

Contents

1. Description
 - 1.1 Background information
 - 1.2 Applications
 - 1.3 Kinetics of cytokine and activation marker expression upon stimulation with CytoStim
 - 1.4 Frequencies of cytokine-producing cells upon stimulation with CytoStim
2. Recommendations for *in vitro* stimulation of T cells with CytoStim
 - 2.1 Reagent requirements
 - 2.2 Sample preparation
 - 2.3 *In vitro* stimulation of T cells
3. Example of detection of IFN-γ-secreting T cells upon restimulation with CytoStim using MACS® IFN-γ Secretion Assay
4. Appendix: Flask and dish sizes for *in vitro* stimulation of T cells

1. Description

Components	200 µL CytoStim, human, or 1 mL CytoStim, human
Capacity	200 µL for stimulation of 10 ⁸ total cells, or 1 mL for stimulation of 5×10 ⁸ total cells.
Product format	CytoStim is supplied in buffer containing stabilizer.
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Background information

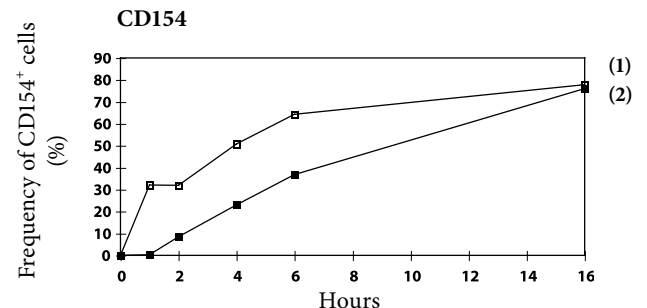
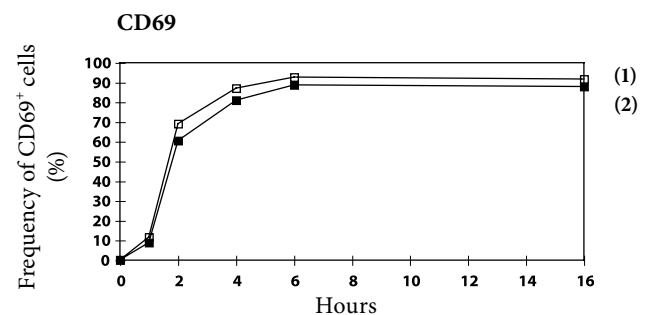
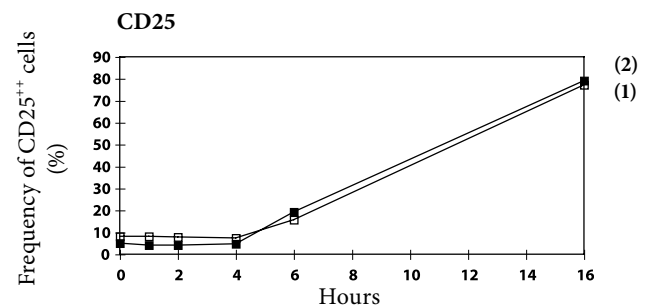
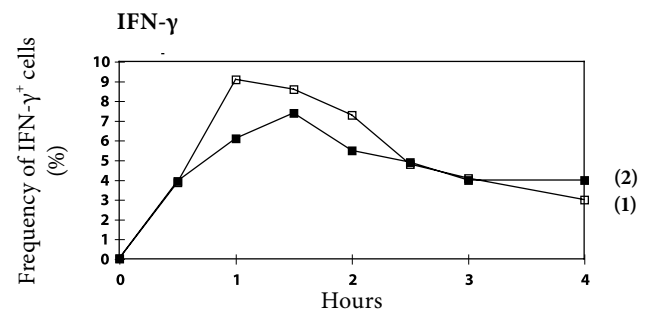
CytoStim was developed for rapid and efficient restimulation of human effector/memory T cells. CytoStim causes activation of T cells by binding the T cell receptor (TCR) and crosslinking it to an major histocompatibility complex (MHC) molecule of an antigen-presenting cell (APC). CytoStim is an antibody-based reagent that acts similar to a superantigen but independently of certain Vβ domains of the TCR. Upon stimulation with CytoStim, CD4⁺ and CD8⁺ cells start to secrete effector cytokines or up-regulate activation markers on their cell surface within a few hours.

1.2 Applications

- Rapid stimulation of T cells as a positive control for cytokine expression.
- Rapid stimulation of T cells as a positive control for expression of activation markers.

1.3 Kinetics of cytokine and activation marker expression upon stimulation with CytoStim

The following expression kinetics of IFN-γ, CD25, CD69, and CD154 upon stimulation with CytoStim were determined from two independent donors, (1) and (2), over several hours. Figures show frequencies of IFN-γ, CD25⁺, CD69⁺, and CD154⁺ cells among viable CD4⁺ lymphocytes.



1.4 Frequencies of cytokine-producing cells upon stimulation with CytoStim

Frequencies of the following cytokine-producing cells among CD4⁺ cells were determined after one hour stimulation with CytoStim in human peripheral blood mononuclear cells (PBMCs) using MACS[®] Cytokine Secretion Assays:

IFN- γ	0.12–9.10%
IL-2	0.08–0.24%
IL-4	0.02–0.04%
IL-5	0.02–0.14%
IL-10	0.15–0.20%
TNF- α	1.00–1.50%

For IL-17-secreting CD4⁺ T cells the maximum frequency was determined after four hours stimulation with CytoStim in human PBMCs using MACS Cytokine Secretion Assays:

IL-17A	0.60–1.40%
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2. Recommendations for *in vitro* stimulation of T cells with CytoStim

2.1 Reagent requirements

- Culture medium, e.g., RPMI 1640 (# 130-091-440) supplemented with 5% human serum.
▲ **Note:** When using CytoStim as a positive control for T cells stimulated with specific antigens, avoid using non-human proteins such as bovine serum albumin (BSA) or fetal bovine serum (FBS). Autologous or human AB serum is recommended.
- (Optional) MACS Cytokine Secretion Assay Kit. For additional reagent and instrument requirements refer to the respective data sheet.
For a detailed product list of MACS Cytokine Secretion Assays see www.miltenyibiotec.com.
- (Optional) Intracellular cytokine staining, e.g., with Anti-IFN- γ -PE (# 130-091-653). For additional reagent requirements refer to the respective data sheet.
- (Optional) Surface staining reagents, such as CD69-FITC (# 130-092-166), CD25-PE (# 130-091-024), CD154-PE (# 130-092-289), or CD154-APC (# 130-092-290).
▲ **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.

2.2 Sample preparation

For activation of T cells, best results are achieved by stimulation of fresh PBMCs, whole blood, or other leukocyte containing single-cell preparations from tissues or cell lines. Alternatively, frozen cell preparations can be used.

When working with anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™.

▲ **Note:** To remove platelets after density gradient separation, resuspend cell pellet in buffer and centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully aspirate supernatant. Repeat washing step.

When working with tissues or lysed blood, prepare a single-cell suspension using standard methods.

For details see the protocols section at www.miltenyibiotec.com/protocols.

It is necessary, that the cell preparation also contains APCs for efficient stimulation of the T cells. When working with purified T cells, APCs need to be added to the culture.

▲ **Note:** PBMCs may be stored overnight. The cells should be resuspended and incubated in culture medium as described in 2.3 steps 1–3, without addition of CytoStim. CytoStim is then added to the culture on the next day.

2.3 *In vitro* stimulation of T cells with CytoStim

▲ Always include a negative control in experiment. The sample should be treated exactly the same as the stimulated sample, except for the addition of CytoStim.

▲ A positive control should also be included in experiment, stimulated with CytoStim.

▲ Do not use media containing any non-human proteins, such as BSA or FBS, because of non-specific stimulation.

1. Wash cells by adding medium and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cells in culture medium at 10⁷ cells/mL. Plate cells in dishes at a density of 5×10⁶ cells/cm² (see 5. Appendix: Flask and dish sizes for *in vitro* T cell stimulation).
3. Add 20 μ L of CytoStim per mL cell suspension. Mix carefully and incubate cells at 37 °C; 5% CO₂.

Cytokine Secretion Assay: Incubate cells for 1–4 hours, depending on the cytokines to be analyzed.

▲ **Note:** Cells can be prepared and placed into culture overnight without addition of CytoStim. CytoStim is then added the next morning for 1–4 hours of stimulation, directly followed by the Cytokine Secretion Assay.

Intracellular cytokine staining: Incubate cells for 2 hours, then add 1 μ g/mL Brefeldin A, and incubate for further 4 hours.

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.

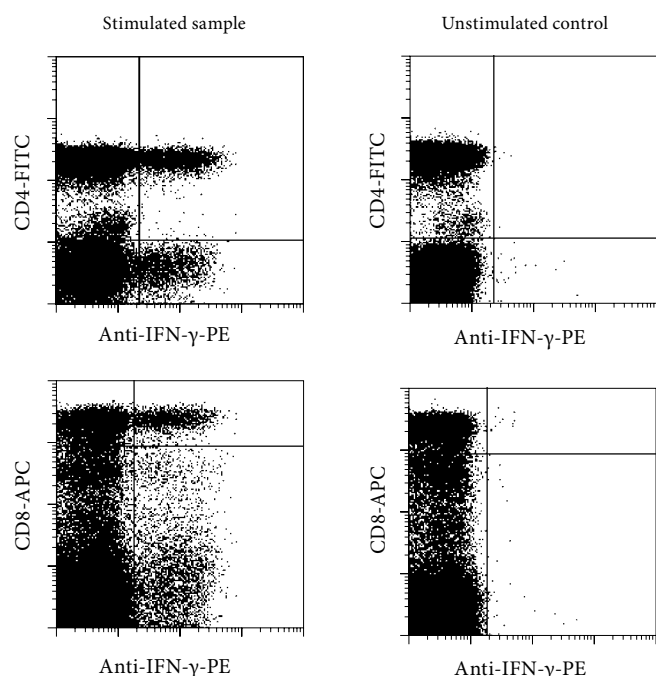
Cytokine Secretion Assay: Refer to the protocol section of the respective data sheet.

Intracellular cytokine staining: When working with fluorochrome-conjugated MACS anti-cytokine antibodies, refer to the protocol section of the respective data sheet.

▲ **Note:** When preparing cells for intracellular cytokine staining, fixed cells may be stored at 2–8 °C for up to one week.

3. Example of detection of IFN- γ -secreting T cells upon restimulation with CytoStim using MACS® IFN- γ Secretion Assay

Human PBMCs were restimulated with CytoStim for one hour. Responding T cells were stained according to their secretion of IFN- γ using the IFN- γ Secretion Assay – Detection Kit (# 130-054-202). T cells were counterstained for CD4 and CD8 expression. IFN- γ -secretion of viable lymphocytes is shown.



5. Appendix: Flask and dish sizes for *in vitro* stimulation of T cells

For *in vitro* stimulation of T cells (see 2.2) 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 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

All protocols and data sheets are available at www.miltenyibiotec.com.

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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