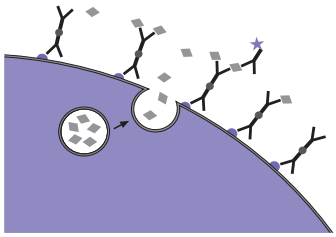
Miltenyi Biotec

TNF-α Secretion Assay
Detection Kit (APC)
human

For 100 tests with 10^6 cells Order no. 130-091-267



Miltenyi Biotec

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

140-000-980.02

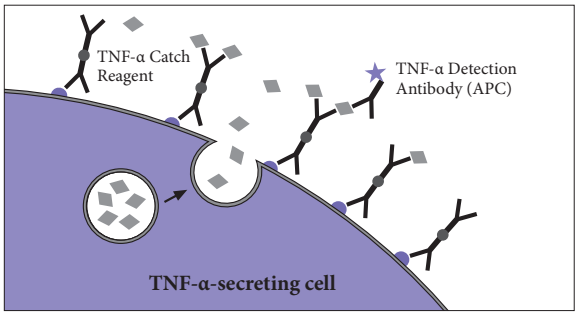
Index

1. Description

Components	1 mL **TNF-α Catch Reagent**: anti-TNF-α mono-clonal antibody (mouse IgG1) conjugated to cell surface specific monoclonal antibody (mouse IgG2a). 1 mL **TNF-α Detection Antibody**: anti-TNF-α monoclonal antibody (human IgG1) conjugated to APC (allophycocyanin).
Size	For 100 tests with 10^6 cells.
Product format	TNF-α Catch Reagent and TNF-α Detection Antibody are supplied in a solution containing 0.1% gelatine and 0.05% sodium azide.
Storage	Store protected from light at 4–8 °C. Do not freeze. The expiration dates are indicated on the vial labels.

1.1 Principle of the TNF-α Secretion Assay

Antigen-specific T cells are analyzed using the TNF-α Secretion Assay starting from whole blood, PBMCs or other leukocyte containing single cell preparations. The cells are restimulated for a short period of time with specific peptide, protein or other antigen preparations.



Subsequently, a TNF-α-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 TNF-α binds to the TNF-α Catch Reagent on the positive, secreting cells. These cells are subsequently labeled with a second TNF-α-specific antibody, the **TNF-α Detection Antibody** conjugated to allophycocyanin (APC) for sensitive detection by flow cytometry. Since viable cells are analyzed, non-specific background can be minimized by dead cell exclusion. This provides highest sensitivity of analysis.

1.2 Background and product applications

The TNF-α Secretion Assay - Detection Kit (APC) is designed for the detection and analysis of viable TNF-α-secreting leukocytes. It can also be used for two color cytokine analysis, a combination of the TNF-α Secretion Assay (APC) with a second Cytokine Secretion Assay (PE), to stain cells for co-expression of two cytokines.

The TNF-α Secretion Assay - Detection Kit is developed for **detection of TNF-α-secreting, antigen-specific T cells**. After re-stimulation with specific antigen *in vitro* secretion of TNF-α is induced.

TNF-α (tumor necrosis factor α) is secreted by activated $CD4^+$ T cells, monocytes, macrophages, NK cells and neutrophils. TNF-α is a mediator of inflammatory immune responses.

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

Examples of applications

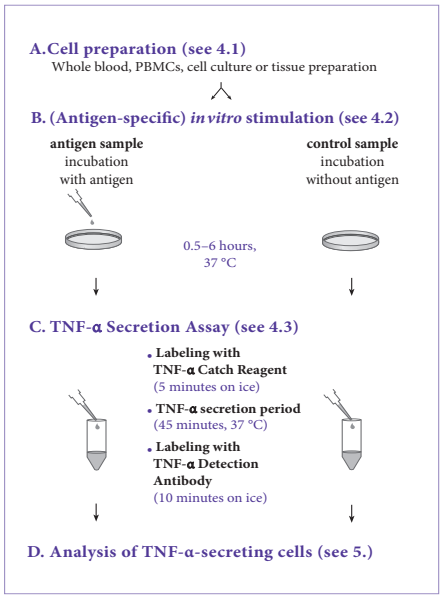
- Combination with a second Cytokine Secretion Assay - Detection Kit (PE) for the analysis of individual cells, which co-express TNF-α and the second cytokine.
- Detection of TNF-α-secreting, antigen-specific T cells for enumeration and phenotypic analysis as well as functional characterization in combination 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 TNF-α-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): Prepare a solution containing PBS (phosphate buffered saline) pH 7.2, 0.5% 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 (4–8 °C).
- **Culture medium**, e.g. RPMI 1640 (# 130-91-440), containing 5% human serum, like autologous or AB serum (do **not** use **BSA** or **FCS** because of non-specific stimulation!).
- **Propidium iodide (PI)** or **7-AAD** for flow-cytometric exclusion of dead cells. 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) or CD4-PE (# 130-091-231) and CD14-PerCP.
- (Optional) Cytokine Secretion Assay - Detection Kit:
  e.g. IFN-γ Secretion Assay (PE) (# 130-054-202), IL-2 Secretion Assay (PE) (# 130-090-487), or IL-4 Secretion Assay (PE) (# 130-054-102).
- Refrigerated centrifuge (4–8 °C).
- Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753).

2. Protocol overview



3. Experimental set-up

3.1 Controls

Negative control

For accurate detection of TNF-α-secreting antigen-specific cells, a negative control sample should always be included. This will provide information about TNF-α 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 0.5–6 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 TNF-α-secreting cells do not allow conclusions on the performance (e.g. sensitivity) of the TNF-α Secretion Assay.

3.2 Kinetics of restimulation and proposed time schedule

Peptides

Upon stimulation with peptide, the cells can be analyzed for TNF-α secretion 0.5–2 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 0.5–2 hours of stimulation, directly followed by the TNF-α Secretion Assay.

Proteins

Upon stimulation with protein, the cells can be analyzed for TNF-α secretion 3–6 hours later.

It is possible to start the stimulation of the cells late in the afternoon, and to perform the TNF-α Secretion Assay the following morning.

Co-stimulation

The addition of co-stimulatory agents like CD28 or CD49d antibody may enhance the response to the antigen. If co-stimulatory 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 TNF-α-secreting cells are stained with APC-conjugated TNF-α Detection Antibodies. To identify cells of interest, counterstaining for T cells with e.g. CD4-FITC (# 130-080-501) or CD4-PE (# 130-091-231) 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)

TNF-α-secreting cells can be analyzed simultaneously for IFN-γ, IL-2, IL-4 or IL-10 production by two color cytokine analysis combining the TNF-α 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 Detection of very low frequencies

(Optional, reagents not included) If the sample contains fewer than 0.01–0.1% of TNF-α-secreting cells, it is possible to enrich these cells magnetically using the TNF-α Secretion Assay – Enrichment and Detection Kit (PE) (# 130-091-269). Alternatively, TNF-α-secreting cells stained with the TNF-α 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 TNF-α Secretion Assay

4.1 Cell preparation

For the detection of cytokine-secreting cells, best results are achieved by starting the assay with fresh PBMCs, or 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., 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 TNF-α 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-murine proteins, like BSA or FCS, because of non-specific stimulation.

Protocol for *in vitro* stimulation

▲ When combining the TNF-α 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/cm² (see 7. Appendix A: Flask and dish sizes for stimulation).
3. Add antigen or control reagent:

   peptide: 0.5–2 hours at 37 °C, 5–7% CO_2, e.g. 1–10 µg/mL
   protein: 3–6 hours at 37 °C, 5–7% CO_2, e.g. 10 µg/mL
   SEB: 0.5–6 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 TNF-α-secreting cells. If ≥ 5% of TNF-α-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 TNF-α 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 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. Pipette off or aspirate supernatant completely.

Labeling cells with TNF-α 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 fewer 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 **TNF-α 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 TNF-α 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**.

TNF-α secretion period

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

Expected number of TNF-α–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 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 TNF-α 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 **TNF-α 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 CD4-PE (# 130-091-231) and CD14-PerCP.
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. Resuspend cell pellet in 0.5 mL of cold buffer and proceed to analysis (see 5.).

5. Detection and analysis of TNF-α-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 fixation of cells in combination with dead cell exclusion the Fixation and Dead Cell Discrimination Kit (# 130-091-163) is recommended. For details on the protocol refer to the data sheet.

▲ 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 Co-expression of TNF-α and IFN-γ by CD4⁺ T cells

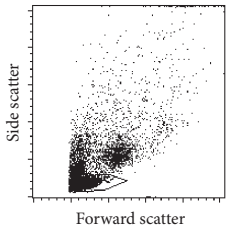
To illustrate the analysis, we describe the detection of TNF-α- and IFN-γ-secreting CD4⁺ T cells by using the TNF-α Secretion Assay (APC) in combination with the IFN-γ Secretion Assay (PE). This description, including how to set gates, should serve as a model for the analysis of your own sample.

1. 10^6 human PBMCs have been restimulated for 4 hours with, 5 µg/mL CMV-lysate.
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 A.).
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 B.).

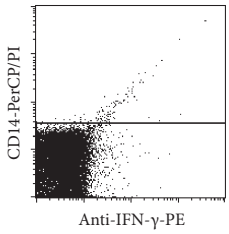
- The **dead cell exclusion** is crucial for the analysis of rare antigen-specific T cells, as antibodies may bind non-specifically to dead cells. This could lead to false positive events.
- The sensitivity of the detection is further enhanced by exclusion of undesired non-T cells, like monocytes which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

9. CD4⁺ T cells were gated in a fluorescence 1 versus fluorescence 4 plot (not shown).
10. For analysis secreted TNF-α (APC) versus secreted IFN-γ (PE) of viable CD4 ⁺ T cells is displayed (plot C.)

A. Lymphocyte gate using FSC versus SSC

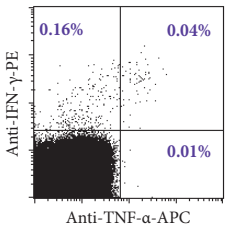


B. Dead cell and monocyte exclusion

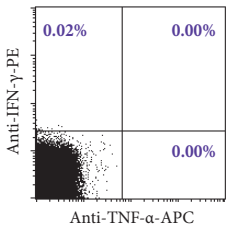


C. TNF-α and IFN-γ-secreting CD4⁺ T cells after stimulation with CMV-lysate

Stimulated sample



Unstimulated control



6. References

1. Manz, R; Assenmacher, M; Pflüger, E; Miltenyi, S; Radbruch, A (1992) Analysis and Sorting of Live cells According to Secreted Molecules Relocated to a Cell-Surface Affinity Matrix. Proc.Natl.Acad.Sci. USA 1921–1925. [139]
2. Assenmacher, M; Löhning, M; Scheffold, A; Manz, RA; Schmitz, J; Radbruch, A. (1998) Sequential production of IL-2, IFN-γ and IL-10 by individual staphylococcal enterotoxin B-activated T helper lymphocytes. Eur. J. Immunol. 28: 1534–1543. [483]
3. Brosterhus, H; Brings, S; Leyendeckers, H; Manz, RA; Miltenyi, S; Radbruch, A; Assenmacher, M; Schmitz, J (1999) Enrichment and detection of live antigen-specific CD4⁺ and CD8⁺ T cells based on cytokine secretion. Eur. J. Immunol. 29: 4053–4059. [573]
4. Oelke, M; Moehrle, U; Chen, JL; Behringer, D; Cerundolo, V; Lindemann, A; Mackensen, A (2000) Generation and purification of CD8⁺ Melan-A-Specific Cytotoxic T Lymphocytes for Adoptive Transfer in Tumor Immunotherapy. Clin. Cancer Res. 6: 1997–2005. [663]
5. Oelke, M; Kurokawa,T; Hentrich, I.; Behringer, D; Cerundolo, V; Lindemann, A; Mackensen, A (2000) Functional Characterization of CD8⁺ Antigen-Specific Cytotoxic T Lymphocytes after Enrichment Based on Cytokine Secretion: Comparison with the MHC-Tetramer Technology. Scand. J. Immunol. 52. 544–549. [970]
6. Bickham, K; Münz, C; Tsang, ML; Larsson, M; Fonteneau, J-F; Bhardwaj, N; Steinmann, R (2001) EBNA1-specific CD4⁺T cells in healthy carriers of Epstein-Barr virus are primarily Th1 in function. J. Clin. Invest. 107: 121–130. [1035]
7. Pittet, MJ; Zippelius, A; Speiser, DE; Assenmacher, M; Guillaume, P; Valmori, D; Lienard, D; Lejeune, F; Cerottini, JC; Romero, P (2001) Ex vivo IFN-γ secretion by circulating CD8 T lymphocytes: Implications of a novel approach for T cell monitoring in infectious malignant diseases. J. Immunol. 166: 7634–7640. [1037]

8. Becker, C; Pohla, H; Frankenberger, F; Schüler, T; Assenmacher, M; Schendel, DJ; Blankenstein, T (2001) Adoptive tumor therapy with T lymphocytes enriched through an IFN-γ capture assay. Nature Medicine 7, 10: 1159–1162. [1207]

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

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/cm^2. Both the dilution and the cell density are important to assure optimum stimulation.

The following table lists culture plate, dish and flask sizes suitable for different T cell numbers. It also indicates the appropriate amount of medium to add.

total cell number	medium volume to add	culture plate	well diameter
0.15×10^7	0.15 mL	96 well	0.64 cm
0.5×10^7	0.5 mL	48 well	1.13 cm
1×10^7	1 mL	24 well	1.6 cm
2×10^7	2 mL	12 well	2.26 cm
5×10^7	5 mL	6 well	3.5 cm

total cell number	medium volume to add	culture dish	dish diameter
4.5×10^7	4.5 mL	small	3.5 cm
10×10^7	10 mL	medium	6 cm
25×10^7	25 mL	large	10 cm
50×10^7	50 mL	extra large	15 cm

total cell number	medium volume to add	culture flask	growth area
12×10^7	12 mL	50 mL	25 cm^2
40×10^7	40 mL	250 mL	75 cm^2
80×10^7	80 mL	720 mL	162 cm^2
120×10^7	120 mL	900 mL	225 cm^2

B: Detection of cytokine-secreting cells from whole blood

B 1. Reagent and instrument requirements

- **Anticoagulant:** sodium heparin
- **Buffer** (degassed): Prepare a solution containing PBS (phosphate buffered saline) pH 7.2, 0.5% 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 (4–8 °C).
- **Culture medium**, e.g. RPMI 1640 (# 130-091-440), 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 CD4-PE (# 130-091-231) 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** for flow-cytometric exclusion of dead cells. For cell fixation and flow-cytometric exclusion of dead cells, the **Fixation and Dead Cell Discrimination Kit** (#130-091-163) is recommended.
- (Optional) Rotation device for tubes: MACSmix Tube Rotator (# 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 0.5–6 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) Co-stimulatory 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 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.

Labeling cells with Cytokine Catch Reagent

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 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 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 CD4-PE (# 130-091-231) 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 device, 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 fixation of cells in combination with dead cell exclusion the Fixation and Dead Cell Discrimination Kit (# 130-091-163) is recommended. For details on the protocol refer to the datasheet.

▲ 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 section 5.

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.

Warning

Reagents contain sodium azide. Sodium azide yields hydrazoic acid under acid conditions, which is extremely toxic. Azide compounds should be diluted with running water before being discarded. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

Legal notices

Limited product warranty

Miltenyi Biotec B.V. & Co. KG and/or its affiliate(s) warrant this product to be free from material defects in workmanship and materials and to conform substantially with Miltenyi Biotec's published specifications for the product at the time of order, under normal use and conditions in accordance with its applicable documentation, for a period beginning on the date of delivery of the product by Miltenyi Biotec or its authorized distributor and ending on the expiration date of the product's applicable shelf life stated on the product label, packaging or documentation (as applicable) or, in the absence thereof, ONE (1) YEAR from date of delivery ("Product Warranty"). Miltenyi Biotec's Product Warranty is provided subject to the warranty terms as set forth in Miltenyi Biotec's General Terms and Conditions for the Sale of Products and Services available on Miltenyi Biotec's website at www.miltenyibiotec.com, as in effect at the time of order ("Product Warranty"). Additional terms may apply. BY USE OF THIS PRODUCT, THE CUSTOMER AGREES TO BE BOUND BY THESE TERMS.
THE CUSTOMER IS SOLELY RESPONSIBLE FOR DETERMINING IF A PRODUCT IS SUITABLE FOR CUSTOMER'S PARTICULAR PURPOSE AND APPLICATION METHODS.

Technical information

The technical information, data, protocols, and other statements provided by Miltenyi Biotec in this document are based on information, tests, or experience which Miltenyi Biotec believes to be reliable, but the accuracy or completeness of such information is not guaranteed. Such technical information and data are intended for persons with knowledge and technical skills sufficient to assess and apply their own informed judgment to the information. Miltenyi Biotec shall not be liable for any technical or editorial errors or omissions contained herein.
All information and specifications are subject to change without prior notice. Please contact Miltenyi Biotec Technical Support or visit www.miltenyibiotec.com for the most up-to-date information on Miltenyi Biotec products.

Licenses

This product and/or its use may be covered by one or more pending or issued patents and/or may have certain limitations. Certain uses may be excluded by separate terms and conditions. Please contact your local Miltenyi Biotec representative or visit Miltenyi Biotec's website at www.miltenyibiotec.com for more information.
The purchase of this product conveys to the customer the non-transferable right to use the purchased amount of the product in research conducted by the customer (whether the customer is an academic or for-profit entity). This product may not be further sold. Additional terms and conditions (including the terms of a Limited Use Label License) may apply.
CUSTOMER'S USE OF THIS PRODUCT MAY REQUIRE ADDITIONAL LICENSES DEPENDING ON THE SPECIFIC APPLICATION. THE CUSTOMER IS SOLELY RESPONSIBLE FOR DETERMINING FOR ITSELF WHETHER IT HAS ALL APPROPRIATE LICENSES IN PLACE. Miltenyi Biotec provides no warranty that customer's use of this product does not and will not infringe intellectual property rights owned by a third party. BY USE OF THIS PRODUCT, THE CUSTOMER AGREES TO BE BOUND BY THESE TERMS.

Trademarks

autoMACS, MACS, MACSmix, and the Miltenyi Biotec logo are registered trademarks or trademarks of Miltenyi Biotec and/or its affiliates in various countries worldwide. All other trademarks mentioned in this publication are the property of their respective owners and are used for identification purposes only.