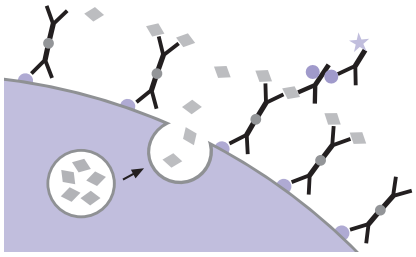
Miltenyi Biotec

GM-CSF Secretion Assay – Detection Kit (PE)

human

For 100 tests with 10^6 cells

Order no. 130-105-755



Miltenyi Biotec

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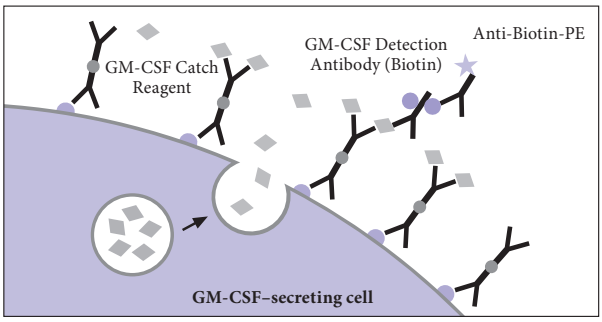
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1. Description

Components	**1 mL GM-CSF Catch Reagent:** anti-GM-CSF monoclonal antibody (isotype: rat IgG2a) conjugated to CD45-specific monoclonal antibodies (mouse IgG2a). **1 mL GM-CSF Detection Antibody (Biotin):** anti-GM-CSF monoclonal antibody (isotype: rat IgG2a) conjugated to biotin. **0.2 mL Anti-Biotin-PE:** monoclonal anti-biotin antibody conjugated to R-phycoerythrin (PE).
Capacity	For 100 tests with 10^6 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.

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1.1 Principle of the GM-CSF Secretion Assay

Antigen-specific T cells are analyzed using the GM-CSF 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 such as CytoStim or specific peptide, protein, or other protein antigen preparations, for example, from *Candida albicans* (*C. albicans*). Subsequently, a GM-CSF–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 GM-CSF binds to the GM-CSF Catch Reagent on the positive, secreting cells. These cells are subsequently labeled with a second GM-CSF–specific antibody, the **GM-CSF Detection Antibody** conjugated to biotin and Anti-Biotin-PE for sensitive detection by flow cytometry.

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

1.2 Background information

Granulocyte macrophage colony–stimulating factor (GM-CSF) is a hematopoietic growth factor that is essential for proliferation and development of granulocyte and monocyte/macrophage progenitors. It also functions as a growth factor for erythroid and megakaryocytic precursor cells in conjunction with erythropoietin. GM-CSF is secreted by various cell types including T cells, macrophages, endothelial cells, and fibroblasts in response to inflammatory stimuli and cytokines. In addition, GM-CSF strongly chemoattracts neutrophils and eosinophils and enhances the effector functions of neutrophils and macrophages.

1.3 Applications

- Detection of viable GM-CSF–secreting leukocytes.
- Detection of viable GM-CSF–secreting leukocytes from whole blood. The GM-CSF Secretion Assay can be started directly from whole blood. For details on the procedure refer to www.miltenyibiotec.com/protocols.
- Detection of GM-CSF–secreting, antigen-specific T cells for enumeration and phenotypic characterization.

- Monitoring and analysis of antigen-specific T cell immunity, for example, in autoimmunity and allergy.
- Analysis of GM-CSF–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.

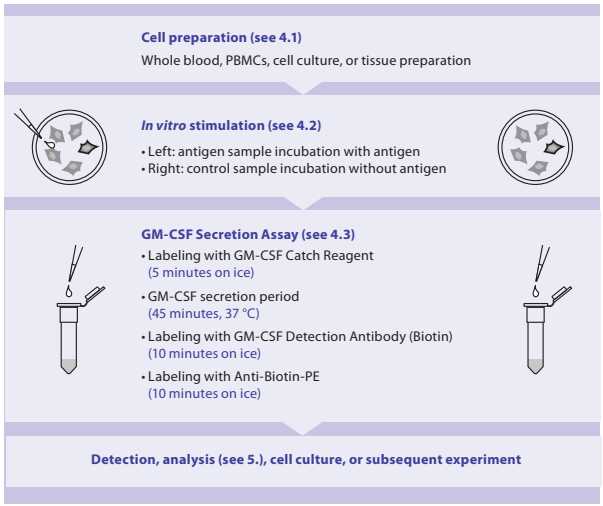
1.4 Reagent and instrument requirements

- 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 with stable glutamin, containing 5% human serum, like autologous or AB 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.

- (Optional) For detection of activated T cells with CD154, the incubation with CD40 pure – functional grade (# 130-094-133) is recommended to avoid downregulation of CD154 expression.
- Propidium Iodide Solution (# 130-093-233) or 7-AAD Staining Solution (# 130-111-568) 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, CD8-FITC, CD14-PerCP-Vio® 700, and CD20-PerCP-Vio 700.
- 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 setup

3.1 Controls

Negative control
For accurate detection of GM-CSF–secreting cells, a negative control sample should always be included. This will provide information about GM-CSF 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 phorbol myristate acetate (PMA) (20 ng/mL) and ionomycin (1 µg/mL) for 4 hours, may be included in the experiment.

3.2 Kinetics of restimulation and proposed time schedule

Peptides
Upon stimulation with peptide, the cells can be analyzed for GM-CSF 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 GM-CSF Secretion Assay.

Proteins
Upon stimulation with protein antigen preparations, for example, from *C. albicans*, the cells can be analyzed for GM-CSF secretion 6–16 hours later. It is possible to start the stimulation of the cells late in the afternoon and perform the GM-CSF Secretion Assay the following morning.

Costimulation
The addition of costimulatory agents like CD28 or CD49d antibodies 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 GM-CSF–secreting cells are stained with biotin-conjugated GM-CSF Detection Antibodies and Anti-Biotin-PE. To identify cells of interest, counterstaining for T cells with, for example, CD4-APC and CD8-FITC is important.

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

▲ The samples should be stained with propidium iodide (PI), for example with Propidium Iodide Solution (# 130-093-233), or 7-AAD Staining Solution (# 130-111-568) 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 and B cells with antibodies conjugated to peridinin chlorophyll protein (PerCP), for example, CD14-PerCP-Vio® 700 and CD20-PerCP-Vio700. These cells together with PI-stained dead cells can then be excluded by gating.

3.4 Two-color cytokine analysis

GM-CSF–secreting cells can be analyzed simultaneously for IFN-γ, IL-2, IL-5, IL-10, IL-17, or TNF-α production by two-color cytokine analysis combining the GM-CSF Secretion Assay – Detection Kit (PE) with, e.g., the IL-2 Secretion Assay – Detection Kit (APC) (# 130-090-763) or the IFN-γ Secretion Assay – Detection Kit (FITC) (# 130-090-433). Detailed protocols are included in the data sheets of the Cytokine Secretion Assay – Detection Kits (APC) or (FITC) and are available at www.miltenyibiotec.com/protocols.

3.5 Combination with peptide-MHC tetramer staining

GM-CSF–secreting cells can be analyzed simultaneously for peptide-MHC tetramers combining the GM-CSF Secretion Assay – Detection Kit (PE) with APC-conjugated peptide-MHC tetramers. A special protocol with detailed recommendations for the experimental setup and the procedure is 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 GM-CSF–secreting cells, it is possible to enrich these cells

magnetically using the GM-CSF Secretion Assay – Cell Enrichment and Detection Kit (PE) (# 130-105-760). 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 GM-CSF 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.

▲ **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 GM-CSF 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 (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, containing 5% human serum, adjust to 10^7 cells/mL and 5×10^6 cells/cm² (refer to 6. Appendix: Flask and dish sizes for *in vitro* stimulation).
3. Add antigen or control reagent:

   PMA/ionomycin: 2–4 hours at 37 °C, 5–7% CO_2, e.g., 20 ng/mL/ 1 µg/mL

   CytoStim: 1–3 hours at 37 °C, 5–7% CO_2, e.g., 20 µL/mL

   Peptide: 3–6 hours at 37 °C, 5–7% CO_2, e.g., 1–10 µg/mL

   Protein preparation: 6–16 hours at 37 °C, 5–7% CO_2, e.g., 20 µg/mL

   For comparison of different experiments, the stimulation time should always be the same (refer to 3.2).

   ▲ **Note:** If CD154 antibodies are used in the labeling step of the cytokine secretion assay to stain activated $CD4^+$ T cells, a CD40-blocking antibody has to be added during the *in vitro* stimulation step to prevent CD154 down-regulation.
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 GM-CSF–secreting cells. If ≥5% of GM-CSF–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 GM-CSF 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)
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 non-specific cell labeling (exception: warm medium during secretion period).

▲ Volumes given below are for up to 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 GM-CSF Secretion Assay, for example, by densitiy gradient centrifugation or by using the Dead Cell Removal Kit (# 130-090-101).



Labeling cells with GM-CSF 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 **GM-CSF Catch Reagent** per 10^6 total cells, mix well and incubate for 5 minutes on ice.



GM-CSF secretion period

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

Expected number of GM-CSF–secreting cells	Dilution	Amount of medium to add per 10^6 total cells
<5%	10^6 cells/mL	1 mL
≥5%	≤10^5 cells/mL	10 mL

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

2. Incubate cells in closed tube for 45 minutes at 37 °C under slow continuous rotation 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 GM-CSF Detection Antibody (Biotin) and Anti-Biotin-PE

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 **GM-CSF Detection Antibody (Biotin)** per 10^6 total cells.
5. (Optional) Add staining antibodies, e.g., CD4-APC and CD8-FITC, according to manufacturer's recommendation.
6. Mix well and incubate for 10 minutes on ice.
7. Add 1 mL of **cold buffer** and centrifuge at 300×g for 10 minutes at 2–8 °C. Aspirate supernatant completely.

8. Resuspend cell pellet in 98 µL of cold buffer per 10^6 total cells.
9. Add 2 µL of **Anti-Biotin-PE** and incubate for 10 minutes on ice.
10. Add 1 mL of **cold** buffer and centrifuge at 300×g for 5 minutes at **2–8° C**. Aspirate supernatant.
11. Proceed to analysis (5.).

5. Detection and analysis of GM-CSF–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.

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

To illustrate the analysis, we describe the detection of GM-CSF–secreting T cells using the GM-CSF 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 have been incubated for 4 hours with and without PMA (20 ng/mL) and ionomycin (1 µg/mL).

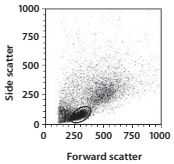
2. The GM-CSF Secretion Assay was performed on the stimulated and the unstimulated sample.
3. Counterstaining of T cells was performed using CD4-APC and CD8-FITC.
4. Monocytes and B cells were stained with CD14-PerCP-Vio® 700 and CD20-PerCP-Vio700.
5. Dead cells were stained with PI, which was added just prior to flow cytometric analysis to a final concentration of 0.5 µg/mL.
6. 250,000 viable cells 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, monocytes, and B cells were excluded according to PI–, CD14-PerCP-Vio700–, and CD20-PerCP-Vio700–staining in a fluorescence 2 (PE) versus fluorescence 3 (PI/PerCP-Vio700) plot (B).

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

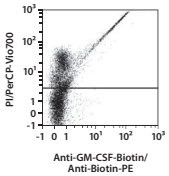
▲ **Note:** The sensitivity of detection is further enhanced by exclusion of undesired non–T cells, like monocytes which may cause non-specific background staining.

9. Analysis of secreted GM-CSF (PE) versus CD4-APC (C) or CD8-FITC (D) staining of viable lymphocytes is displayed. CD4 is downregulated due to PMA and ionomycin stimulation.

A) Lymphocyte gate

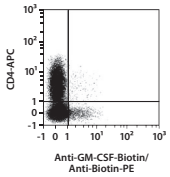


B) B cell, monocyte, and dead cell exclusion



C) GM-CSF–secreting CD4⁺ T cells

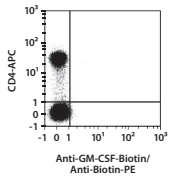
Stimulated sample



0.72% of the total CD4⁺ T cell population secrete GM-CSF (see formula below).

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

Unstimulated control sample

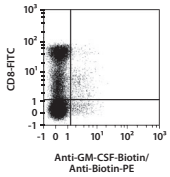


0.02% of the total CD4⁺ T cell population secrete GM-CSF.

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D) GM-CSF–secreting CD8⁺ T cells

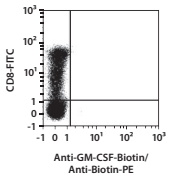
Stimulated sample



4.32% of the total CD8⁺ T cell population secrete GM-CSF (see formula below).

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

Unstimulated control sample



0.17% of the total CD8⁺ T cell population secrete GM-CSF.

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6. Appendix: 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 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

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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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