining reagents such as CD4-FITC or CD8-FITC and CD45R/B220-PerCP™.
- Refrigerated centrifuge (4-8°C).
- Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753).

2. Protocol overview



3. Experimental set-up

3.1 Controls

Negative control
For accurate detection of antigen-specific cells secreting IL-4, a negative control sample should always be included. This will provide information about IL-4 secretion unrelated to the in vitro stimulation with the specific antigen, e.g. due to ongoing in vivo immune response (see section 5.). The control sample should be treated exactly the same as the antigen-stimulated sample except for the addition of antigen, or by using a control antigen.
When working with immunized mice, it could be relevant to include an experiment analyzing cells of a non-immunized mouse (see section 5.).

Positive control
When setting up a new experiment, it is recommended to include a positive control. As a positive control, a sample stimulated with the superantigen Staphylococcal Enterotoxin B (Sigma, St. Louis, USA) 10 µg/ml for 3-16 hours, may be included in the experiment.

3.2 Kinetics of restimulation and proposed time schedule

Peptides
Upon stimulation with peptide, the cells can be analyzed for IL-4 secretion 3-6 hours after onset of stimulation.

Proteins
Upon stimulation with protein, the cells can be analyzed for IL-4 secretion 6-16 hours later.

It is possible to start the stimulation of the cells late in the afternoon, and to perform the IL-4 Secretion Assay the following morning.

Costimulation
The addition of costimulatory agents like CD28 antibody may enhance the response to the antigen. If costimulatory agents are added to the antigen sample, they also have to be included in the control sample.

3.3 Counterstaining of cytokine secreting cells

The IL-4 secreting cells are stained with PE-conjugated Mouse IL-4 Detection Antibodies. To identify cells of interest, counterstaining for T cells with e.g. CD4-FITC or CD8-FITC is important.

▲ Upon activation of T cells, TCR and some associated molecules, like CD3, might be down-regulated.

▲ The samples should be stained with propidium iodide (PI) or 7-AAD prior to acquisition, to exclude dead cells from analysis. This will reduce non-specific background staining and increase sensitivity.

▲ For optimal sensitivity, we recommend labeling of undesired non-T cells such as B cells with antibodies conjugated to PerCP™, e.g. CD45R/B220-PerCP™. These cells can then be excluded together with PI stained dead cells by gating.

3.4 Detection of very low frequencies

(Optional, reagents not included) If the sample contains less than 0.01-0.1% of IL-4 secreting cells, it is possible to enrich these cells magnetically with the Mouse IL-4 Secretion Assay – Enrichment and Detection Kit (# 130-090-515). Thereby it is possible to detect antigen-specific T cells down to frequencies as low as 0.0001% (1 in 10^6).

4. Protocol for the Mouse IL-4 Secretion Assay

4.1 Cell preparation

Mouse spleen preparation
Prepare fresh mouse spleen cells or other leukocyte containing single cell preparations under sterile conditions according to standard protocols. Avoid excess of dead cells.

4.2 In vitro stimulation

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

▲ Do **not use** media containing any non-murine proteins, like BSA or FCS, because of non-specific stimulation.



Protocol for in vitro stimulation

1. Wash cells by adding medium, centrifuge at 200xg for 10 minutes. Pipette off supernatant.
2. Resuspend cells in culture medium at 10^7 cells/ml and $5x10^6$ cells/cm^2 (see 7. Appendix: Flask and dish sizes for stimulation).
3. Add antigen or control reagent:
   peptide: 3-6 hours at 37°C, 7% CO_2, e.g. 1-10 µg/ml
   protein: 6-16 hours at 37°C, 7% CO_2, e.g. 10 µg/ml
   SEB: 3-16 hours at 37°C, 5-7% CO_2, e.g. 10 µg/ml
   For comparison of different experiments, the stimulation time should be kept constant (see 3.2).
4. Collect cells carefully by using a cell scraper, or by pipetting up and down when working with smaller volumes. 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-4 secreting cells. If ≥ 5% of IL-4 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 avoids non-specific staining of cells not secreting IL-4 during this period.

▲ For each test with 10^6 total cells, prepare:

50 ml of **cold buffer** (4-8°C)

100 µl of **cold medium** (4-8°C)

1 ml (or 10 ml; see table below) of **warm medium** (37°C).

▲ Work fast, keep the cells cold, use pre-cooled solutions which will prevent capping of antibodies on the cell surface and a non-specific cell labeling (exception: warm medium during secretion period).

▲ Volumes shown below are for 10^6 total cells. When working with less 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 $2x10^6$ 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. Pipette off or aspirate supernatant.



Labeling cells with Mouse IL-4 Catch Reagent

1. Use 10^6 total cells in a 2 ml closable tube per sample.

   ▲ **Note:** For larger cell numbers, scale up all volumes accordingly. For less than 10^6 cells, use same volumes.

2. Wash cells once by adding 2 ml of **cold buffer**, centrifuge at 300xg for 10 minutes at **4-8°C**, pipette off supernatant completely.

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

3. Repeat wash step, pipette off supernatant completely.
4. Resuspend cell pellet in 90 µl of **cold medium** per 10^6 total cells.
5. Add 10 µl of **Mouse IL-4 Catch Reagent** per 10^6 total cells, mix well and incubate for 5 minutes **on ice**.



IL-4 secretion period

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

Expected number of IL-4 secreting cells	Dilution	Amount of medium to add per 10^6 total cells
< 5 %	10^6 cells/ml	1 ml
≥ 5 %	≤ 10^5 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.

2. Incubate cells in (closed) tube for 45 minutes at 37°C under slow continuous rotation by 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 Mouse IL-4 Detection Antibody

1. Put the tube **on ice**.
2. Wash the cells by filling up the tube with **cold buffer**, centrifuge at 300xg for 10 minutes at **4-8°C**. Pipette off supernatant completely.
3. Repeat wash step, pipette off supernatant completely.
4. Resuspend cell pellet in 90 µl of **cold buffer** per 10^6 total cells.
5. Add 10 µl of **Mouse IL-4 Detection Antibody (PE)** per 10^6 total cells.
6. (Optional) Add additional staining antibodies, e.g. CD4-FITC or CD8-FITC and B220-PerCP™.
7. Mix well and incubate for 10 minutes **on ice**.
8. Wash cells by adding 2 ml of **cold buffer**, centrifuge at 300xg for 10 minutes at **4-8°C**. Pipette off supernatant completely.
9. Resuspend the cells in 500 µl of cold buffer and proceed to analysis (see section 5.).

5. Detection and analysis of IL-4 secreting T cells

▲ Add propidium iodide (PI) or 7-AAD to a final concentration of 0.5 µg/ml **just prior** to acquisition for exclusion of dead cells from flow cytometric analysis. Incubating with PI for longer periods will affect the viability of the cells.
Do not fix the cells when using PI or 7-AAD.

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

- Acquire $2x10^5$ events from each sample.

To illustrate the analysis, we describe the detection of IL-4 secreting T cells by using the Mouse IL-4 Secretion Assay. The detailed description, including how to set gates, may serve as a model for the analysis of your own sample.

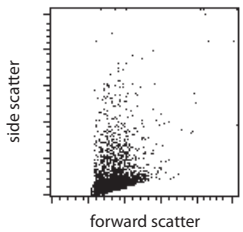
1. BALB/c mice were intraperitoneally (i.p.) immunized with 100 µg Keyhole limpet hemocyanin (KLH) in incomplete Freund´s adjuvant with 200 ng Pertussis Toxin. 200 ng Pertussis Toxin in PBS was i.p. injected again 24 hours later.
2. After 18 weeks 10^7 mouse spleen cells of the immunized mouse were incubated in vitro for 16 hours with or without 100 µg/ml KLH.
3. The Mouse IL-4 Secretion Assay was performed on the stimulated and the unstimulated sample.

4. **Counterstaining of T cells** was performed by using CD4-FITC.
5. **B lymphocytes** were stained with CD45R/B220-PerCP™.
6. **Dead cells** were stained with propidium iodide (PI), which was added **just prior** to flow cytometric analysis to a final concentration of **0.5 µg/ml**.
7. 200,000 viable cells were acquired by flow cytometry, from the stimulated and the unstimulated samples.
8. A **lymphocyte gate** based on forward and side scatter (FSC/SSC) properties was activated prior to further gating to exclude B cells and debris (see A.).
9. Dead cells and B cells were excluded according to PI- and CD45R/B220-PerCP™-staining in a fluorescence 2 versus fluorescence 3 plot (see B.).

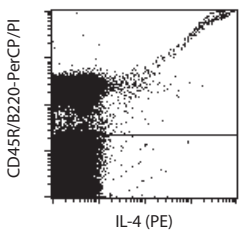
   The **dead cell exclusion** is crucial for the analysis of rare antigen-specific T cells, as immunoglobulins may bind non-specifically to dead cells. This could lead to false positive events.

   The sensitivity of the detection will further be enhanced by exclusion of undesired non-T cells which may cause non-specific background staining.
10. For analysis IL-4 (PE) versus CD4-FITC staining of viable lymphocytes is displayed (see C.).

A. Lymphocyte gate using FSC versus SSC

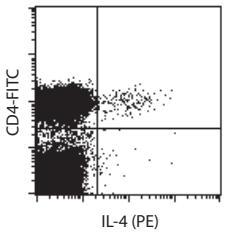


B. Dead cell and B cell exclusion



C. IL-4 secreting CD4⁺ T cells

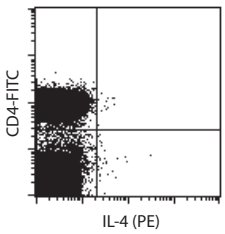
Splenocytes **restimulated with KLH** from a mouse immunized with KLH



0.360% of the total CD4⁺ T cell population secrete IL-4 (see formula below).

$$\%\ \text{IL-4}^+\ \text{cells among CD4}^+ = \frac{\#\ \text{of IL-4}^+\text{CD4}^+\ \text{cells in the analyzed sample}}{\#\ \text{of total CD4}^+\ \text{cells in the analyzed sample}} \times 100$$

Unstimulated splenocytes from a mouse immunized with KLH



0.027% of the total CD4⁺ T cell population secrete IL-4.

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For further references visit our website **www.miltenyibiotec.com**.

7. Appendix: Flask and dish sizes for stimulation

For (antigen-specific) stimulation (see 4.2) the cells should be resuspended in culture medium at 10^7 cells/ml and $5x10^6$ 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
0.5×10^7	0.5 ml	48 well	1.13 cm
1×10^7	1 ml	24 well	1.6 cm
2×10^7	2 ml	12 well	2.26 cm
5×10^7	5 ml	6 well	3.5 cm

total cell number	medium volume to add	culture dish	dish diameter
4.5×10^7	4.5 ml	small	3.5 cm
10×10^7	10 ml	medium	6 cm
25×10^7	25 ml	large	10 cm
50×10^7	50 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

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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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