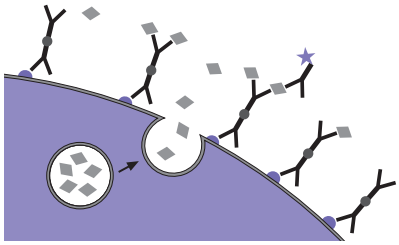
Miltenyi Biotec

IFN-γ Secretion Assay – Detection Kit (PE)

human

For 100 tests with 10^6 cells

Order no. 130-054-202



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**

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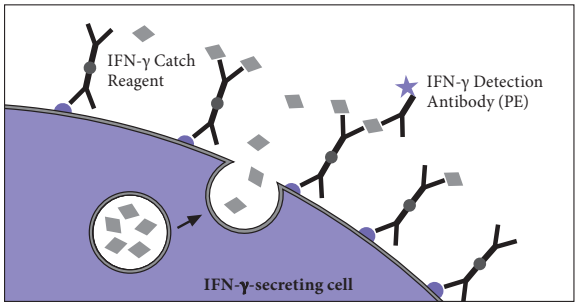
Contents

1. Description

Components	**1 mL IFN-γ Catch Reagent:** anti-IFN-γ monoclonal antibody (mouse IgG1) conjugated to CD45-specific monoclonal antibody (mouse IgG2a). **1 mL IFN-γ Detection Antibody (PE):** anti-IFN-γ monoclonal antibody (mouse IgG1) conjugated to R-phycoerythrin (PE).
Size	For 100 tests with 10^6 cells.
Product format	IFN-γ Catch Reagent and IFN-γ Detection Antibody 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.



1.1 Principle of the IFN-γ Secretion Assay

Antigen-specific T cells are analyzed using the IFN-γ Secretion Assay starting from whole blood, peripheral blood mononuclear cells (PBMCs), or other leukocyte containing single cell preparations. The cells 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 are subsequently labeled with a second IFN-γ-specific antibody, the **IFN-γ Detection Antibody** conjugated to R-phycoerythrin (PE) for sensitive detection by flow cytometry. Since viable cells

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are analyzed, non-specific background can be minimized by dead cell exclusion. This provides highest sensitivity of analysis.

1.2 Background

The IFN-γ Secretion Assay – Detection Kit is designed for the detection and analysis of viable IFN-γ-secreting leukocytes. It is specially developed for the **detection and isolation of 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 NK cells upon activation. Quantitative analysis of antigen-specific T cell populations can provide important information on the natural course of immune responses.

1.3 Applications

- Detection of viable IFN-γ-secreting leukocytes.
- Detection of IFN-γ-secreting, antigen-specific T cells for enumeration and phenotypic characterization.
- Monitoring and analysis of antigen-specific T cell immunity, for example, in infection, autoimmunity, cancer, allergy, or alloreactivity.
- Analysis of IFN-γ-secreting cells for determination of 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: 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 1640 (# 130-091-440), containing 5% human serum, like autologous or AB serum (do not use BSA or FCS because of non-specific stimulation!).
- Propidium iodide (PI) or 7-AAD for flow cytometric exclusion of dead cells without fixation.
- Refrigerated centrifuge (2–8 °C).
- Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753).
- (Optional) CytoStim (# 130-092-172, # 130-092-173), CMV pp65 – Recombinant Protein (# 130-091-823, # 130-091-824), or PepTivator – CMV pp65 (# 130-093-435, # 130-093-438) for restimulation of human T cells. For details see the respective data sheet. For more information about other antigens see www.miltenyibiotec.com.
- (Optional) Staining reagents such as CD4-FITC (# 130-080-501) or CD8-FITC (# 130-080-601) and CD14-PerCP.

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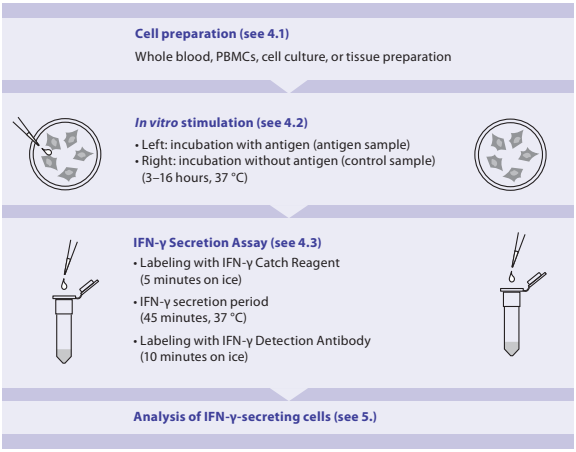
2. Protocol overview



3. Experimental setup

3.1 Controls

Negative control
For accurate detection of IFN-γ-secreting antigen-specific cells, a negative control sample should always be included. This will provide information about IFN-γ secretion unrelated to the specific antigen-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 antigen, or by using a control antigen.

Positive control
When setting up a new experiment, it is recommended to include a positive control. As a positive control, a sample stimulated with CytoStim (# 130-092-172, # 130-092-173) 20 µL/mL for 1–3 hours, or with the superantigen Staphylococcal Enterotoxin B (Sigma) 1 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 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 later.
It is possible to prepare the cells first and take them into culture overnight, but without adding the antigen (see 4.2 step 2.). Peptide is then added the next morning for 3 hours of stimulation, directly followed by the IFN-γ Secretion Assay.

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 or CD49d 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 PE-conjugated IFN-γ Detection Antibodies. To identify cells of interest, counterstaining for T cells with, for example, CD4-FITC (# 130-080-501) or CD8-FITC (# 130-080-601) 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 monocytes with antibodies conjugated to Peridinin-chlorophyll-protein (PerCP), e.g., CD14-PerCP. These cells together with PI stained dead cells can then be excluded by gating.

3.4 Two-color cytokine analysis

IFN-γ-secreting cells can be analyzed simultaneously, for example, for IL-2 or IL-10 production by two-color cytokine analysis combining the IFN-γ Secretion Assay with the IL-2 Secretion Assay – Detection Kit (APC) (# 130-090-763) or 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.

3.5 Combination with peptide-MHC tetramer staining

IFN-γ-secreting cells can be analyzed simultaneously for peptide-MHC tetramers combining the IFN-γ Secretion Assay –Detection Kit (PE) with APC-conjugated peptide-MHC tetramers. For combination with PE-conjugated peptide-MHC tetramers the IFN-γ Secretion Assay – Detection Kit (APC) (# 130-090-762) and the IFN-γ Secretion Assay – Detection Kit (FITC) (# 130-090-433) are available. Detailed recommendations for the experimental setup and the procedure are included in the data sheets of the Cytokine Secretion Assay – Detection Kits (APC) and are available at www.miltenyibiotec.com/protocols.

3.6 Detection with 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 IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (PE) (# 130-054-201). 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 IFN-γ 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 IFN-γ 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. A **positive control** may also be included (see 3.1).

▲ 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 10^7 cells/mL and 5×10^6 cells/cm² (see 7. Appendix A: Flask and 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
CytoStim:	1–3 hours at 37 °C, 5–7% CO_2, e.g. 20 µg/mL
SEB:	3–16 hours at 37 °C, 5–7% CO_2, e.g. 1 µg/mL

   For comparison of different experiments, the stimulation time should always be the same (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.

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4.3 Cytokine Secretion Assay

General considerations

▲ The assay is optimized for cell samples containing <5% of total IFN-γ-secreting cells. If ≥5% 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 prevents 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** (2–8 °C)
100 µL of **cold medium** (2–8 °C)
1 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 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 IFN-γ Secretion Assay, e.g., by densitiy gradient centrifugation or by using the Dead Cell Removal Kit (# 130-090-101).

Labeling cells with IFN-γ Catch Reagent

1. Use 10^6 total cells in a 2 mL closable tube per sample.
2. Wash cells by adding 1–2 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 **IFN-γ Catch Reagent** 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
<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.

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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 IFN-γ 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 **IFN-γ Detection Antibody (PE)** per 10^6 total cells.
5. (Optional) Add additional staining reagents, for example, 10 µL of CD4-APC (# 130-091-232) or 10 µL of CD8-FITC (# 130-080-601) and CD14-PerCP.
6. Mix well and incubate for 10 minutes **on ice**.
7. Wash cells by adding 2 mL of **cold buffer** and centrifuge at 300×g for 10 minutes at **2–8 °C**, aspirate supernatant.
8. 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 to exclude 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 viable cells from each sample.

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

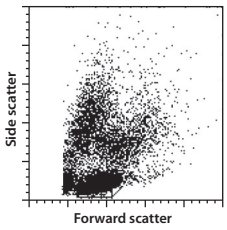
1. 10^7 human PBMC of a CMV$^+$ donor have been restimulated for 4 hours with and without the peptide pool PepTivator – CMV pp65.
2. The IFN-γ Secretion Assay was performed on the stimulated and the unstimulated sample.
3. **Counterstaining of T cells** was performed using CD4-APC and CD8-FITC.

4. **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.
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 (FSC/SSC) properties was activated prior to further gating to exclude monocytes and debris (see A.).
7. Dead cells were excluded according to PI-staining in a fluorescence 2 (PE) versus fluorescence 3 (PI) plot (see B.).

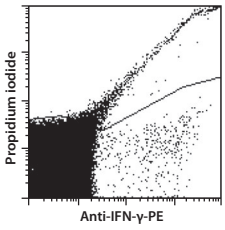
- The **dead cell exclusion** is crucial for the analysis of rare antigen-specific T cells, as dead cells may bind non-specifically to antibodies or MicroBeads. This could lead to false positive events.
- The sensitivity of detection can be further enhanced by exclusion of undesired non–T cells, like monocytes which may cause non-specific background staining.

8. Analysis of secreted IFN-γ (PE) versus CD4-APC and CD8-FITC staining by viable lymphocytes is displayed (see C.).

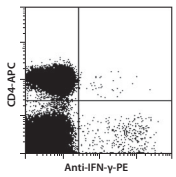
A. Lymphocyte gate using FSC versus SSC



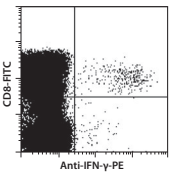
B. Dead cell exclusion



C. Antigen-specific T cells stained for secreted IFN-γ
Samples stimulated with PepTivator – CMV pp65



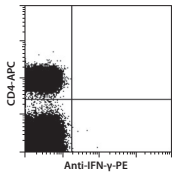
0.12% of the total CD4$^+$ T cell population secrete IFN-γ (see formula below).



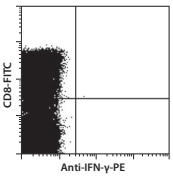
1.4% of the total CD8$^+$ T cell population secrete IFN-γ (see formula below).

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

Unstimulated control sample



≤0.003% of the total CD4$^+$ T cell population secrete IFN-γ.



≤0.004% of the total CD8$^+$ T cell population secrete IFN-γ.

6. References

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

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 10^7 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 cm²
40×10^7	40 mL	250 mL	75 cm²
80×10^7	80 mL	720 mL	162 cm²
120×10^7	120 mL	900 mL	225 cm²

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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