User Manual

Epitope Mapping Peptide Sets (EMPS)

for Antigen-Specific Stimulation of T-Lymphocytes

Table of Contents

1 Introduction 1

1.1. EMPS Reagents 2

1.2. Storage and Handling 2

2 Experimental Protocols 3

2.1. Dissolving EMPS Peptides 3

2.2. Cell Stimulation Assays 4

2.2.1 Intracellular cytokine staining (ICS) Protocol 4

2.2.2 ELISpot Protocol 5

2.3. References / Further Reading 6

3 Contact Us 7

4 Product Use & Liability 7

Please read the entire Manual before starting your Experiments!
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1 Introduction

EMPS (Epitope mapping peptide set) is a peptide based tool which enables fast identification of T-cell epitopes within an antigen while consuming minimal amounts of material.

EMPS consists of overlapping peptides that are arranged along the amino acid sequence of the antigen in such a way that T-cell stimulation is optimized while the chance of missing T-cell epitopes is minimized (PepMix™ concept). These peptides are pooled according to a matrix design generating several subpools (Figure 1). In addition, the set also includes all single peptides which can be used for a quick verification of the obtained results from subpool testing and/or for the following investigations.

The Matrix pool design arranges each peptide in a square matrix and subpools would comprise peptides from a single row or a single column. According to this layout, each peptide is present in exactly two subpools. The tested subpools showing cell stimulation would pinpoint the peptides in the intersection on the layout.

Pool No.	I	II	III	IV	V	VI	VII	VIII
IX	1	2	3	4	5	6	7	8
X	9	10	11	12	13	14	15	16
XI	17	18	19	20	21	22	23	24
XII	25	26	27	28	29	30	31	32
XIII	33	34	35	36	37	38	39	40
XIV	41	42	43	44	45	46	47	48
XV	49	50	51	52	53	54	55	56
XVI	57	58	59	60	61	62	63	64

*Figure 1: Example for **Matrix pool** design.*

In the example shown, 64 peptides (numbers) are pooled into 16 **subpools (roman numerals) according to the matrix design**. In this example, pools V and XIII elicit a positive T-cell response. Only peptide 37 is present in both subpools and therefore is the peptide containing the epitope responsible for the positive T-cell response. Thus, instead of 64 single peptides, only 18 subpools have to be tested thereby reducing material consumption considerably.

A typical procedure for epitope mapping using **EMPS** consists of 2 steps:

1. First, Matrix subpools are used in T-cell stimulation assays like ELISpot or intracellular cytokine staining (ICS) /flow cytometry. The alignment of the results to the pooling matrix design allows afterwards the identification of peptides harboring putative epitopes.
2. In a second step, these peptides can be analyzed exclusively as single peptides and thus confirming the existence of an epitope.

This manual contains in the following exemplary protocols for the stimulation and detection of the stimulated antigen-specific T-cells using both, ELISpot and ICS/flow cytometry techniques. The protocols suggest the usage of donor peripheral blood mononuclear cells (PBMCs). PBMCs may be freshly prepared from anticoagulated whole blood by density gradient centrifugation or taken from cryopreserved stocks. The protocols will need minor adjustments if suspensions containing T-cells of other origin are used.

1.1 EMPS Reagents

Unless otherwise indicated, **EM peptides** contain 15 amino-acid peptides spanning the complete amino acid sequence of the indicated protein antigen with an 11 amino acid-overlap between adjacent peptides. Approx. 5 µg of each peptide are contained as single peptide and -in addition- in the respective matrix pools.

All peptides were chemically synthesized and analyzed by LC-MS (major peak by LC-MS is guaranteed to be peptide of interest - determined at 220 nm).

1.2 Storage and Handling

The optimum storage temperature for freeze-dried **EM peptides** is -20°C or below. At this temperature **EM peptides** are stable for at least 6 months from the date of purchase. Dissolving the reagents will reduce their long-term stability.

2 Experimental Protocols

Note: The following procedure is provided as a guideline only. The optimal experimental conditions will vary depending on your samples and instruments. The optimal experimental conditions must be established by the user. No warranty or guarantee of performance using this procedure with your target enzyme can be made or is implied.

2.1 Dissolving EMPS Peptides

EM peptide stock solution can be prepared by dissolving the peptides in dimethyl-sulfoxide (DMSO) at room temperature. We recommend adding DMSO step-wise, 10 µl at a time, to the freeze-dried **EM peptides** until it has dissolved completely. Typically, 25 µl of DMSO are sufficient to dissolve the material provided. Sonication may help accelerate the process but avoid heating of **EM peptides.** If required, aliquots should be prepared immediately and stored at -20°C or below. Stock solution may be further diluted with supplemented media or buffer for immediate use in cell stimulation assays (compare protocol examples below).

Note: To avoid loss or cross-contamination of EM-peptides please carefully open the tube and directly dissolve the peptides after the cap is removed. Weak centrifugation of the plates before opening may help to settle the freeze-dried peptides on the bottom of the vessel.

To avoid cell toxicity, the final concentration of DMSO should be below 1% (v/v) in cell stimulation assays. This has to be considered when making EM peptide stock solution in DMSO. Avoid repeated thawing and freezing of dissolved EM peptides.

2.2 Cell Stimulation Assays

ICS/flow cytometry and ELISpot can be used in combination with **EMPS** to identify T cell epitopes. Exemplary protocols of the assays are provided in the following sections.

For each protocol we recommend a minimum of 1 µg/mL final concentration of each peptide per test for stimulating PBMCs.

2.2.1 Intracellular cytokine staining (ICS) Protocol

Resuspend your cell preparation in suitable media, e.g. "supplemented" 1640 RPMI containing 2 mmol/l L-Glutamine, 10% (v/v) heat-inactivated fetal calf serum. Antibiotics may not be required for short term stimulation.

Cell stimulation in 96-well cell culture plates

1. Pipette 100 µl of supplemented media containing one test volume of peptide solution (double working concentration) and 20 µg/ml Brefeldin A (double working concentration) into each well of a sterile 96-well plate. We recommend to use round bottom polypropylene 96-well plates to reduce cell adhesion to the wells. For unstimulated control, pipette 100 µl of supplemented media containing a corresponding amount of DMSO and Brefeldin A into wells. We recommend preparing at least two replicates (two wells) for each experimental condition and for positive and negative controls.

Note: Brefeldin A can be added to cells also 2 hours after adding peptides if you perform a parallel stimulation with another stimulant (*e.g.* proteins, bacterial or viral lysates) which require internal protein processing and thus a time-delayed interruption of protein transport to the Golgi apparatus leading to blockage of cytokine secretion.

2. Add 100 µl of cell suspension containing ~200,000 cells (2 x 10^6 cells/ml) to each well.
3. Close the plate lid and place the plate in a standard cell culture incubator (37°C, H_2O-saturated 5% CO_2-atmosphere) for cell stimulation. Cells can be stimulated for 6 to 16 hours depending on your scientific question.
4. After the stimulation, centrifuge the plate for 6 min at 300 x g and 4°C. Carefully discard the supernatant by performing a gentle but firm swing with the plate.
5. Resuspend the cell pellets in 200 µl PBS containing 0.5% (w/v) bovine serum albumin.
6. Centrifuge the plate again and discard the supernatant as described in step 4.
7. Proceed with surface staining, cell permeabilization and intracellular staining according to your usual protocol or refer to the manufacturer's standard protocols.

Data analysis

Activated T-cells downregulate the T-cell receptor, which should be considered when setting gates. Gates must be designed to include events with lower expression of CD3, CD4 or CD8 than standard gating of non-activated samples. During data acquisition live selection gates that exclude these activated cells should be avoided.

2.2.2 ELISpot Protocol

Day 1 (sterile condition)

1. For coating the ELISpot plate*, dilute the capture antibody of your choice (*e.g.* anti human IFNγ Mab 1-DK1) in buffer (DPBS) and pipette the solution into each well (*e.g.* 1µg/well).
2. Seal the plate and incubate at 4°C overnight (coated plates should be used within one week).
3. Thaw the cells to be stimulated if they are cryopreserved and not freshly isolated from blood. Thawed cells can be placed in a 37°C incubator with 5% CO_2 and in appropriate medium to rest overnight.

Day 2 (sterile condition)

4. Wash the plate with DPBS several times to remove unbound antibodies.
5. Add blocking medium (*e.g.* containing 2% HSA) to each well of the ELISpot plate and incubate for at least 1 hour in the incubator.
6. While blocking, wash overnight rested cells with PBS or medium to remove cell debris and count viable cells.
7. Empty the ELISpot plate by discarding the blocking medium.
8. Add 100 µl of cell suspension containing between 100,000 to 300,000 cells (1-3 x 10^6 cells/ml) to each well.
9. Add 100 µl stimulant solution (double working concentration) to each well.
10. Incubate the plate for at least 16 hours in the humidified incubator at 37°C with 5% CO_2 for cell stimulation.

Day 3 (non-sterile condition**)**

11. Wash the plate several times with appropriate buffer (*e.g.* with PBS containing low amounts of mild detergent like 0.05% Tween 20 to remove attached cells).
12. Add a suitable detection antibody of your choice (*e.g.* anti human IFNγ Mab7-B6-1) to the wells at a concentration of e.g. 1µg/ml and incubate for 2 hours.
13. Wash the plate with buffer several times like described in step 11.
14. If biotinylated antibodies are used, a Streptavidin-coupled enzyme is added at this step (*e.g.* ExtraAvidin-Alkaline Phosphatase at a dilution of 1:1000) and incubated for 1 hour at room temperature in the dark.
15. Wash the plate with buffer several times like described in step 11.
16. Add appropriate substrate to the wells and stop the reaction when spots start to develop. If using Alkaline Phosphatase you might add 100 µl BCIP/NBT at this step to each well and stop the reaction after 3 to 10 minutes by flashing the wells with tap water. (Alternatively, detection antibodies coupled directly to a reporter enzyme can be used at step 12. Like this, step 13 and 14 can be omitted and the color reaction will depend on the enzyme/substrate used.)
17. ELISpot plates should be left to dry before analysis using an ELISpot plate reader.

We recommend making triplicates for each condition (well). For the negative control (unstimulated cells) you might even do six replicates.

To stimulate cells you might use an additional positive control like a CEFT PepMix™.

*Nitrocellulose as well as PVDF membrane plates can be used; please note that for PVDF plates a pre-wetting step with Ethanol is recommended. After membrane activation, several washing steps using sterile water are required to remove the activator.

2.3 References / Further Reading

T-cell stimulation/ICS flow cytometry

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T-cell stimulation/ELISpot

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