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1. Description

Components	6 nmol/peptide PepTivator™ Melan-A/MART-1, human or 60 nmol/peptide PepTivator™ Melan-A/MART-1, human: Pool of lyophilized peptides, consisting mainly of 15-mer sequences with 11 amino acids (aa) overlap, covering the complete sequence of the human Melan-A/MART-1 protein (Swiss-Prot Acc. no. Q16655).
Capacity	6 nmol (approximately 10 µg) per peptide for stimulation of up to 10 ⁸ total cells or 60 nmol (approximately 100 µg) per peptide for stimulation of up to 10 ⁹ total cells.
Product format	Lyophilized peptides containing stabilizer.
Purity	Each peptide >75% (HPLC), low endotoxin.
Storage	Store lyophilized product at -20 °C. The expiration date is indicated on the vial label.

This product contains no preservative and is sterile filtered; always handle under aseptic conditions.

1.1 Background information

Melanoma antigen recognized by T-cells (MART-1) belongs to the tumor-associated antigens of the group of differentiation antigens. Its expression is restricted to normal melanocytes but it is also present in melanoma. CD4⁺ and CD8⁺ T lymphocytes recognizing Melan-A/MART-1 have been identified in melanoma patients. Therefore Melan-A/MART-1 represents a potential target for immunotherapy of melanoma.¹

The PepTivator™ Melan-A/MART-1 is specially developed for efficient *in vitro* stimulation of Melan-A/MART-1-specific CD4⁺ and CD8⁺ T cells, as peptides of 15 amino acid length with 11 amino acid overlap represent the optimized solution for stimulating both CD4⁺ and CD8⁺ T cells in various applications. Stimulation of T cells with PepTivator Melan-A/MART-1 causes the secretion of effector cytokines and upregulation of activation markers, which then allows the detection or isolation of Melan-A/MART-1-specific T cells. Quantitative, phenotypical, or functional analysis of Melan-A/MART-1-specific T cell immunity can provide important information on the natural course of immune responses in healthy or immunocompromised individuals.

1.2 Applications

- Detection and analysis of Melan-A/MART-1-specific CD4⁺ and CD8⁺ effector/memory T cells, for example, in PBMCs, by MACS® Cytokine Secretion Assays, intracellular cytokine staining, or other technologies.
- Isolation of viable Melan-A/MART-1-specific CD4⁺ T cells with the CD154 MicroBead Kit.
- Isolation of viable Melan-A/MART-1-specific CD4⁺ and CD8⁺ T cells using MACS Cytokine Secretion Assay – Cell Enrichment and Detection Kits or the CD137 MicroBead Kit for *in vitro* generation of T cell lines/clones for research on tumor immunotherapy.
- Generation of Melan-A/MART-1-specific CD4⁺ and CD8⁺ effector/memory T cells from naive T cell populations for research on immunotherapy and vaccination.
- Pulsing of antigen-presenting cells for research on dendritic cell vaccination.

2. Recommendations for *in vitro* restimulation of Melan-A/MART-1-specific T cells with PepTivator™ Melan-A/MART-1

2.1 Cell preparation

For induction of cytokine secretion by Melan-A/MART-1-specific 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.

▲ **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.

▲ **Note:** PBMCs may be stored overnight. The cells should be resuspended and incubated in culture medium as described in 2.4, steps 1–3, but without addition of antigen. The antigen is then added to the culture on the next day.

2.2 Reagent requirements

- Culture medium, e.g., RPMI 1640 (# 130-091-440) containing 5% human serum, e.g., autologous or AB serum.
▲ **Note:** Do not use BSA or FCS because of non-specific stimulation.
- (Optional) Cytokine Secretion Assay Kit. For additional reagent and instrument requirements refer to the data sheet of the respective Cytokine Secretion Assay.
- (Optional) Intracellular cytokine staining, e.g., with Anti-IFN- γ -PE (# 130-091-653). For additional reagent requirements refer to the respective data sheet. For more information on other fluorochrome-conjugates see www.miltenyibiotec.com.
- (Optional) Intracellular cytokine staining of activated CD4⁺ T cells by using, for example, the CD154/IFN- γ /CD4 Detection Kit (# 130-092-814).
- (Optional) CD154 MicroBead Kit (# 130-092-658). For details see the CD154 MicroBead Kit data sheet.
- (Optional) CD137 MicroBead Kit (# 130-093-476). For details see the CD137 MicroBead Kit data sheet.
- (Optional) CytoStim for restimulation of human T cells (# 130-092-172, # 130-092-173). For details see the CytoStim data sheet.

2.3 Recommendations for reconstitution of PepTivator™ Melan-A/MART-1

1. For reconstitution of the lyophilized peptide pool take the vial from –20 °C and warm-up to room temperature.
▲ **Note:** Do not open the vial by removing the rubber-stopper.
2. To dissolve the 6 nmol PepTivator™ Melan-A/MART-1 fill a sterile syringe (0.5 mL) with 200 μ L of sterile water. To dissolve the 60 nmol PepTivator Melan-A/MART-1 fill a sterile syringe (5 mL) with 2 mL of sterile water.
3. Slowly inject the water with a sterile needle through the center of the rubber-stopper into the vial containing the lyophilized peptide pool.

4. Vortex the solution to completely dissolve the lyophilized peptide pool.
The concentration of the stock solution of PepTivator Melan-A/MART-1 is 30 nmol (approximately 50 μ g) of each peptide per mL.
5. Remove the rubber-stopper and aspirate the stock solution with a pipette.
6. To avoid repeated freeze-thaw cycles prepare working aliquots from the stock solution.
7. Store the working aliquots at –80 °C.

2.4 *In vitro* restimulation of Melan-A/MART-1-specific T cells

▲ Always include a negative control (without antigen) in the experiment. A positive control (e.g. CytoStim) may also be included.

1. Wash cells by adding medium, centrifuge at 300×g for 10 minutes. Aspirate supernatant.
2. Resuspend cells in culture medium at 10⁷ cells/mL. Plate cells in dishes at a density of 5×10⁶ cells/cm² (see 4. Appendix: Flask and dish sizes for *in vitro* T cell stimulation).
3. Mix the reconstituted PepTivator Melan-A/MART-1 thoroughly. Add 20 μ L of PepTivator Melan-A/MART-1 stock solution per mL cell suspension. Mix carefully and incubate cells at 37 °C and 5% CO₂.

The final concentration of PepTivator Melan-A/MART-1 in the cell suspension is 0.6 nmol (approximately 1 μ g) of each peptide/mL.

Cytokine Secretion Assay: Incubate cells for 3–6 hours.

CD154 MicroBead Kit: Incubate cells for 4–16 hours.

CD137 MicroBead Kit: Incubate cells for 16–24 hours.

Intracellular cytokine staining antibodies or kits, e.g., CD154/IFN- γ /CD4 Detection Kit: 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.

To proceed with the Cytokine Secretion Assay, the CD154 or CD137 MicroBead Kits, or intracellular cytokine staining, please refer to the respective data sheet.

▲ **Note:** When preparing cells for **intracellular cytokine staining**, fixed cells may be stored at 2–8 °C for up to 1 week.

3. Reference

1. Boon, T. *et al.* (2006) Human T cell responses against melanoma. *Annu. Rev. Immunol.* 24: 175–208.

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

For *in vitro* T cell stimulation (see 2.4) 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.

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