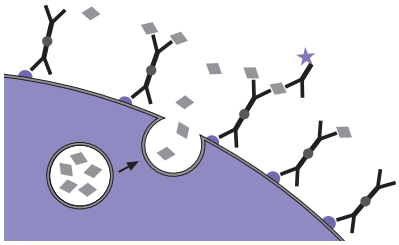
Miltenyi Biotec

IFN-γ Secretion Assay – Detection Kit (APC)

human

For 100 tests with 10^6 cells

Order no. 130-090-762



Miltenyi Biotec

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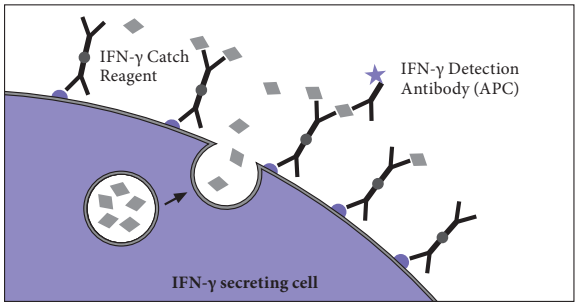
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1. Description

Components	1 mL **IFN-γ Catch Reagent**: anti-IFN-γ monoclonal antibody (mouse IgG1) conjugated to cell surface specific monoclonal antibody (mouse IgG2a). 1 mL **IFN-γ Detection Antibody:** anti-IFN-γ monoclonal antibody (mouse 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 °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, PBMC 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 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.

1.2 Background and product applications

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

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

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

The 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 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).

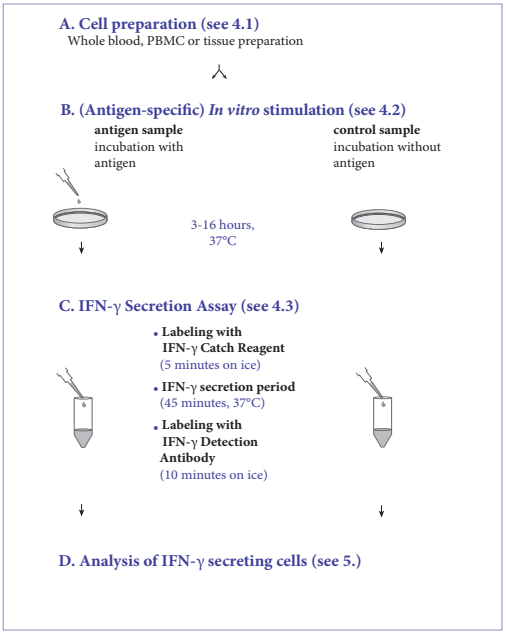
- Detection of IFN-γ secreting, antigen-specific T cells for enumeration and phenotypic analysis as well as functional characterization in combination with other Cytokine Secretion Assays (PE).
- Enumeration and phenotypic analyses of antigen-specific T cell immunity in infection, autoimmunity, cancer, 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 requirement

- **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 (EDTA), adjust pH to 7.5, fill up to 1000 mL with dd H_2O.
- Culture medium, e.g. RPMI 1640 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** to exclude dead cells from analysis.

- (Optional) Staining reagents such as CD4-FITC (# 130-080-501) or CD8-FITC (# 130-080-601) and CD14-PerCP™.
- (Optional) Cytokine Secretion Assay - Detection Kit: e.g. IL-2 Secretion Assay (PE) (# 130-090-487), IL-4 Secretion Assay (PE) (# 130-054-102), or IL-10 Secretion Assay (PE) (# 130-090-434).
- Refrigerated centrifuge (4–8 °C).
- Rotation device for tubes: MACSmix (# 130-090-753).

2. Protocol overview



3. Experimental set-up

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 e.g. 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 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 addition of 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 APC-conjugated IFN-γ Detection Antibodies. To identify cells of interest, counterstaining for T cells with e.g. 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 PerCP™, e.g.

CD14-PerCP™. These cells can then be excluded together with PI stained dead cells by gating.

3.4 Two color cytokine analysis (see also 5.1)

IFN-γ secreting cells can be analyzed simultaneously for IL-2, IL-4 or IL-10 production by two color cytokine analysis combining the IFN-γ Secretion Assay (APC) with the respective 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 Combination with peptide-MHC tetramer staining (see also 5.2)

The IFN-γ Secretion Assay (APC) can be performed in conjunction with peptide-MHC tetramer (PE) labeling for analysis of both functionality and specificity of antigen-specific T cells.

3.5.1 Antigen sample for combination with peptide-MHC tetramer staining

Since stimulation with antigen can strongly downregulate TCR expression on the specific T cells, tetramer labeling should be performed prior to in vitro stimulation and the IFN-γ Secretion Assay.

First, the cells are labeled with the specific peptide-MHC tetramers, followed by in vitro stimulation for 2 hours with specific peptide (see 4.2). Then the IFN-γ Secretion Assay is performed according to the standard protocol (see 4.3).

3.5.2 Negative controls for combination with peptide -MHC tetramer staining

A negative control sample should **always** be included in the experiment (see 3.1).
Depending on the peptide-MHC tetramer, tetramer labeling of the cells (a) may, or (b) may not induce cytokine secretion.
If the stimulatory effect of the peptide-MHC tetramer is unknown, both types of negative controls should be performed initially.

(a) Cytokine secretion is induced:
For the negative control sample the cells should be cultured for 2 hours without addition of the specific peptide or the peptide-MHC tetramer. Then the IFN-γ Secretion Assay is performed followed by peptide-MHC tetramer labeling.
In contrast, the antigen samples are labeled with peptide-MHC tetramers prior to the IFN-γ Secretion Assay (see 3.5.1).

(b) No cytokine secretion is induced:
Cells from all samples, from the antigen sample as well as from the negative control sample, are first labeled with peptide-MHC tetramers. Subsequently, cells from all samples are incubated for 2 hours with or without (negative control) addition of antigen followed by performing the IFN-γ Secretion Assay.

3.6 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 IFN-γ Secretion Assay – Enrichment and

Detection Kit (PE) (# 130-054-201). Alternatively, IFN-γ secreting cells stained with the 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 IFN-γ Secretion Assay

4.1 Cell preparation

For the detection of cytokine secreting cells, best results are achieved by starting the assay with fresh PBMC, or other leukocyte containing single cell preparations from tissues or cell lines. Alternatively, frozen cell preparations can be used.

▲ **Note:** PBMC may be stored overnight. The cells should be resuspended and incubated in culture medium as described in 4.2 step 2, but without addition of antigen. The antigen is then added to the culture on 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, see 7. Appendix B: **Detection of cytokine secreting cells from human whole blood**. This special protocol is also available from our website www. miltenyibiotec.com.

4.2 (Antigen specific) *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, like BSA or FCS because of non-specific stimulation.



Protocol for *in vitro* stimulation

▲ When combining the IFN-γ Secretion Assay with peptide-MHC tetramer staining, the tetramer labeling should be performed prior to the stimulation of the cells (see 3.5).

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/cm^2 (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
   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.

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** (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 completely.



Labeling cells with 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 by adding 1-2 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. 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.

4. Add 10 µL of **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 **IFN-γ Catch Reagent** with the same volume of the second **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
< 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 a closed tube for 45 minutes at 37 °C under slow continuous rotation by using the MACSmix (# 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**, centrifuge at 300×g for 10 minutes at **4–8 °C**. Pipette off supernatant completely.

   ▲ **Note**: If the volume of the cell suspension was higher than the volume of added buffer, repeat wash step.

3. 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.

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

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

5. (Optional) Add additional staining reagents, e.g. 10 µL of CD4-FITC (# 130-080-501) or 10 µL of CD8-FITC (# 130-080-601) and CD14-PerCP^{TM}.
6. Mix well and incubate for 10 minutes **on ice**.
7. Wash cells by adding 2 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at **4–8 °C**, pipette off 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 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 viable cells 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 IFN-γ Secretion Assay (APC) in combination with the IL-2 Secretion Assay (PE) (# 130-090-487). This description, including how to set gates, should serve as a model for the analysis of your own sample.

1. 10^6 human PBMC of a CMV$^+$ donor have been restimulated for 16 hours with, and for the control sample, without CMV-lysate (5 µg/mL; Biowhittaker).
2. The two color Cytokine Secretion Assay was performed on the stimulated and the control sample.
3. **Counterstaining of T cells** was performed by using CD4-FITC.

4. **Monocytes** were stained with CD14-PerCP™.
5. **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.
6. 200,000 viable cells were acquired by flow cytometry, from the stimulated as well as from the control sample.
7. A **lymphocyte gate** based on forward and side scatter (FSC/SSC) properties was activated prior to further gating to exclude monocytes and debris (plot 1.).
8. Dead cells and monocytes were excluded according to PI and CD14-PerCP™ staining in a fluorescence 2 (PE) versus fluorescence 3 (PerCP™) plot (plot 2.).

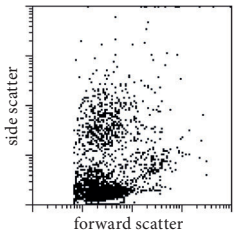
- The **dead cell exclusion** is crucial for the analysis of rare antigen-specific T cells, as antibodies may bind non-specifically to dead cells. This could lead to false positive events.
- The sensitivity of the detection is further enhanced by exclusion of undesired non-T cells, like monocytes which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

9. $CD4^+$ T cells were gated in a fluorescence 1 versus fluorescence 2 plot (not shown).
10. For analysis secreted IFN-γ (APC) versus secreted IL-2 (PE) of viable $CD4^+$ T cells is displayed (plot 3.).

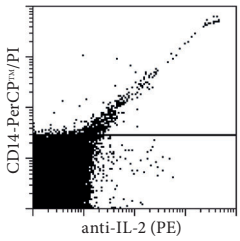
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5. Detection and analysis of IFN-γ secreting T cells

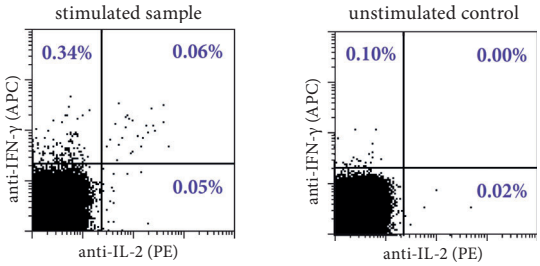
1. Lymphocyte gate using FSC versus SSC



2. Dead cell and monocyte exclusion



3. IFN-γ and IL-2 secreting $CD4^+$ T cells after stimulation with CMV-lysate



5.2 IFN-γ secretion of peptide-MHC tetramer labeled cells

1. 10^6 PBMC have been incubated for 1 h with CMV-$pp65_{495\text{-}503}$ HLA-A2 tetramers conjugated to phycoerythrin (PE) at 8 °C.
2. The pp65-HLA-A2 tetramer labeled cells have been stimulated with pp65-peptide for 2 h at 37 °C (plot A.).

   As control samples (see 3.5) pp65-HLA-A2 tetramer labeled cells have been incubated without peptide for 3 h at 37 °C (plot B.) and cells without peptide-MHC tetramer staining have been stimulated with pp65-peptide (plot C.).
3. The samples have been stained for IFN-γ secretion using the IFN-γ Secretion Assay - Detection Kit (APC).
4. **Counterstaining of T cells** was performed by using CD8-FITC.
5. **Monocytes** were stained with CD14-PerCP™.
6. **Dead cells** were stained with propidium iodide (PI).
7. 200,000 viable cells were acquired by flow cytometry, from the stimulated as well as from the control sample.
8. A **lymphocyte gate** based on forward and side scatter (FSC/SSC) properties was activated prior to further gating to exclude monocytes and debris (plot 1).
9. Dead cells and monocytes were excluded according to PI and CD14-PerCP™ staining in a fluorescence 2 versus fluorescence 3 plot (plot 2).

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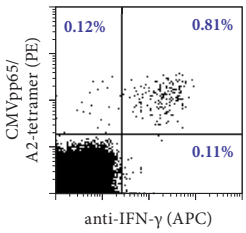
5. Detection and analysis of IFN-γ secreting T cells

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

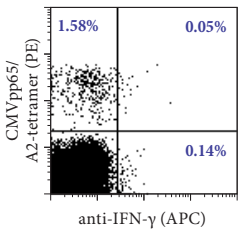
10. $CD8^+$ T cells were gated in a fluorescence 1 versus fluorescence 2 plot (not shown).
11. For analysis, secreted IFN-γ (APC) versus pp65-HLA-A2 tetramer (PE) of gated, viable $CD8^+$ T cells are displayed (for details on gating, see 5.1).

Secretion of IFN-γ by CMV-peptide-specific CD8⁺ T cells

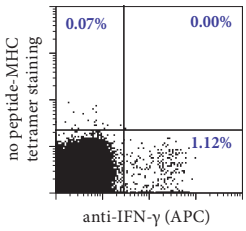
A. stimulated sample



B. unstimulated control



C. stimulated sample without peptide-MHC tetramer staining



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

7. Appendix

7. Appendix

A: Flask and dish sizes for stimulation

For in vitro stimulation (see 4.2 step 2.) the cells should be resuspended in culture medium, containing 5% of human serum, at 10^7 cells/mL and 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
0.5×10^7	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 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

B: Detection of cytokine secreting cells from whole blood

B2. Protocol

B 2.1 (Antigen-specific) *in vitro* stimulation

B 2.2 Cytokine Secretion Assay

B 2.3 Detection and analysis of cytokine secreting cells

The following special protocol can be used in combination with one of the Cytokine Secretion Assay - Detection Kits for human cells.

B 1. Reagent and instrument requirements

- **Cytokine Secretion Assay, for example:**

  IFN-γ Secretion Assay - Detection Kit (PE*) (# 130-054-202)
  IFN-γ Secretion Assay - Detection Kit (FITC) (# 130-090-433)
  IFN-γ Secretion Assay - Detection Kit (APC*) (# 130-090-762)
  IL-2 Secretion Assay - Detection Kit (PE*) (# 130-090-487)
  IL-2 Secretion Assay - Detection Kit (APC*) (# 130-090-763)
  IL-4 Secretion Assay - Detection Kit (PE*) (# 130-054-102)
  IL-10 Secretion Assay - Detection Kit (PE*) (# 130-090-434)
  IL-10 Secretion Assay - Detection Kit (APC*) (# 130-090-761)

- **Anticoagulant:** sodium heparin
- **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 (EDTA), adjust pH to 7.5, fill up to 1000 mL with dd H_2O.

- **Culture medium**, e.g. RPMI 1640 containing 10% of human serum, like autologous serum or AB serum.

  ▲ **Note:** Do **not use BSA** or **FCS** because of non-specific stimulation.

- **Erythrocyte lysing solution (1×):**
- prepare freshly from 10× stock solution.
- **10× stock solution**: 41.4 g NH_4Cl (1.55 M), 5 g $KHCO_3$ (100 mM), 1 mL 0.5 M EDTA (1 mM), adjust pH to 7.3, fill up to 500 mL with dd H_2O.

  ▲ **Note:** Do **not use** FACS Lysing solution™.

- (Optional) **Staining reagents**: CD4-FITC (# 130-080-501) or CD8-FITC (# 130-080-601) and CD14-PerCP™.

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

  ▲ **Note**: For optimal sensitivity, we recommend labeling of undesired non-T cells such as monocytes with antibodies conjugated to PerCP™, e.g. CD14-PerCP™. These cells can then be excluded together with PI stained dead cells by gating.

- **Propidium iodide** (PI) or **7-AAD** to exclude dead cells from analysis.

- (Optional) Rotation device for tubes: MACSmix (# 130-090-753).

B 2. Protocol

B 2.1 (Antigen-specific) *in vitro* stimulation

▲ The peripheral blood should not be older than 20 hours and should be supplemented with anticoagulant **sodium heparin**. **Do not use EDTA or ACD**. Lymphocyte activation and secretion of cytokines requires calcium, and is consequently inhibited by chelating anticoagulants.

▲ **Note**: Whole blood may be stored over night at room temperature.

▲ **Always** include **a negative control** sample in the experiment. **A positive control** with e.g. Staphylococcal Enterotoxin B (SEB) may be included in the experiment (see also detailed protocol provided with the Cytokine Secretion Assay Kits).

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



Protocol for *in vitro* stimulation

1. Start with **250 µL of fresh, sodium heparinized, human blood** (containing about 5×10^5 lymphocytes) in a 15 mL conical polypropylene tube.
2. Add the antigen or, as a positive control, 1 µg/mL SEB for 3–16 hours at 37 °C, 5–7% CO_2 (for details on the kinetics of cytokine secretion and on concentrations of antigen to add, refer to Cytokine

Secretion Assay data sheet, 3.1-3.2).

3. A negative control sample, treated exactly the same as the antigen-stimulated sample, but without addition of antigen, should always be included in the experiment.
4. (Optional) Costimulatory agents like CD28 and CD49d antibodies may be added.

B 2.2 Cytokine Secretion Assay

▲ This protocol is optimized for cell samples containing < 20% of total cytokine secreting cells. If ≥ 20% of cytokine 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. The dilution prevents non-specific staining of cells not secreting cytokines during this period.

▲ For each sample with 250 µL whole blood prepare:

50 mL of **cold buffer** (4–8 °C)
100 µL of **cold medium** (4–8 °C)
5 mL of **warm medium** (37 °C)
5 mL of **erythrocyte lysing solution** (room temperature).

▲ 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 and room temperature during lysing step).

▲ Do not remove supernatant by decanting. This will lead to cell loss and incorrect incubation volumes. Pipette off or aspirate supernatant.

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

▲ Higher temperatures and longer incubation times for staining should be avoided. This will lead to non-specific cell labeling.

1. Wash cells by adding 10 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at 4–8 °C, pipette off supernatant carefully.

   ▲ **Note**: Be careful, leukocytes will appear on top of the loose red cell pellet.

2. Resuspend pellet in 80 µL of **cold medium**.
3. Add 20 µL of **Cytokine Catch Reagent**, mix well and incubate for 5 minutes **on ice**.



Cytokine secretion period

1. Add 5 mL of **warm medium** (37 °C) to dilute the cells.

   ▲ **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 (# 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 Cytokine Detection Antibody

1. Put the tube **on ice**.
2. Wash cells by adding 10 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at **4–8 °C**, pipette off supernatant carefully.
3. Resuspend cell pellet in 80 µL of **cold buffer**.
4. Add 20 µL of **Cytokine Detection Antibody**.
5. (Optional) Add additional staining reagents, e.g. 10 µL of CD4-FITC (# 130-080-501) or CD8-FITC (# 130-080-601) and CD14-PerCP™.
6. Mix well and incubate for 10 minutes **on ice**.



Lysis of erythrocytes

1. Add 5 mL of **erythrocyte lysing solution**.
2. Mix gently and incubate for 10 minutes at room temperature. Rotate tube continuously using the MACSmix, or turn tube several times during incubation.
3. Centrifuge cells at 300×g for 10 minutes at **room temperature**, pipette off supernatant completely.
4. Wash cells by adding 10 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at 4–8 °C, pipette off supernatant.

5. Resuspend the cells in 500 µL of **cold buffer**, and proceed to flow cytometric analysis (see detailed protocol).

B 2.3 Detection and analysis of cytokine secreting 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. Incubation 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.

▲ For details on analysis please refer to the detailed protocol provided with the Cytokine Secretion Assay Kits.

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.

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