For further information refer to our website
www.miltenyibiotec.com

For technical questions, please contact your local subsidiary or distributor.

Technical Support Team, Germany:
Phone: +49 2204 8306-830
macstec@miltenyibiotec.de

Miltenyi Biotec

Miltenyi Biotec GmbH
Friedrich-Ebert-Straße 68
51429 Bergisch Gladbach
Germany
Phone +49 2204 8306-0
Fax +49 2204 85197
macs@miltenyibiotec.de

Miltenyi Biotec Inc.
2303 Lindbergh Street
Auburn, CA 95602-9562, USA
Phone 800 FOR MACS
Phone +1 530 888 8871
Fax +1 530 888 8925
macs@miltenyibiotec.com

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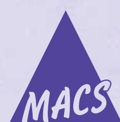
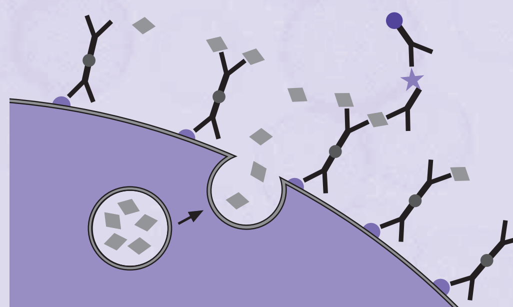


Miltenyi Biotec

IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (PE)

human

For 50 tests with 10^7 cells   Order no. 130-054-201



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1. Description

Components

1 mL IFN-γ Catch Reagent:
anti-IFN-γ monoclonal antibody (isotype: mouse IgG1) conjugated to cell surface specific monoclonal antibody (isotype: mouse IgG2a).

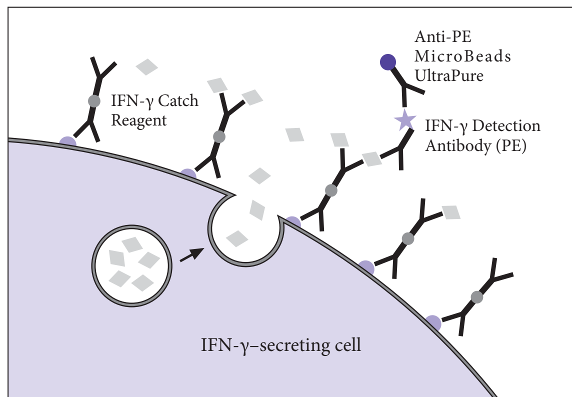
1 mL IFN-γ Detection Antibody:
anti-IFN-γ monoclonal antibody (isotype: mouse IgG1) conjugated to PE (R-phycoerythrin).

1 mL Anti-PE MicroBeads UltraPure:
colloidal superparamagnetic MicroBeads UltraPure conjugated to monoclonal mouse anti-PE antibody (isotype: mouse IgG1).

Capacity For 50 tests with 10^7 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 date is indicated on the vial label.



1.1 Principle of the IFN-γ Secretion Assay

Antigen-specific T cells are analyzed and isolated 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 a polyclonal stimulus or specific peptide, protein, or other protein antigen preparations, for example, from *Candida albicans* (*C. albicans*).

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

1. Description

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.

The IFN-γ–secreting cells can now be magnetically labeled with **Anti-PE MicroBeads UltraPure** and enriched over a MACS® Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled cells are retained in the MACS Column while the unlabeled cells run through. After the column has been removed from the magnetic field, the magnetically retained cells can be eluted as positively selected cell fraction, enriched for cytokine secreting cells. The cells can now be used for cell culture or analysis. 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

The IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (PE) has been designed for the detection, isolation and analysis of viable IFN-γ secreting leukocytes. It has been 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. Enrichment of the antigen-specific T cells via MACS Technology

increases the sensitivity of analysis, allowing detection of frequencies as low as one in a million cells.

The enrichment of cytokine-producing cells also enables further functional characterization of the antigen-specific cells and downstream experiments, as well as the expansion of antigen-specific cells allowing research on potential future immunotherapeutical applications.

1.3 Applications

- Enrichment and detection of viable IFN-γ–secreting leukocytes.
- Enrichment and detection of IFN-γ–secreting, antigen-specific T cells for enumeration and phenotypic analysis as well as for expansion and functional characterization.
- Monitoring and analysis of antigen-specific T cell immunity, e.g., in infection, autoimmunity, cancer, allergy, or alloreactivity.
- Isolation and expansion of antigen-specific T cells for research in immunotherapy.
- Enrichment and analysis of IFN-γ–secreting cells for determination of functional antigens in disease and for T cell receptor (TCR) epitope mapping.
- Analysis or cloning of TCR repertoire of antigen-specific T cells.

1. Description

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.

  ▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as human serum albumin, human serum, or fetal bovine serum (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.
- Culture medium, e.g., RPMI 1640, containing 5% human serum (do not use BSA or FBS because of non-specific stimulation!)
- (Optional) Cell stimulation reagents, for example, PepTivator® Peptide Pools for restimulation of human T cells. For more information refer to www.miltenyibiotec.com/peptivators.
- Propidium Iodide Solution (# 130-093-233) or 7-AAD for flow cytometric exclusion of dead cells without fixation. For cell fixation and flow cytometric exclusion of dead cells, the Fixation and Dead Cell Discrimination Kit (# 130-091-163) is recommended.
- (Optional) Staining reagents such as CD4-FITC (# 130-080-501), CD8-FITC (# 130-080-601), and CD14-PerCP-Vio700™ (# 130-097-539).

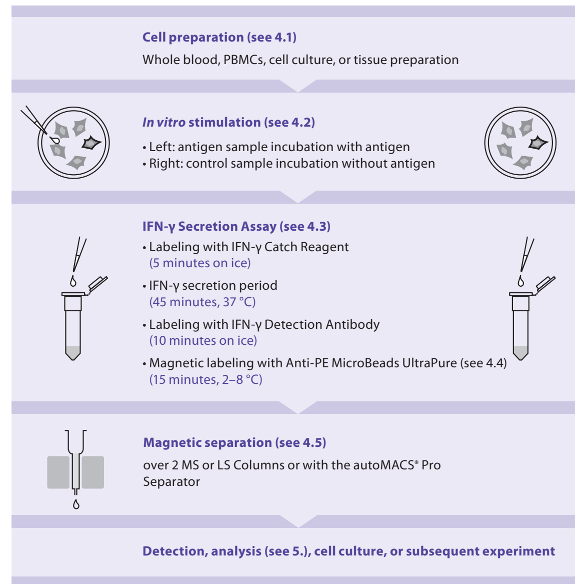
- MACS Columns and MACS Separators:

Column	Max. number of labeled cells	Max. number of total cells	Separator
Positive selection			
MS	10^7	2×10^8	MiniMACS, OctoMACS, VarioMACS, SuperMACS II
LS	10^8	2×10^9	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II
autoMACS	2×10^8	4×10^9	autoMACS Pro

▲ **Note:** Column adapters are required to insert certain columns into the VarioMACS™ or SuperMACS™ II Separators. For details refer to the respective MACS Separator data sheet.

- Refrigerated centrifuge (2–8 °C).
- Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753).
- (Optional) Pre-Separation Filters, 30 µm (# 130-041-407) to remove cell clumps.

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, 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 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 antigen preparations, for example, from *C. albicans*, 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.

▲ Do not use PE-tandem conjugates. They may also be recognized by Anti-PE MicroBeads UltraPure.

▲ 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), for example with Propidium Iodide Solution (# 130-093-233), 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), for example, CD14-PerCP (# 130-094-969). 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 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 from our website www.miltenyibiotec.com/protocols.

3.5 Combination with peptide-MHC tetramer staining

IFN-γ-secreting cells can be analyzed simultaneously for peptide-MHC tetramers by combining the IFN-γ Secretion Assay (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 from our website www.miltenyibiotec.com/protocols.

3.6 Detection without prior enrichment

(Optional, reagents not included) If the sample contains more than 0.01–0.1% of IFN-γ–secreting cells, you may be able to analyze IFN-γ–secreting cells without prior enrichment (refer also to: IFN-γ Secretion Assay – Detection Kit (PE), # 130-054-202). The assay can also be performed directly starting from whole blood. For details on the procedure refer to www.miltenyibiotec.com/protocols.

4. Protocol for the IFN-γ Secretion Assay

4.1 Cell preparation

For the detection and isolation 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 over night. 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.

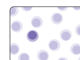
▲ **Note:** Remove platelets after density gradient separation. Resuspend cell pellet, fill tube with buffer and mix. Centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully remove supernatant.

Special protocols for whole blood: You can start the IFN-γ Secretion Assay directly from whole blood. For details on the procedure, refer to 7. Appendix B: Detection and enrichment of cytokine secreting cells from human whole blood. This special protocol is also available from our website www. miltenyibiotec.com/protocols.

4.2 *In vitro* stimulation

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

▲ Do not use media containing any non-human proteins, such as BSA or FBS, 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, e.g., RPMI 1640 (# 130-091-440), 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 *in vitro* stimulation).
3. Add antigen or control reagent:
   CytoStim: 4–6 hours at 37 °C, 5–7% CO2, e.g. 20 µL/mL
   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 (refer to 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 (refer to table below). The dilution prevents non-specific staining of cells not secreting IFN-γ during this period.

▲ For each test with 10^7 total cells, prepare:
100 mL of **cold buffer** (2–8 °C)
100 µL of **cold medium** (2–8 °C)
10 mL (or 100 mL; refer to table below) of **warm medium** (37 °C).

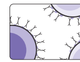
▲ Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and a non-specific cell labeling (exception: warm medium during secretion period).

▲ Volumes shown below are for 10^7 total cells. When working with fewer than 10^7 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 2×10^7 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 completely.

▲ 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 IFN-γ Secretion Assay, e.g., by density gradient centrifugation or by using the Dead Cell Removal Kit (# 130-090-101).

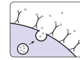
4. Protocol for the IFN-γ Secretion Assay



Labeling cells with IFN-γ Catch Reagent

1. Use 10^7 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.

   ▲ **Note:** Do not remove supernatant by decanting. This will lead to cell loss and incorrect incubation volumes.
3. Resuspend cell pellet in 80 µL of cold medium per 10^7 total cells.
4. Add 20 µL of IFN-γ Catch Reagent per 10^7 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:

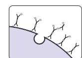
Expected number of IFN-γ–secreting cells	Dilution	Amount of medium to add per 10^7 total cells
<5%	10^6 cells/mL	10 mL
≥5%	≤10^5 cells/mL	100 mL

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

4. Protocol for the IFN-γ Secretion Assay

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.



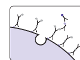
Labeling cells with IFN-γ Detection Antibody

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 added buffer, repeat wash step.
3. Resuspend cell pellet in 80 µL of **cold buffer** per 10^7 total cells.
4. Add 20 µL of **IFN-γ Detection Antibody (PE)** per 10^7 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 (# 130-094-969).
6. Mix well and incubate for 10 minutes **on ice**.
7. Wash cells by adding 10 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at 2–8 °C, aspirate supernatant completely.
8. Proceed to magnetic labeling (4.4).

4. Protocol for the IFN-γ Secretion Assay

4.4 Magnetic labeling



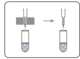
Magnetic labeling with Anti-PE MicroBeads UltraPure

▲ The recommended incubation temperature is 2–8 °C. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic separation. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, 30 µm, # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

1. Resuspend cell pellet in 80 µL of **cold buffer** per 10^7 total cells.
2. Add 20 µL of **Anti-PE MicroBeads UltraPure** per 10^7 total cells.
3. Mix well and incubate for 15 minutes at 2–8 °C.
4. Wash cells by adding 10 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at 2–8 °C. Aspirate supernatant completely
5. Resuspend cell pellet in 500 µL of **cold buffer**. For higher cell numbers than 5×10^7 use a dilution of 10^8 cells/mL.
6. (Optional) Take an aliquot for flow cytometric analysis and cell count of the fraction before enrichment.
7. Proceed to magnetic separation (4.5).

4.5 Magnetic separation



Magnetic separation using MS or LS Columns

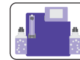
▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of IFN-γ–secreting cells. For details refer to the table in section 1.4.

▲ Always wait until the column reservoir is empty before proceeding to the next step.

▲ When enriching antigen-specific T cells, **always perform two consecutive column runs** to achieve best results.

1. Place column in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.
2. Prepare column by rinsing with the appropriate amount of buffer:
   MS: 500 µL LS: 3 mL
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with the appropriate amount of buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty.
   MS: 3×500 µL LS: 3×3 mL

5. Remove column from the separator and place it on a suitable collection tube.
   ▲ **Note:** To perform a second column run, you may elute the cells directly from the first onto the second, equilibrated column instead of a collection tube.
6. Pipette the appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
   MS: 1 mL LS: 5 mL
7. To increase the purity of IFN-γ–secreting cells, the eluted fraction can be enriched over a second MS or LS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.
   ▲ **Note:** For subsequent cell culture, the cells can also be eluted with medium. If part of the cells are analyzed by flow cytometry, the medium should not contain phenol red.
8. Proceed to analysis (refer to section 5), cell culture, or other subsequent experiment.



Magnetic separation with the autoMACS® Pro Separator

▲ Refer to the user manual for instructions on how to use the autoMACS® Pro Separator.

▲ Buffers used for operating the autoMACS Pro Separator should have a temperature of ≥10 °C.

▲ Program choice depends on the isolation strategy, the strength of magnetic labeling, and the frequency of magnetically labeled cells. For details refer to the section describing the cell separation programs in the user manual.

1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.
3. For a standard separation choose the following program:
   Positive selection: Posseld
   Collect positive fraction in row C of the tube rack.
4. Proceed to analysis (refer to section 5), cell culture, or other subsequent experiment.

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.

▲ **Note:** Acquire 2×10^5 viable cells from each sample.

▲ **Note:** For enumeration of low frequent IFN-γ–secreting cells, acquire all of the positive fraction. For preparative purposes, acquire an aliquot of the positive fraction to determine the performance of the cell enrichment.

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 PBMCs of a CMV$^+$ donor have been incubated for 16 hours with and without CMV lysate (5 µg/mL).
2. The IFN-γ Secretion Assay was performed on the stimulated and the unstimulated sample.
3. Counterstaining of T cells was performed using CD4-FITC.
4. Monocytes were stained with CD14-PerCP.
5. Dead cells were stained with PI, which was added just prior to flow cytometric analysis to a final concentration of 0.5 µg/mL.
6. 200,000 viable cells of the fractions before enrichment and the complete enriched fractions were acquired by flow cytometry using the MACSQuant® Analyzer, from the stimulated as well as from the unstimulated samples.
7. A lymphocyte gate based on forward scatter (FSC) and side scatter (SSC) properties was activated prior to further gating to exclude monocytes and debris (A).

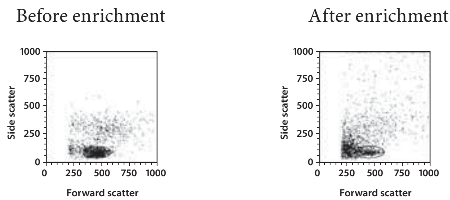
8. Dead cells and monocytes were excluded according to PI- and CD14-PerCP–staining in channel B2 (PE) versus channel R1 (PerCP).

   ▲ **Note:** 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.

   ▲ **Note:** The sensitivity of detection is further enhanced by exclusion of undesired non-T cells, like monocytes which may cause non-specific background staining.

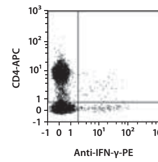
9. Analysis of secreted IFN-γ (PE) versus CD4-APC staining by viable lymphocytes is displayed (B).

A) Lymphocyte gate

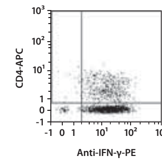


B) Antigen-specific CD4⁺ T cells stained for secreted IFN-γ

Sample stimulted with CMV lysate



0.257% of the total CD4⁺ T cell population secrete IFN-γ (see formula below).



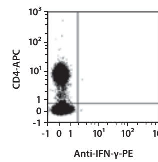
The IFN-γ–secreting CD4⁺ T cells have been enriched to 97.1%.

719 IFN-γ⁺CD4⁺ T cells were enriched from 10^6 CD4⁺ cells (= 0.072%; see formula below).

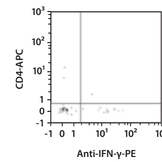
$$\%\ \text{IFN-}\gamma^+ \text{ cells among CD4}^+ = \frac{\#\text{ of IFN-}\gamma^+\text{CD4}^+\text{ cells in the analyzed sample}}{\#\text{ of total CD4}^+\text{ cells in the analyzed sample}} \times 100$$

$$\%\ \text{IFN-}\gamma^+ \text{ cells among CD4}^+ = \frac{\text{abs. }\#\text{ of IFN-}\gamma^+\text{CD4}^+\text{ cells in the enriched fraction}}{\text{abs. }\#\text{ of total CD4}^+\text{ cells before enrichment}} \times 100$$

Unstimulated control sample



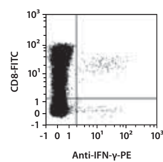
0.006% of the total CD4⁺ T cell population secrete IFN-γ.



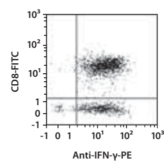
≤2 IFN-γ⁺CD4⁺ T cells were enriched from 10^6 CD4⁺ cells (≤ 0.0002%).

C) Antigen-specific CD8⁺ T cells stained for secreted IFN-γ

Sample stimulted with CMV lysate



0.257% of the total CD8⁺ T cell population secrete IFN-γ (see formula below).



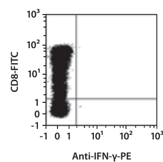
The IFN-γ–secreting CD8⁺ T cells have been enriched to 98.9%.

2953 IFN-γ⁺CD8⁺ T cells were enriched from 10^6 CD8⁺ cells (= 0.295%; see formula below).

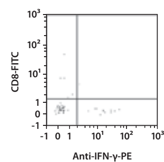
$$\%\ \text{IFN-}\gamma^+ \text{ cells among CD8}^+ = \frac{\#\text{ of IFN-}\gamma^+\text{CD8}^+\text{ cells in the analyzed sample}}{\#\text{ of total CD8}^+\text{ cells in the analyzed sample}} \times 100$$

$$\%\ \text{IFN-}\gamma^+ \text{ cells among CD8}^+ = \frac{\text{abs. }\#\text{ of IFN-}\gamma^+\text{CD8}^+\text{ cells in the enriched fraction}}{\text{abs. }\#\text{ of total CD8}^+\text{ cells before enrichment}} \times 100$$

Unstimulated control sample



0.006% of the total CD8⁺ T cell population secrete IFN-γ.



≤1 IFN-γ⁺CD8⁺ T cells were enriched from 10^6 CD8⁺ cells (≤ 0.0001%).

6. References

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7. Appendix:

A: Flask and dish sizes for *in vitro* stimulation

For *in vitro* stimulation (refer to 4.2) 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
0.50×10^7	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

B: Detection and enrichment of cytokine-secreting cells from whole blood

B1. Reagent and instrument requirements

B2. Protocol

- **B 2.1 (Antigen-specific) *in vitro* stimulation**
- **B 2.2 Cytokine Secretion Assay**
- **B 2.3 Magnetic labeling**
- **B 2.4 Magnetic separation**

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

B 1. Reagent and instrument requirements

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

  IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (PE) (# 130-054-201)

  IL-2 Secretion Assay – Cell Enrichment and Detection Kit (PE) (# 130-090-488)

  IL-4 Secretion Assay – Cell Enrichment and Detection Kit (PE) (# 130-054-101)

  IL-10 Secretion Assay – Cell Enrichment and Detection Kit (PE) (# 130-090-435)

- **Anticoagulant**: sodium heparin
- 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.

  ▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as human serum albumin, human serum, or fetal bovine serum (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- Culture medium, e.g., RPMI 1640, containing 5% human serum (do not use BSA or FBS because of non-specific stimulation!).
- **Erythrocyte lysing solution (1×):**

  Prepare freshly from Red Blood Cell Lysis Solution 10× (130-094-183).

- (Optional) **Staining reagents**: CD4-FITC (# 130-080-501) or CD8-FITC (# 130-080-601) and CD14-PerCP (# 130-094-969).

  ▲ **Note:** Do not use PE-tandem conjugates. They may also be recognized by Anti-PE MicroBeads UltraPure.

  ▲ **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 (# 130-094-969). These cells can then be excluded together with PI-stained dead cells by gating.

- **Propidium Iodide Solution** or **7-AAD** to exclude dead cells from analysis.
- MACS Columns and MACS Separators:

Column	Max. number of labeled cells	Max. number of total cells	Separator
MS	10^7	2×10^8	MiniMACS, OctoMACS, VarioMACS, SuperMACS II
autoMACS	2×10^8	4×10^9	autoMACS Pro

  ▲ **Note:** Column adapters are required to insert certain columns into VarioMACS™ Separator or SuperMACS™ Separator. For details, see MACS Separator data sheets.

- (Optional) Rotation device for tubes: MACSmix Tube Rotator (# 130-090-753)
- (Optional) Pre-Separation Filters, 30 µm (# 130-041-407) to remove cell clumps.

B 2. Protocol

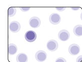
B 2.1 (Antigen-specific) *in vitro* stimulation

▲ 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 overnight 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 FBS because of non-specific stimulation.



Protocol for *in vitro* stimulation

1. Start with **5 mL of fresh, sodium heparinized, human blood** (containing about 10^7 lymphocytes) in a 50 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 <5% of total cytokine secreting cells. If ≥5% 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 avoids non-specific staining of cells not secreting cytokines during this period.

▲ For each sample with 5 mL whole blood prepare:

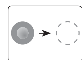
100 mL of **cold buffer** (4–8 °C)
200 µL of **cold medium** (4–8 °C)
7 mL of **warm medium** (37 °C)
45 mL of **erythrocyte lysing solution** (room temperature).

▲ 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.

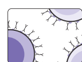
▲ Dead cells may bind non-specifically to MACS MicroBeads or antibodies. Therefore, when working with cell preparations containing large amounts of dead cells, they should be removed before starting the Cytokine Secretion Assay, e.g. by density gradient centrifugation or by using the Dead Cell Removal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

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



Lysis of erythrocytes

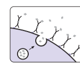
1. After stimulation add 45 mL of erythrocyte lysing solution to 5 mL whole blood sample.
2. Mix gently and incubate for 10 minutes at **room temperature**. Rotate tube continuously using the MACSmix Tube Rotator (# 130-090-753), or turn tube several times during incubation.
3. Centrifuge cells at 300×g for 10 minutes at **room temperature**, remove supernatant **completely**.



Labeling cells with Cytokine Catch Reagent

1. Resuspend cell pellet in 15 mL of **cold buffer**, and transfer into a 15 mL conical propylene tube.

2. Centrifuge at 300×g for 10 minutes at **2–8 °C**. Pipette off supernatant completely.
3. Resuspend pellet in 160 µL of **cold medium**.
4. Add 40 µL of **Cytokine Catch Reagent**, mix well and incubate for 5 minutes **on ice**.

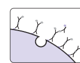


Cytokine secretion period

1. Add 7 mL of **warm medium** (37 °C) to dilute the cells.

   ▲ **Note:** For frequencies of cytokine secreting cells ≥ 5% 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 using the MACSmix Tube Rotator, 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.

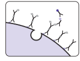


Labeling cells with Cytokine Detection Antibody

1. Put the tube **on ice**.
2. Wash cells by adding 8 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at **2–8 °C**. Pipette off supernatant completely.
3. Resuspend cell pellet in 160 µL of **cold buffer**.

4. Add 40 µL of **Cytokine Detection Antibody (PE)**.
5. (Optional) Add additional staining reagents, e.g. 20 µL of CD4-FITC (# 130-080-501) or CD8-FITC (# 130-080-601) and CD14-PerCP (# 130-094-969).
6. Mix well and incubate for 10 minutes **on ice**.
7. Wash cells by adding 10 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at **2–8 °C**. Pipette off supernatant completely.

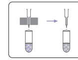
B 2.3 Magnetic labeling



Magnetic labeling with Anti-PE MicroBeads UltraPure

1. Resuspend cell pellet in 160 µL of **cold buffer**.
2. Add 40 µL of **Anti–PE MicroBeads UltraPure**, mix well and incubate for 15 minutes at **2–8 °C**.
   ▲ **Note:** Incubate in refrigerator at 2–8 °C; do not work on ice during this step.
3. Wash cells by adding 10 mL of **cold buffer**, centrifuge at 300×g for 10 minutes at 2–8 °C. Pipette off supernatant completely.
4. Resuspend cell pellet in 500 µL of **cold buffer**.
5. (Optional) Take an aliquot for flow cytometric analysis and cell count of the fraction before enrichment.
6. Proceed to magnetic separation.

B 2.4 Magnetic separation

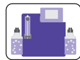


Magnetic separation using MS Columns

▲ To achieve highest purities, perform two consecutive column runs.

1. Prepare **two MS Columns** per sample by rinsing with 500 µL **cold buffer**, discard effluent.
2. Place first column into the magnetic field of a suitable MACS Separator. For details refer to MS Column data sheet.
3. (Optional) Pass cells through Pre-Separation Filters, 30 µm (# 130-041-407) to remove clumps.
4. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
5. Wash column with 3×500 µL of cold buffer. Collect unlabeled cells that pass through and combine with the effluent from step 4.
   ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.
6. Remove first column from separator, place second column into the separator, and put the first column on top of the second one.
7. Pipette 1 mL of cold buffer on top of the first column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger; directly onto the second column. Collect unlabeled cells that pass through.

8. Wash second column with 3×500 µL of cold buffer. Collect unlabeled cells that pass through and combine with the effluent from step 5.
   ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.
9. Remove second column from separator, place column on a suitable collection tube.
10. Pipette 500 µL of cold buffer on top of the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger.
    ▲ **Note:** For subsequent cell culture, the cells can also be eluted with medium. If part of the cells are analyzed by flow cytometry, the medium should **not contain** phenol red.
11. Proceed to flow cytometric analysis (see detailed protocol), cell culture, or other subsequent experiment.



Magnetic separation with the autoMACS® Pro Separator

▲ Refer to the user manual for instructions on how to use the autoMACS® Pro Separator.

▲ Buffers used for operating the autoMACS Pro Separator should have a temperature of ≥10 °C.

▲ Program choice depends on the isolation strategy, the strength of magnetic labeling, and the frequency of magnetically labeled cells. For details refer to the section describing the cell separation programs in the user manual.

1. Prepare and prime the instrument.

2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.
3. For a standard separation choose the following program:

   Positive selection: Posseld
   Collect positive fraction in row C of the tube rack.
4. Proceed to flow cytometric analysis (see detailed protocol), cell culture or other subsequent experiment.

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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