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## 1. Description

<b>Components</b>	<b>6 nmol/peptide PepTivator® A. fumigatus pmp20 – research grade:</b> Pool of lyophilized peptides, consisting mainly of 15-mer sequences with 11 amino acids (aa) overlap, covering the sequence of the <i>Aspergillus fumigatus</i> pmp20 protein (UniProtKB Acc. no. O43099).
<b>Capacity</b>	6 nmol (approximately 10 µg) per peptide for stimulation of up to 10 <sup>8</sup> total cells.
<b>Product format</b>	Lyophilized peptides containing stabilizer.
<b>Purity</b>	Average purity of peptides >70% (HPLC).
<b>Storage</b>	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

*Aspergillus fumigatus* is a common saprophytic fungus. Colonies of the fungus produce numerous airborne conidia, which are constantly inhaled by humans. In immunocompetent individuals conidia are normally eliminated without any side effects. *Aspergillus fumigatus* can cause allergic bronchopulmonary aspergillosis and can induce severe and fatal invasive infections in the respiratory tract of immunocompromised patients, for example, after hematopoietic stem cell transplantation. Besides alveolar macrophages and neutrophils, it is suggested that CD4<sup>+</sup> T cells play a central role in prevention and elimination of the infection. The putative peroxiredoxin pmp20 also known as Asp f 3 is a described allergen and discussed to induce protective immunity against fungal infection in animal vaccination models.

PepTivator® Peptide Pools have been specially developed for efficient *in vitro* stimulation of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as peptides of 15 amino acid length with 11 amino acid overlap represent the optimized solution for stimulating both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in various applications. Stimulation of T cells with PepTivator Peptide Pools causes the secretion of effector cytokines and upregulation of activation markers, which then allows the detection or isolation of T cells specific for the respective antigen. Quantitative, phenotypical, or functional analysis of antigen-specific T cell immunity can provide important information on the natural course of immune responses.

### 1.2 Applications

- Detection and analysis of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> effector/memory T cells, for example, in PBMCs, by MACS® Cytokine Secretion Assays, intracellular cytokine staining, or other technologies.
- Isolation of viable antigen-specific CD4<sup>+</sup> T cells with the CD154 MicroBead Kit.
- Isolation of viable antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> 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.
- Generation of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> effector/memory T cells from naive T cell populations.
- Pulsing of antigen-presenting cells.

## 2. Recommendations for *in vitro* restimulation of antigen-specific T cells with PepTivator® Peptide Pools

### 2.1 Cell preparation

For induction of cytokine secretion by antigen-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.

For details about cell preparation refer to the protocols section at [www.miltenyibiotec.com/protocols](http://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.
- (Optional) Antibodies or kits for intracellular cytokine staining, e.g., Anti-IFN- $\gamma$ -PE (# 130-091-653), CD154/IFN- $\gamma$ /CD4 Detection Kit (# 130-092-814), or IL-17 Secretion Assay – Cell Enrichment and Detection Kit (PE), human (# 130-094-542). For more information on other fluorochrome-conjugates refer to [www.miltenyibiotec.com/antibodies](http://www.miltenyibiotec.com/antibodies).
- (Optional) CD154 MicroBead Kit (# 130-092-658) or CD137 MicroBead Kit (# 130-093-476).
- (Optional) CytoStim for restimulation of human T cells (# 130-092-172, # 130-092-173).

## 2.3 Recommendations for reconstitution of PepTivator® Peptide Pools

- For reconstitution of the lyophilized peptide pool take the vial from  $-20^{\circ}\text{C}$  and warm-up to room temperature.  
▲ **Note:** Do not open the vial by removing the rubber plug.
- To dissolve the PepTivator® Peptide Pool fill a sterile syringe (0.5 mL) with 200  $\mu\text{L}$  of sterile water.
- Slowly inject the water with a sterile needle through the center of the rubber plug into the vial containing the lyophilized peptide pool.
- Vortex the solution to completely dissolve the lyophilized peptide pool.  
The concentration of the stock solution of PepTivator Peptides is 30 nmol (approximately 50  $\mu\text{g}$ ) of each peptide per mL.
- Remove the rubber plug and aspirate the stock solution with a pipette.
- To avoid repeated freeze-thaw cycles prepare working aliquots from the stock solution.
- Store the working aliquots at  $-80^{\circ}\text{C}$ .

## 2.4 Recommendations for *in vitro* restimulation of antigen-specific T cells

▲ *Aspergillus fumigatus* pmp20-specific T cells are expected to be present only in certain individuals. Their frequency may be low compared to T cells with other specificities. The given protocol for *in vitro* T cell stimulation thus may only serve as a guideline.

▲ Magnetic enrichment of stimulated antigen-specific T cells according to cytokine secretion, e.g. IL-17, using the MACS Secretion Assay Technology or according to expression of activation marker, e.g. CD154, will enhance the sensitivity to detect rare cells.

▲ 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.

- Wash cells by adding medium, centrifuge at  $300\times g$  for 10 minutes. Aspirate supernatant.
- Resuspend cells in culture medium at  $10^7$  cells/mL. Plate cells in dishes at a density of  $5\times 10^6$  cells/ $\text{cm}^2$  (refer to 3. Appendix: Flask and dish sizes for *in vitro* T cell stimulation).

- Mix the reconstituted PepTivator thoroughly. Add 20  $\mu\text{L}$  of PepTivator stock solution per mL cell suspension. Mix carefully and incubate cells at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ .

The final concentration of PepTivator in the cell suspension is 0.6 nmol (approximately 1  $\mu\text{g}$ ) 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- $\gamma$ /CD4 Detection Kit:** Incubate cells for 2 hours, then add 1  $\mu\text{g}/\text{mL}$  brefeldin A, and incubate for further 4 hours.

- 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  $2-8^{\circ}\text{C}$  for up to 1 week.

## 3. 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  $5\times 10^6$  cells/ $\text{cm}^2$ . 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 cell numbers. It also indicates the appropriate amount of medium to add.

Total cell number	Medium volume to add	Culture plate	Well diameter
$0.15\times 10^7$	0.15 mL	96 well	0.64 cm
$0.50\times 10^7$	0.50 mL	48 well	1.13 cm
$1.00\times 10^7$	1.00 mL	24 well	1.60 cm
$2.00\times 10^7$	2.00 mL	12 well	2.26 cm
$5.00\times 10^7$	5.00 mL	6 well	3.50 cm
Total cell number	Medium volume to add	Culture dish	Dish diameter
$4.5\times 10^7$	4.5 mL	small	3.5 cm
$10.0\times 10^7$	10.0 mL	medium	6 cm
$25.0\times 10^7$	25.0 mL	large	10 cm
$50.0\times 10^7$	50.0 mL	extra large	15 cm
Total cell number	Medium volume to add	Culture flask	Growth area
$12\times 10^7$	12 mL	50 mL	25 $\text{cm}^2$
$40\times 10^7$	40 mL	250 mL	75 $\text{cm}^2$
$80\times 10^7$	80 mL	720 mL	162 $\text{cm}^2$
$120\times 10^7$	120 mL	900 mL	225 $\text{cm}^2$

Refer to [www.miltenyibiotec.com](http://www.miltenyibiotec.com) for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit [www.miltenyibiotec.com/local](http://www.miltenyibiotec.com/local) to find your nearest Miltenyi Biotec contact.

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