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1. Description

Components	6 nmol/peptide PepTivator® MAGE-A3, human or 60 nmol/peptide PepTivator® MAGE-A3, human: Pool of lyophilized peptides, consisting mainly of 15-mer sequences with 11 amino acids (aa) overlap, covering the complete sequence of the human MAGE-A3 protein (Swiss-Prot Acc. no. P43357).
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-associated antigen 3 (MAGE-A3) belongs to the cancer-testis family antigens. Expression is limited to male germ cells in normal tissue. It is aberrantly expressed in tumors of several types, such as melanoma, head and neck squamous cell carcinoma, lung carcinoma, breast carcinoma, and hematological malignancies. MAGE-A3 is described to be an attractive antigen to monitor anti-tumor T cell responses and to be used for immunotherapeutic interventions.^{1,2}

The PepTivator® MAGE-A3 has been specially developed for efficient *in vitro* stimulation of MAGE-A3-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 MAGE-A3 causes the secretion of effector cytokines and upregulation of activation markers, which then allows the detection or isolation of MAGE-A3-specific T cells. Quantitative, phenotypical, or functional analysis of MAGE-A3-specific T cell immunity can provide important information on the natural course of immune responses in diseased individuals.

1.2 Applications

- Detection and analysis of MAGE-A3-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 MAGE-A3-specific CD4⁺ T cells with the CD154 MicroBead Kit.
- Isolation of viable MAGE-A3-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 MAGE-A3-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, for example, dendritic cell vaccination.

2. Recommendations for *in vitro* restimulation of MAGE-A3-specific T cells with PepTivator® MAGE-A3

2.1 Cell preparation

For induction of cytokine secretion by 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.

For details about cell preparation refer to the protocols section at www.miltenyibiotec.com/protocols.

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 bovine serum albumin (BSA) or fetal bovine serum (FBS) 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 refer to 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 refer to the CD154 MicroBead Kit data sheet.
- (Optional) CD137 MicroBead Kit (# 130-093-476). For details refer to the CD137 MicroBead Kit data sheet.
- (Optional) CytoStim for restimulation of human T cells (# 130-092-172, # 130-092-173). For details refer to the CytoStim data sheet.

2.3 Recommendations for reconstitution of PepTivator® MAGE-A3

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 plug.
2. To dissolve the 6 nmol PepTivator® MAGE-A3 fill a sterile syringe (0.5 mL) with 200 μ L of sterile water. To dissolve the 60 nmol PepTivator MAGE-A3 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 plug 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 MAGE-A3 is 30 nmol (approximately 50 μ g) of each peptide per mL.
5. Remove the rubber plug 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 Recommendations for *in vitro* restimulation of antigen-specific T cells

▲ MAGE-A3-specific T cells are expected to be present only in certain individuals. Their frequency may be very low compared to T cells with other specificities. The given protocol for *in vitro* T cell stimulation thus may only serve as a guideline and is based on experiences using other PepTivator products, for example, PepTivator CMV pp65.

▲ Always include a negative control (without antigen) in the experiment. As a positive control, a sample stimulated with, e.g. CytoStim, may also be included.

1. Wash cells by adding medium, centrifuge at 300 \times 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 \times 10⁶ cells/cm² (refer to 4. Appendix: Flask and dish sizes for *in vitro* T cell stimulation).
3. Mix the reconstituted PepTivator thoroughly. Add 20 μ L of PepTivator stock solution per mL cell suspension. Mix carefully and incubate cells at 37 °C and 5% CO₂.

The final concentration of PepTivator 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. References

1. Boon, T. *et al.* (2006) Human T cell responses against melanoma. *Annu. Rev. Immunol* 24: 175–208.
2. Goodyear, O.C. *et al.* (2008) Differential pattern of CD4⁺ and CD8⁺ T-cell immunity to MAGE-A3/A2/A3 in patients with monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma. *Blood* 112: 3362–3372.

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

For *in vitro* T cell stimulation (refer to 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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