Miltenyi Biotec

CMV IE-1 – Recombinant Protein
human

Order no. 130-092-137

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1. Description

Components	**2.5 mg CMV IE-1 – Recombinant Protein, human:** full-length protein (Swiss-Prot Acc. no. P13202).
Capacity	1 mL for loading of up to 10^8 DCs.
Product format	The recombinant protein is supplied in a solution of 1× phosphate-buffered saline (PBS).
Purity	>95% as determined by SDS-PAGE analysis. Low endotoxin.
Transport	At –70 °C.
Storage	CMV IE-1 – Recombinant Protein should be stored at –70 °C. The expiration date is indicated on the vial label. Aliquots should be stored at –70 °C. Avoid repeated freeze-thaw cycles.

1.1 Background information

Human cytomegalovirus (CMV) is a member of the herpes virus group belonging to the subfamily of beta-herpes viruses. Between 50–85% of human adults are infected with CMV. Once infected, the virus persists in the organism. The infection is asymptomatic in healthy individuals, but in immunocompromised patients CMV can cause severe diseases.
CMV IE-1 (immediate early protein 1), also known as UL123, is a non-structural protein, which is one of the first CMV antigens expressed in an infected cell, and predominantly induces a $CD8^+$ T cell response. IE-1–specific T cells occur in infected individuals at frequencies comparable to those of pp65-specific $CD8^+$ T cells.[1] Both CMV antigens, IE-1 and pp65, are considered to be dominant T cell targets.
CMV IE-1 – Recombinant Protein is specially developed for efficient loading of monocyte derived dendritic cells (MoDCs) for subsequent restimulation of IE-1–specific $CD4^+$ and $CD8^+$ T cells. The effector cytokines produced after stimulation allow identification and analysis of IE-1–specific T cells.

1.2 Applications

- Restimulation of IE-1–specific T cells using MoDCs pulsed with recombinant IE-1 protein.

2. Recommendations for *in vitro* restimulation of IE-1–specific T cells

2.1 Sample preparation

For induction of cytokine secretion, presentation of the recombinant protein via professional antigen-presenting cells (APCs), e.g. monocyte derived dendritic cells (MoDCs), is necessary. Addition of the recombinant protein directly to peripheral blood mononuclear cell (PBMC) samples is not recommended. For pulsing, the use of immature MoDCs prepared using the Mo-DC Generation Toolbox I or II is recommended. Alternatively, APCs can be generated *in vitro* from human monocytes accordingly to Dauer, M. *et al.* [2]

2.2 Reagent requirements

- Culture media, for example, RPMI 1640 (# 130-091-440) containing 5% human serum, e.g., autologous or AB serum, and X-VIVO 15® (Cambrex).

  ▲ **Note:** Do not use bovine serum albumin (BSA) or fetal bovine serum (FBS) because of non-specific stimulation.
- Mo-DC Generation Toolbox I (# 130-093-568) or Mo-DC Generation Toolbox II (# 130-095-044) including separation reagents, media, and cytokines for generation of MoDCs.
- (Optional) Reagents for the isolation of monocytes, for example, CD14 MicroBeads (# 130-050-201) or the Monocyte Isolation Kit II (# 130-091-153).
- (Optional) Cytokines for MoDC generation, for example, Human GM-CSF (# 130-093-866), Human IL-4 (# 130-093-921), or Human TNF-α (# 130-094-024).
- (Optional) Antibodies for flow cytometric analysis, e.g., CD4-FITC (# 130-080-501), CD8-FITC (# 130-080-601), CD154-APC (# 130-092-290), or Anti-IFN-γ-PE (# 130-091-653). For more information about antibodies refer to www.miltenyibiotec.com
- (Optional) Cytokine Secretion Assay Kit. For additional reagent and instrument requirements refer to the data sheet of the respective Cytokine Secretion Assay Kit.
- (Optional) T cells for restimulation can be isolated using, e.g., the Pan T Cell Isolation Kit II (# 130-091-156).
- (Optional) Inside Stain Kit (# 130-090-477).

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2.3 *In vitro* restimulation of IE-1–specific T cells

Protocol overview
1) Isolation of monocytes
2) Generation of immature MoDCs
3) Pulsing of immature MoDCs with CMV IE-1 Recombinant Protein and maturation
4) Restimulation of T cells
5) Analysis of T cells, e.g., by intracellular cytokine staining

▲ Always include unpulsed MoDCs as a negative control in the experiment.

2.3.1 Pulsing of MoDCs with CMV IE-1 – Recombinant Protein and maturation

1. Resuspend up to 3×10^6 immature MoDCs in 1 mL X-VIVO 15 cell culture medium.
2. Add 10 µL of CMV IE-1 Recominant Protein (25 µg).
3. Add, e.g., 1000 IU TNF-α for DC maturation.
4. Mix carefully and incubate cells for 24 hours at 37 °C and 5% CO_2.

2.3.2 Restimulation of T cells

1. Harvest pulsed and maturated MoDCs.
2. Wash and resuspend to a final concentration of 10^7 cells/mL in RPMI 1640 medium supplemented with L-Glutamine and 5% human AB serum.
3. For restimulation mix one MoDC per 3–10 T cells, e.g., 3×10^5 MoDCs (30 µL) for restimulation of 7×10^5 T cells (70 µL).
4. Incubate cells for 2 hours.
5. Add brefeldin A and further cultivate for 4 hours.
6. Harvest and wash cells.
7. Proceed to intracellular cytokine staining (refer to 2.3.3). Alternatively proceed with MACS® Cytokine Secretion Assay, for a detailed protocol refer to the respective data sheet.

2.3.3 Protocol for intracellular staining in suspension

1. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
2. (Optional) Counterstain for cell surface antigens that are sensitive to fixation, according to the manufacturer's recommendations. Then wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend 10^7 cells in 500 µL of buffer.
4. Add 500 µL of Inside Fix. Mix well and incubate for 20 minutes at room temperature.
5. Centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
6. Wash cells by adding 1 mL of buffer and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
   ▲ **Note:** Fixed cells may be stored at 4–8 °C for up to 1 week.
7. (Optional) Counterstain for cell surface antigens that are sensitive to permeabilization according to manufacturer´s recommendations. Then wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely
8. Wash cells by adding 1 mL of Inside Perm and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
9. Resuspend cells in 90 µL of Inside Perm. Add antibodies for intracellular cytokine staining, e.g., 10 µL of Anti-IFN-γ antibodies.
10. (Optional) Add additional staining antibodies to the solution, e.g., CD137 or CD154.
11. Mix well and incubate for 10 minutes in the dark at room temperature.
12. Wash cells by adding 1 mL of Inside Perm and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
13. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry. Store cells at 4–8 °C in the dark until analysis. Mix well before flow cytometric data acquisition.
    ▲ **Note:** Samples may be stored at 4–8 °C in the dark for up to 24 hours.
    ▲ **Note:** Do not use propidium iodide (PI) or 7-AAD staining.

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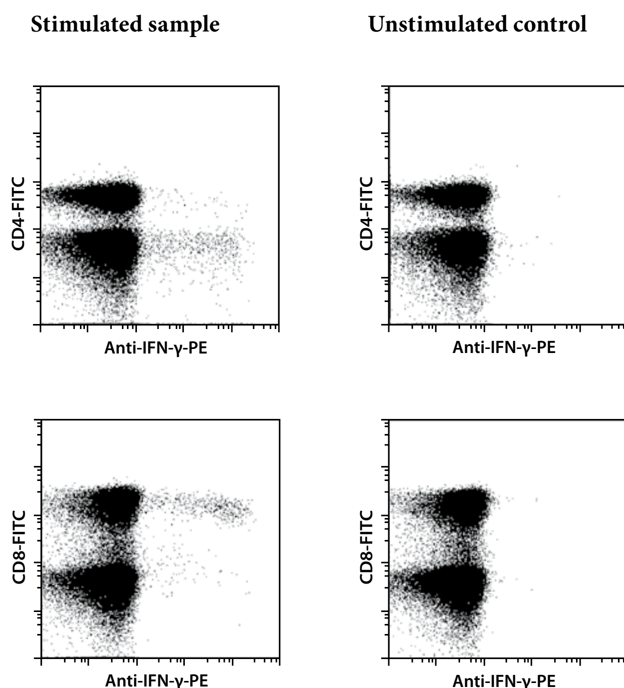
3. Example: Detection of IE-1–specific T cells by intracellular cytokine staining

10^6 human T cells from a CMV$^+$ donor were restimulated for 6 hours using unloaded *in vitro* generated DCs or *in vitro* generated DCs loaded with CMV IE-1 – Recombinant Protein. After 2 hours of restimulation 1 µg/mL of brefeldin A was added. Cells were harvested, fixed, permeabilized, and IE-1–specific cells were intracellularly stained with Anti-IFN-γ-PE (# 130-091-653). T cells were counterstained for CD4 and CD8 expression. IFN-γ production of lymphocytes is shown.

Cells were analyzed by flow cytometry using the MACSQuant® Analyzer. A lymphocyte gate based on forward and side scatter properties was activated. Cell debris was excluded from the analysis.



4. References

1. Kern, F. *et al.* (1999) Target Structures of the CD8$^+$-T-Cell Response to Human Cytomegalovirus: the 72-Kilodalton Major Immediate-Early Protein Revisited. J. Virol. 73: 8179–8184.
2. Dauer, M. *et al.* (2003) Mature Dendritic Cells Derived from Human Monocytes Within 48 Hours: A Novel Strategy for Dendritic Cell Differentiation from Blood Precursors. J. Immunol. 170: 4069–4076.

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

For *in vitro* T cell stimulation (refer to 2.3) 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

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

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