User Manual

PepMix Peptide Pools

For antigen-specific stimulation of T-lymphocytes

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Please read the entire manual before starting your experiments!
Carefully note the handling and storage conditions.
For research use only.

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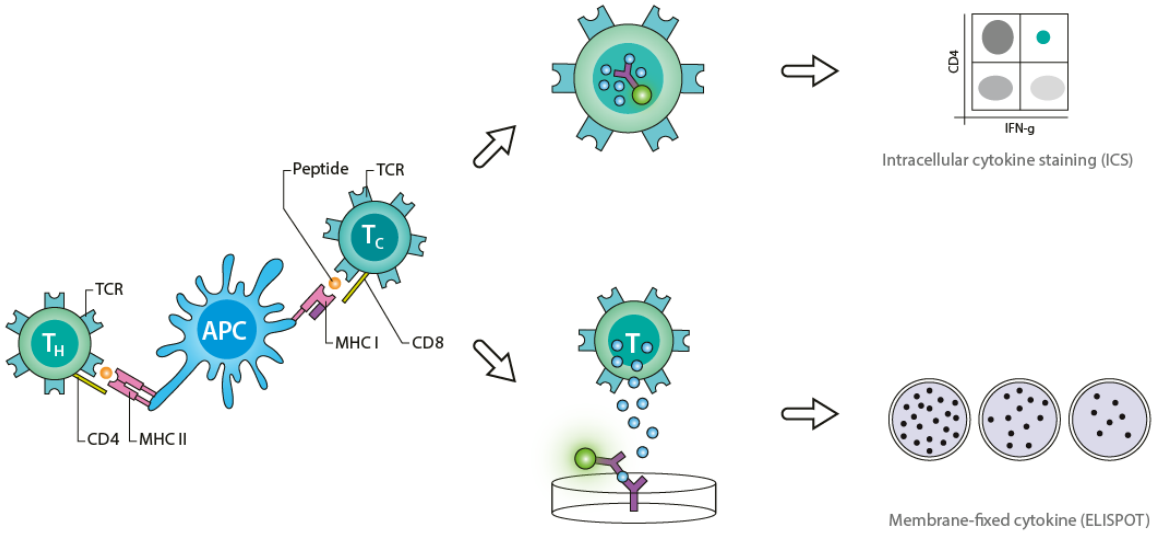
1. Introduction

We arrange overlapping peptides along the amino acid sequence of proteins of interest in such a way that T cell stimulation is optimal while minimizing the chance of missing T cell epitopes. You can use PepMixes in lieu of proteins (e.g. recombinant proteins) or pathogen lysates for antigen-specific stimulation of T cells. They provide more effective stimulation than protein antigens because processing through the external pathway of antigen presentation is not required. PepMix peptide pools are being used globally for various purposes in basic and clinical research such as detection, enumeration, and functional profiling of antigen-specific T cells, proliferation studies, and T cell expansion.

The provided protocols for Intracellular Cytokine Staining (ICS) and ELISpot detail the stimulation of T cells within suspensions of peripheral blood mononuclear cells (PBMCs). PBMCs can be freshly prepared from anticoagulated (preferably heparinized) whole blood by density gradient centrifugation. The protocols are provided as an illustration for utilizing PepMix, the experimental procedures will need adjusting if whole blood or suspensions containing T cells of other origin are used.



PepMixes allow stimulation of antigen-specifc T cells in functional immunoassays such as ELISPOT and ICS.

2. PepMix Peptide Pools

Unless otherwise specified, PepMixes contain peptides of 15 amino acids length spanning the complete sequence of the indicated protein with an 11 amino acid- overlap between adjacent peptides. Each vial contains 25 µg (approximately 15 nmol) of each peptide, sufficient to stimulate up to 2.5 x 10^8 cells. Each peptide is chemically synthesized, purified, and analyzed by LC-MS before it is added to the PepMix.

2.1 Storage & Handling

The optimal storage temperature for a freeze-dried PepMix peptide pool is -20°C or below. At this temperature, the PepMix is stable for at least 6 months from the date of purchase. Dissolving the reagents will reduce their long-term stability.

2.2 Dissolving freeze-dried PepMix Peptide Pools

A stock solution of PepMix can be prepared through dissolving the peptides in dimethyl sulfoxide (DMSO) at room temperature. Typically, using 50 µl of DMSO should suffice to dissolve the contents provided in a vial containing 25 µg per peptide. However, the amount can be increased if lower concentrations are acceptable, as demonstrated on the subsequent page.

Supposing it is necessary, aliquots should be prepared right away, and any remaining stock solutions kept at -20°C or below. The stock solution can be diluted further using supplemented media to be used immediately for cell stimulation assays.

Avoid repeated thawing and freezing of dissolved PepMix peptide pools!

To avoid cell toxicity, the final concentration of DMSO should be below 1% (v/v) in cell stimulation assays!

Dissolving PepMixes

Calculation Examples for PepMixes

1

Freeze-dried PepMix

Standard	15 nmol (approx. 25 µg)
Large Scale	120 nmol (approx. 200 µg)

2

Aliquot and store stock solution at ≤ -20°C

Prepare stock solution:
Add DMSO and vortex to dissolve peptides
100% DMSO!

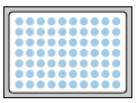
Standard	125 µl DMSO (0,2 mg/ml stock solution)
Large Scale	1000 µl DMSO (0,2 mg/ml stock solution)

3

Prepare peptide working solution:
Dilute in aequous solution (e.g. medium) and vortex
1% DMSO!

Standard	4µl stock solution + 396 µl medium (2 µg/ml working solution)
Large Scale	4 µl stock solution + 396 µl medium (2 µg/ml working solution)

4



Dilute further while mixing with cell suspension for antigen-specific stimulation
0,5 % DMSO!

Standard	100 µl peptide working solution + 100 µl cell suspension (1 µg/ml final peptide concentration)
Large Scale	100 µl peptide working solution + 100 µl cell suspension (1 µg/ml final peptide concentration)

Final DMSO concentration in the assay should be < 1 % to avoid cell toxicity!

3. Experimental Protocols

The following procedures are provided only as guidelines. The optimal experimental conditions will vary depending on the sample used.

3.1 ELISpot Assay

This protocol is designed for stimulation of PBMCs. You may need to adjust it for use with other material. PBMCs can be freshly isolated from whole blood or buffy coats. Alternatively, cryopreserved PBMC samples may be used. In that case, a resting step allowing the cells to recover may be useful (see below).

Please perform all procedures until the overnight incubation under sterile conditions!

3.1.1 Required Reagents

Use freshly prepared solutions or obtain frozen stock aliquots.

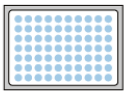
Component	We recommend
Blocking medium	X-Vivo 15 + 2% HSA
Capture antibody	Anti-human IFN-γ mAb (1-D1K) (Mabtech)
Capture antibody buffer	Dulbecco's phosphate buffered saline (DPBS)
Dimethyl-sulfoxide (DMSO)	Use anhydrous DMSO for long term storage of stock solution
Enzyme solution	Enzyme/ Buffer 1:1000 solution 69 Well Plate 10ml DPBS + BSA 0.5% + 10ul ExtrAvidin-Alkaline Phosphatase
Detection antibody	Anti-human IFNγ Mab7-B6-1 (Mabtech)
Detection antibody buffer	DPBS + 0.5 % BSA
Streptavidin-coupled enzyme (optional)	ExtrAvidin-Alkaline Phosphatase, 1:1000
Enzyme substrate	BCIP/NBT
Ethanol	35% (for optional prewetting see 4.3)
Human serum albumin (HSA)	
ELISpot medium	OpTmizer (Gibco)
Phosphate buffered saline (PBS)	

Component	We recommend
Positive control	CEFX Ultra SuperStim Pool 1µg/ml in DMSO
Wash buffer	PBS + 0.05% Tween

3.1.2 Assay Protocol

1. Add 100µl of cell suspension containing 100,000 to 300,000 cells (1-3 x 10^6 cells/ml) to each well of the coated ELISpot plate (coated with capture antibody). In addition, add 100µl stimulant solution (double final concentration) to each well.
2. Incubate the plate for at least 16 hours in the humidified incubator at 37°C with 5% CO_2 for cell stimulation.
3. Wash the plate several times to remove attached cells.
4. Add a suitable detection antibody of your choice to the wells at a desired concentration (e.g. 1µg/ml).
5. Incubate for 2 hours in the humidified incubator (37°C, 5% CO_2).
6. Wash the plate several times.
7. If you use biotinylated antibodies, add a Streptavidin-coupled enzyme at this step and incubate for 1 hour at room temperature in the dark.
8. Wash the plate several times.
9. Add 100µl appropriate substrate to the wells and wait for spots to develop color (usually after 3-7min).
10. Stop the reaction by flashing the wells with tap water.
11. ELISpot plates should be left to dry before analysis.
12. Count the spots preferably by using an ELISpot reader.

Quickguide: ELISpot

1			Add PepMix (100 µl) Cell suspension (100 µl)
2	≥ 16 h		Incubate 37° C 5 % CO_2
3	7 x 1 min		Wash 1x PBST (200 µl) 1x Aqua dest (200 µl) 5x PBST (200 µl)
4			Add detection antibody (60 µl)
5	2 h		Incubate 37° C 5 % CO_2
6	5 x 1 min		Wash 5x PBST (200 µl)
7	1 h		Add enzyme (100 µl) Incubate (room temperature, in the dark)
8	5 x 1 min		Wash 3x PBST (200 µl) 2x PBS (200 µl)
9	3–7 min		Add substrate (100 µl) Incubate until color development
10	2 min		Wash with running tab water
11	Until dry		Air dry
12			Count spots

3.2 Intracellular Cytokine Staining (ICS)

The total volume suitable for PBMC stimulation in a standard 96-well round-bottom plate is 200µl.
You may stimulate between 0.2 x 10^6 and 0.5 x 10^6 cells in this volume for example. We recommend adjusting your cell concentration to 2 x 10^6 cells/ml, in order to dispense 0.2 x 10^6 cells in 100µl cell suspension.

Please perform all procedures until the end of the incubation time under sterile conditions!

3.2.1 Additional Reagents

Component	We recommend
Positive control	CEFX Ultra SuperStim Pool 1µg/ml in DMSO

3.2.2 Assay Protocol

We recommend using:

- a minimum of 1 µg/mL final concentration of each peptide per test for stimulating PBMCs
- a resting of cryopreserved PBMCs over night or at least for 4 hours in assay medium
- 200,000 cells per well
- a final volume of 200 µl per well for stimulation
- Brefeldin A for inhibiting protein transport
- round bottom polypropylene 96-well plates to reduce cell adhesion to the wells

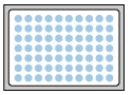
Adjust your cell preparation to 2 x 10^6 cells/ml in suitable assay medium, e.g. 1640 RPMI containing 2 mmol/l L-Glutamine, 5% (v/v) human male AB serum, 1% MEM Non-Essential Amino Acids Solution (100x), and 1mM Sodium Pyruvate Solution (100mM). Antibiotics may not be required for short-term stimulation.

Prepare a double concentrated PepMix solution from your PepMix stock solution in assay medium and supplement with 20 µg/ml Brefeldin A (double working concentration).

1. Pipette 100 µl of peptide solution with 20 µg/ml Brefeldin A (double working concentration) into each well of a sterile 96-well plate.
   For an unstimulated control pipette 100 µl of assay medium containing a corresponding amount of DMSO and Brefeldin A into wells.
   Prepare 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 100µl of cell suspension containing 200,000 cells 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.

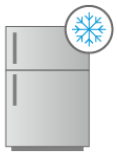
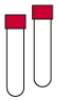
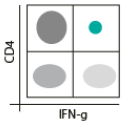
4. After the stimulation, centrifuge the plate for 6 min at 300 x g and 4°C. Carefully discard the supernatant by performing a gentle but firm swing with the plate.
5. Resuspend the cell pellets in 200 µl PBS containing 0.5% (w/v) bovine serum albumin.
6. Centrifuge the plate again and discard the supernatant as described in step 4.
7. Perform surface staining for immunophenotyping in a final volume of 50 µl for 20 min at 4°C in the dark.
   Use at least antibodies against CD3, CD4, and CD8 to distinguish between cytotoxic T lymphocytes and T helper cells.
   The antibodies should be suitable for the subsequent permeabilization and fixation procedure.
   Verify necessary antibody concentrations by antibody titration.
8. After staining, add 150µl PBS containing 0.5% (w/v) bovine serum albumin and 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.
9. Resuspend the cell pellets in 200µl PBS containing 0.5% (w/v) bovine serum albumin.
10. Centrifuge the plate again and discard the supernatant as described in step 8.
11. Cells are permeabilized for intracellular staining with a fixation buffer containing methanol and saponin e.g. with 50µL of BD Cytofix/Cytoperm™ per well for 20 min at 4°C in the dark.
12. Add 150µl per well of a wash buffer that maintains the permeabilized state of the cells, e.g. BD Perm/Wash™ buffer and 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.
13. Resuspend the cell pellets in 200µl wash buffer.
14. Centrifuge the plate again and discard the supernatant as described in step 12.
15. For intracellular staining antibodies suitable for cytokines of your interest are diluted in wash buffer and 50 µl of a master mix is added to the cells per well.
    Mix thoroughly but avoid foaming due to saponin in the buffer.
    Incubate for 30 min at 4°C in the dark.
16. After intracellular staining, add 150µl of wash buffer and centrifuge the plate for 6 min at 300 x g and 4°C. Carefully discard the supernatant.
17. Resuspend the cell pellets in 200µl buffer for flow cytometry, e.g. Dulbecco's modified phosphate buffered saline (D-PBS) with 0.5% (w/v) bovine serum albumin (BSA).
18. Centrifuge the plate again and discard the supernatant as described in step 16.
19. Resuspend the cell pellets in 100µl buffer for flow cytometry and proceed with flow cytometry according to your instrument.

Quickguide: ICS (Part 1)

1			Add Peptide-Brefeldin (100 µl) Cell suspension (100 µl)
2	≥ 16 h		Incubate 37° C 5 % CO_2
3	6 min		Wash Resuspend cells in PBS (+BSA) (200 µl) Centrifuge (6 min, 300 g) Carefully remove buffer
4			Stain with (extracellular) antibody cocktail (50 µl)
5	20 min		Incubate 4° C
6	2 x 6 min		Wash twice 1. Resuspend cells in PBS (+BSA) (150 µl) Centrifuge (6 min, 300 g) Carefully remove buffer 2. Resuspend cells in PBS (+BSA) (200 µl) Centrifuge (6 min, 300 g) Carefully remove buffer
7			Add Cytofix/Cytoperm™ (50 µl) for fixation and permeabilization
8	20 min		Incubate 4° C
9	6 min		Wash Add Perm/Wash™ Buffer (150 µl) Centrifuge (6 min, 300g) Carefully remove buffer

Go to Part 2

Quickguide: ICS (Part 2)

10	6 min		Wash Resuspend cells in Perm/Wash™ buffer (200 µl) Centrifuge (6 min, 300 g) Carefully remove buffer
11			Stain with (extracellular) antibody cocktail (50 µl)
12	30 min		Incubate 4° C
13	6 min		Wash Add Perm/Wash™ Buffer (150 µl) Centrifuge (6 min, 300 g) Carefully remove buffer
14	6 min		Wash Resuspend cells in Perm/Wash™ buffer (200 µl) Centrifuge (6 min, 300 g) Carefully remove buffer
15			Resuspend cells in PBS (+ BSA) (100 µl)
16			Analyze with FACS

4. Hints and Comments

4.1 Dissolving Peptides

We recommend adding DMSO gradually, 10µl at a time, to the freeze-dried PepMix until it has dissolved completely. Vortexing and sonication may help accelerate the process. If you need to add solvent for further dilution, also do that gradually, again using vortexing and sonication if required.
Avoid heating your PepMix!
Please also have a look at our FAQs for further information.

4.2 Washing Cells from the Plate

Add one incubation step with water within the washing procedure to burst the cells.

4.3 Coating of ELISpot Plates

For coating the ELISpot plate*, dilute the capture antibody of your choice in DPBS buffer and pipette the solution into the wells at e.g. 1µg/well. Seal the plate and incubate at 4°C overnight. Wash the ELISpot plate with DPBS several times to remove unbound antibodies. Add blocking medium to each well of the ELISpot plate and incubate for at least 1 hour in the incubator. Empty the ELISpot plate by discarding the blocking medium.

*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.

4.4 Experimental Design

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

5. References/Further Reading

5.1 T cell Stimulation/ICS

1. Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides
   Kern at al., Eur J Immunol. (2000).
2. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry
   Maecker et al., J Immunol Methods. (2001)
3. Analysis of antigen-specific T cell responses with synthetic peptides--what kind of peptide for which purpose?
   Kiecker et al., Hum Immunol. (2004)

5.2 T cell Stimulation/ELISpot

1. Guidelines for the automated evaluation of ELISpot assays
   Janetzki et al., Nat Protoc. (2015)
2. Enzyme-Linked ImmunoSpot (ELISpot) for Single-Cell Analysis
   Janetzki et al., Methods Mol Biol. (2015)

5.3 PepMix in the Scientific Literature

1. A linear DNA encoding the SARS-CoV-2 receptor binding domain elicits potent immune response and neutralizing antibodies in domestic cats
   Conforti et al., Molecular Therapy (2023)
2. Immunogenicity and safety of a two-dose regimen with hepatitis E virus vaccine in healthy adults in rural Bangladesh: A randomized, double-blind, controlled, phase 2/pilot trial
   Øverbø et al., Vaccine (2023)
3. SARS-CoV-2 Omicron BA.4/BA.5 Mutations in Spike Leading to T Cell Escape in Recently Vaccinated Individuals
   Emmelot et al., Viruses (2023)
4. Non-Mutated Nucleophosmin 1 Is Recognized by the CD8+ T Lymphocytes of an AML Patient after the Transplantation of Hematopoietic Stem Cells from an HLA-Haploidentical Donor
   Nemeckova et al., Current Oncology (2023)
5. A phase I study of an adenoviral vector delivering a MUC1/CD40-ligand fusion protein in patients with advanced adenocarcinoma
   Tan et al., Nature Communications (2022)
6. Local delivery of low-dose anti–CTLA-4 to the melanoma lymphatic basin leads to systemic Tregreduction and effector T cell activation
   van Pul et al., Science Immunology
7. Age-related Differences in Immune Reactions to SARS-CoV-2 Spike and Nucleocapsid Antigens
   Morhart et al,. Vaccines (2023)

Find more references

6. Contact Us

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