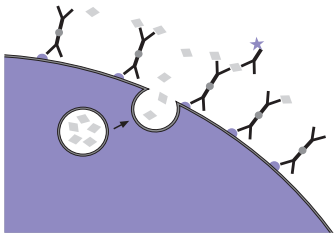
Miltenyi Biotec

Mouse IFN-γ Secretion Assay

Detection Kit (APC)

For 100 tests with 10^6 cells Order no. 130-090-984



Miltenyi Biotec

Miltenyi Biotec B.V. & Co. KG
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macsde@miltenyi.com, **www.miltenyibiotec.com**

140-000-761.03

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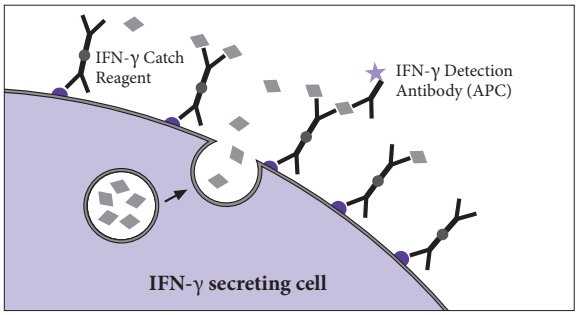
1. Description

Components	1 mL **Mouse IFN-γ Catch Reagent**: anti-IFN-γ monoclonal antibody (rat IgG1) conjugated to cell surface specific monoclonal antibody (rat IgG2b). 1 mL **Mouse IFN-γ Detection Antibody**: anti-IFN-γ monoclonal antibody (rat IgG1) conjugated to APC (allophycocyanin).
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 4–8 °C. Do not freeze. The expiration dates are indicated on the vial labels.

1.1 Principle of the Mouse IFN-γ Secretion Assay

For analysis of murine antigen-specific T cells using the Mouse IFN-γ 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.



Subsequently, an IFN-γ-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 IFN-γ binds to the IFN-γ Catch Reagent on the positive, secreting cells. These cells can subsequently be labeled with a second IFN-γ-specific antibody, the **Mouse IFN-γ Detection Antibody** conjugated to allophycocyanin (APC) 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.

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1.2 Background and product applications

The Mouse IFN-γ Secretion Assay - Detection Kit (APC) is designed for the detection and analysis of viable IFN-γ secreting murine leukocytes. It is specifically developed for :

(1) Two color cytokine analysis, a combination of the Mouse IFN-γ Secretion Assay (APC) with a second Mouse Cytokine Secretion Assay (PE), to stain cells for coexpression of two cytokines.

(2) Direct correlation of the Mouse IFN-γ Secretion Assay (APC) and peptide-MHC tetramer (PE) staining of T cells.

The Mouse IFN-γ Secretion Assay - Detection Kit is developed for **detection of IFN-γ secreting, antigen-specific T cells**. After restimulation with specific antigen in vitro secretion of IFN-γ is induced.

IFN-γ is predominantly secreted by activated $CD4^+$ and $CD8^+$ memory and effector T cells and by activated NK cells.

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

Examples of applications

- Combination with a second Mouse Cytokine Secretion Assay - Detection Kit (PE) for the analysis of individual cells, which coexpress IFN-γ and the second cytokine.
- Staining of IFN-γ secreting cells in combination with peptide-MHC tetramers conjugated to phycoerythrin (PE).

- Detection of IFN-γ secreting, antigen-specific T cells for enumeration and phenotypic analysis as well as functional characterization in combination with other Mouse Cytokine Secretion Assays (PE).
- Enumeration and phenotypic analyses of antigen-specific T cell immunity in infection, autoimmunity, cancer, allergy or alloreactivity.
- Analysis of viable IFN-γ secreting leukocytes to determine functional antigens in disease and for T cell receptor (TCR) epitope mapping.
- Analysis of TCR repertoire of antigen-specific T cells.

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™.

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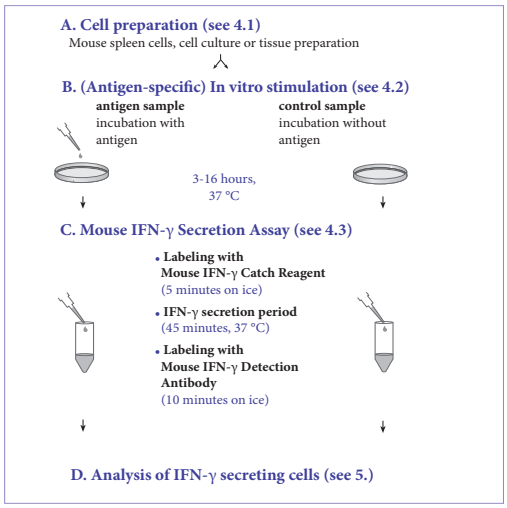
- Refrigerated centrifuge (4–8 °C).
- Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753).

2. Protocol overview



3. Experimental set-up

3.1 Controls

Negative control
For accurate detection of antigen-specific cells secreting IFN-γ, a negative control sample should always be included. This will provide information about IFN-γ secretion unrelated to the in vitro stimulation with the specific antigen, e.g. due to ongoing in vivo immune response. 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.

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.

▲ **Note:** Mitogens like PHA or PMA/Ionomycin are not recommended for stimulation of a positive control, as the resulting high frequencies of IFN-γ secreting cells do not allow conclusions on the performance (e.g. sensitivity) of the Mouse IFN-γ Secretion Assay.

3.2 Kinetics of restimulation and proposed time schedule

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

Proteins
Upon stimulation with protein, the cells can be analyzed for IFN-γ secretion 6–16 hours later.
It is possible to start the stimulation of the cells late in the afternoon, and to perform the IFN-γ 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 IFN-γ secreting cells are stained with APC-conjugated Mouse IFN-γ 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 Two colour cytokine analysis (see also 5.1)

IFN-γ secreting cells can be analyzed simultaneously for IL-2, IL-4, IL-5 or IL-10 production by two color cytokine analysis combining the Mouse IFN-γ Secretion Assay (APC) with the respective Mouse Cytokine Secretion Assay - Detection Kit (PE).
For details on the procedure, please refer to the standard protocol, paying attention to the steps marked with ★ (see 4.3).

3.5 Detection of very low frequencies

(Optional, reagents not included) If the sample contains less than 0.01-0.1% of IFN-γ secreting cells, it is possible to enrich these cells magnetically using the Mouse IFN-γ Secretion Assay – Enrichment and Detection Kit (PE) (# 130-090-517). Alternatively, IFN-γ secreting cells stained with the Mouse IFN-γ Secretion Assay (APC) can be enriched by using Anti-APC MicroBeads (# 130-090-855). 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 IFN-γ 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 200×g for 10 minutes. Pipette off supernatant.
2. Resuspend cells in culture medium at 10^7 cells/mL and 5×10^6 cells/cm^2 (see 7. Appendix: Flask/dish sizes for stimulation).
3. Add antigen or control reagent:

peptide:	3–6	hours at 37 °C,	5–7% CO_2, e.g.	1–10 µg/mL
protein:	6–16	hours at 37 °C,	5–7% 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 < 2% of total IFN-γ secreting cells. If ≥ 2 % of IFN-γ 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 IFN-γ 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 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. Pipette off or aspirate supernatant.

Labeling cells with Mouse IFN-γ 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 300×g 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.

   ★ For **two color cytokine analysis** resuspend the cells in 80 µL of **cold medium** per 10^6 total cells.

5. Add 10 µL of **Mouse IFN-γ Catch Reagent** per 10^6 total cells, mix well and incubate for 5 minutes **on ice**.

   ★ For **two color cytokine analysis** it is important to pre-mix the **Mouse IFN-γ Catch Reagent** with the same volume of the second **Mouse Cytokine Catch Reagent** before adding to the cells. Add 20 µL of this cocktail per 10^6 total cells, mix well and incubate for 5 minutes **on ice**.

IFN-γ secretion period

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

Expected number of IFN-γ secreting cells	Dilution	Amount of medium to add per 10^6 total cells
< 2%	10^6 cells/mL	1 mL
2-20%	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 IFN-γ 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.

   ★ For **two color cytokine analysis** resuspend the cells in 80 µL of **cold buffer** per 10^6 total cells.

5. Add 10 µL of **Mouse IFN-γ Detection Antibody (APC)** per 10^6 total cells.

   ★ For **two color cytokine analysis** add additionaly 10 µL of the second **Mouse Cytokine Detection Antibody (PE)** per 10^6 total cells.

6. (Optional) Add additional staining antibodies, e.g. CD4-FITC or CD8-FITC and CD45R/B220-PerCP.
7. Mix well and incubate for 10 minutes **on ice**.
8. Wash cells by adding 2 mL of **cold buffer**, centrifuge at 300×g 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 IFN-γ 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 2×10^5 events from each sample.

5.1 Coexpression of IFN-γ and IL-2 by CD4⁺ T cells

To illustrate the analysis, we describe the detection of IFN-γ and IL-2 secreting CD4⁺ T cells by using the Mouse IFN-γ Secretion Assay (APC) in combination with the Mouse IL-2 Secretion Assay (PE) (# 130-090-479). This description, including how to set gates, should serve as a model for the analysis of your own sample.

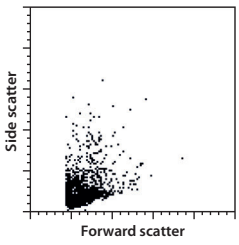
1. BALB/c mice were intraperitoneally (i.p.) immunized with 100 µg Keyhole limpet hemocyanin (KLH) in incomplete Freund´s adjuvant with 200 ng Pertussis Toxin. 200 ng Pertussis Toxin in PBS was i.p. injected again 24 hours later.
2. After 3 weeks (2 to 20 weeks possible) 10^7 mouse spleen cells of the immunized mouse were incubated in vitro for 16 hours with or without 100 µg/mL KLH.
3. The two color Cytokine Secretion Assay was performed on the stimulated and the unstimulated sample from the KLH-immunized mouse.
4. **Counterstaining of T cells** was performed with CD4-FITC.
5. **Blymphocytes** 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 plot 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 plot 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.

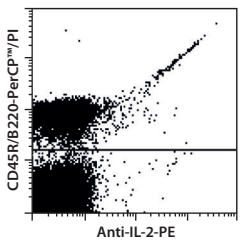
The sensitivity of the detection will further be enhanced by exclusion of undesired non-T cells which may cause non-specific background staining.

10. CD4⁺ cells were gated based on fluorescence 1 properties (not shown).
11. For analysis secreted IFN-γ (APC) versus secreted IL-2 (PE) of viable CD4⁺ cells is displayed (see plots C.).

A. Lymphocyte gate using FSC versus SSC

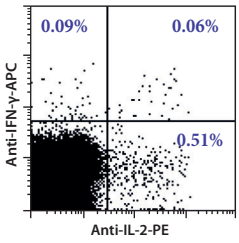


B. Dead cell and B cell exclusion

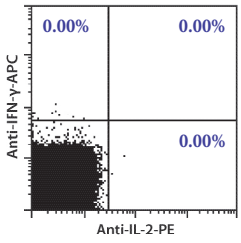


C. IFN-γ and IL-2 secreting CD4⁺ T cells from a mouse immunized with KLH

stimulated sample



unstimulated control



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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 step 2.) the cells should be resuspended in culture medium at 10^7 cells/ml and 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
0.5×10^7	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

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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