Miltenyi Biotec

PepTivator® GAD65 – research grade
human

6 nmol/peptide 130-096-769

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1. Description

Components	**6 nmol/peptide PepTivator® GAD65 – research grade, human** Pool of lyophilized peptides, consisting mainly of 15-mer sequences with 11 amino acids (aa) overlap, covering the sequence of the glutamate decarboxylase (GAD) 65 protein (UniProtKB Acc. no. Q05329).
Capacity	6 nmol (approximately 10 µg) per peptide for stimulation of up to 10^8 total cells.
Product format	Lyophilized peptides containing stabilizer.
Purity	Average purity of peptides >70% (HPLC).
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

Type 1 diabetes mellitus is an autoimmune disease characterized by autoreactive T cells, which infiltrate the pancreatic islets and destroy the insulin-producing β-cells resulting in a disorder of glucose homeostasis. Glutamic acid decarboxylase (GAD) 65 is expressed by the pancreatic β-cells and appears to be an important autoantigen involved in the onset and pathogenesis of the disease.

PepTivator® Peptide Pools have been specially developed for efficient *in vitro* stimulation of antigen–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 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 $CD4^+$ and $CD8^+$ effector/memory T cells, for example, in PBMCs, by MACS® Cytokine Secretion Assays, intracellular cytokine staining, or other technologies.
- Isolation of viable antigen–specific $CD4^+$ T cells with the CD154 MicroBead Kit.
- Isolation of viable antigen–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.
- Generation of antigen–specific $CD4^+$ and $CD8^+$ 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.

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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-γ-PE (# 130-091-653) or CD154/IFN-γ/CD4 Detection Kit (# 130-092-814). For more information on other fluorochrome-conjugates refer to 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

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 PepTivator® Peptide Pool fill a sterile syringe (0.5 mL) with 200 µL 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 Peptides is 30 nmol (approximately 50 µg) 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

▲ GAD65-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 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.

1. Wash cells by adding medium, centrifuge at 300×g for 10 minutes. Aspirate supernatant.
2. Resuspend cells in culture medium at 10^7 cells/mL. Plate cells in dishes at a density of 5×10^6 cells/cm² (refer to 3. Appendix: Flask and dish sizes for *in vitro* T cell stimulation).
3. Mix the reconstituted PepTivator thoroughly. Add 20 µL of PepTivator stock solution per mL cell suspension. Mix carefully and incubate cells at 37 °C and 5% CO_2.

   The final concentration of PepTivator in the cell suspension is 0.6 nmol (approximately 1 µg) 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 µg/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. 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 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

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