Innovative Peptide Solutions

Protocol

PepMix™

for Antigen-Specific Stimulation of T-Lymphocytes

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Product Use & Liability

1. Introduction

PepMix™ reagents are used for the antigen-specific stimulation of T-cells. Overlapping peptides are arranged along the amino acid sequence of proteins of interest in such a way that T-cell stimulation is optimized while the chance of missing T-cell epitopes is minimized. PepMix™ reagents can be used in lieu of proteins (e.g. recombinant proteins) or pathogen lysates. They provide more effective stimulation than protein antigens because processing through the external pathway of antigen presentation is not required. PepMix™ reagents are being used globally for a variety of purposes in basic and clinical research. These include the detection, enumeration, or functional profiling of antigen-specific T-cells, proliferation studies and T-cell expansion.

The provided protocols describe the stimulation of T-cells within suspensions of peripheral blood mononuclear cells (PBMC). PBMCs may be freshly prepared from anticoagulated (preferably heparinized) whole blood by density gradient centrifugation. The protocols are examples showing how PepMix™ reagents can be used. They will need adjusting if suspensions containing T-cells of other origin are used.

2. Reagents

2.1. PepMix™.

Unless otherwise indicated, PepMix™ reagents 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. 25 µg (15 nmol) of each peptide are contained. All peptides were chemically synthesized, purified, and analyzed by LC-MS.

2.2. Reagents required but not supplied:

2.2.1. T-cell activation assays followed by ICS or proliferation assays

- Dimethyl-sulfoxide (DMSO) for dissolving the PepMix™ reagent
- Ficoll for PBMC preparation
- Phosphate buffered saline (PBS) for washing cells
- Cell culture media

- Bovine serum albumin, fetal calf serum, or human AB serum for supplementing buffers/media
- Penicillin/Streptomycin (for long term cell culture only)
- Brefeldin A (BFA) to prevent cytokine secretion
- Staphylococcus Enterotoxin B (SEB) as a positive stimulation control (or alternative)

Recommended stock solutions (use freshly prepared or from frozen aliquots)

Reagent	**Solvent**	**Concentration**
BFA	Ethanol (95%)	5 mg/ml
SEB	DMSO	1 mg/ml

2.2.2. ELISpot assay

- Capture antibody for coating the ELISpot plate
- Dulbecco's phosphate buffered saline (DPBS) for PBMC preparation
- Human serum albumin for preparing blocking media
- ELISpot media (e.g. OpTmizer, Gibco)
- Positive control stimulant, for example the CEFX PepMix™.
- Bovine serum albumin, fetal calf serum, or human AB serum for supplementing buffers/media

Recommended stock solutions (use freshly prepared or from frozen aliquots)

Reagent	solvent	Concentration
SEB	DMSO	1 mg/ml

3. Storage and Handling

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

4. Dissolving freeze-dried PepMix™ reagents

PepMix™ stock solution should be prepared, ideally by dissolving the reagents in dimethyl-sulfoxide (DMSO) at room temperature. We recommend adding DMSO step-wise, 10 µl at a time, to the freeze-dried **PepMix™** reagent until it has dissolved completely. Typically 50 µl of DMSO are sufficient to dissolve the material provided in one vial of 25 tests. Vortexing and sonication may help accelerate the process. Avoid heating PepMix™ reagents! If required aliquots should be prepared immediately and, like any remaining stock solution, be stored at -20°C or below. Stock solution may be further diluted with supplemented media for immediate use in cell stimulation assays *(See section 4.2).*

Note: In order to avoid cell toxicity the final concentration of DMSO should be below 1% (v/v) in cell stimulation assays. This should be considered when making a PepMix™ stock solution in DMSO. One test-worth of a PepMix™ reagent (typically sold as 25 tests per vial) is sufficient for PBMC stimulation in a final reaction volume of 1 ml. Higher concentrations may be required for whole blood or other materials. A range of experimental protocols can be found in the literature. **Avoid repeated thawing and freezing of dissolved PepMix™ reagents.**

5. Cell Stimulation assays

PBMC should be resuspended in cell culture media. We suggest supplemented RPMI 1640 containing 2 mmol/l L-Glutamine and 10% (v/v) heat-inactivated fetal calf serum (‘complete media’). Antibiotics may be required for long-term stimulation protocols (e.g. proliferation assays) but not necessarily for short-term stimulation.

The cell suspension should be adjusted to $5x10^6$ cells/ml so that 200 µl of cell suspension contain 10^6 cells.

5.1 Cell stimulation in single tubes with 1 ml final reaction volume

We recommend sterile polyethylene or polypropylene tubes with caps (4.5 – 15 mL). To minimize differences between the tubes with respect to the time that cells are in contact with the reagents we recommend placing all required reagents in each tube before cell suspension is added.

5.1.1.The assay in brief:

- PepMix™, positive and negative stimulation solutions are added to the respective tubes in a volume of 100µl each.
- BFA working solution is added to each tube in a volume of 100µl.
- Cell suspension is added in a volume of 200µl.
- In addition, 600µl of complete media is added to adjust the final volume.
- The final assay volume is 1000µl.
- The incubation time is 6-16 hours.

Note: All procedures until the end of the incubation time are to be performed under sterile conditions.

5.1.2. Preparing the stimulation assay

1. Prepare PepMix™ working solution in complete media. Dissolve the contents of one PepMix™ vial containing 25 tests in 50 µl of DMSO (stock solution, see section 4.1). If less than 25 tests are required, store unused stock solution in suitable aliquots at -20°C or below. Prepare a PepMix™ working solution by diluting the required amount of PepMix™ stock solution 1:50 with complete media. Add 100 µl of PepMix™ working solution to each tube requiring stimulation.
2. Prepare negative control solution by diluting DMSO 1:50 in complete media. Add 100 µl of negative control solution to each tube designated a negative control.

3. Prepare positive control solution by diluting your positive control in complete media. If you wish to use, for example, staphylococcus enterotoxin B (SEB) at a final concentration of 1 µg/ml and your stock is 1 mg/ml, dilute your SEB stock solution 1:100 in complete media. Add 100µl of positive control solution to each tube designated a positive control.
4. Prepare BFA working solution from BFA stock solution (usually prepared in ethanol and stored at -80°C). We recommend using BFA at 10 µg/ml final concentration. As with negative and positive stimulation controls, you may add BFA stock solution in supplemented media as well. If your BFA stock solution is 5 mg/ml, dilute it 1:50 in complete media. Add 100µl of BFA solution to each tube. Different concentrations of your stock solution require you to adjust the dilution step.

   Note: Brefeldin A working solution should be added later (at least one hour later) if you are performing stimulation with a protein, or a bacterial or viral lysate in parallel to your peptide stimulation, since, unlike peptides, such antigen preparations may require processing by antigen-presenting cells, which would be disrupted by BFA if is added too early. In this case, defer the preparation and addition of BFA working solution until that time and proceed with the next step.

5.1.3. Performing the stimulation assay

1. Add 200 µl of cell suspension to each tube and mix by gently pipetting up and down. Add 600 µl of complete media to each tube and mix again.
2. Incubate in a standard incubator (37°C, humidified 5% CO_2-atmosphere). Caps can be put on each tube but must not be closed tightly during the incubation process to allow gas exchange. Some labs recommend positioning the rack with tubes at a 5° slant from the horizontal (the tubes should be almost horizontal to ensure maximum gas exchange). Cells can be stimulated for 6 to 16 hours depending on your scientific question. Consider the time required for upregulation of your activation marker of interest.
3. If BFA was not added immediately, freshly prepare BFA working solution and add 100 µl of BFA working solution to each tube at the desired time.

Note: Always use the same conditions to ensure comparability between assays. Some laboratories perform the assay in a total volume of 500 µl for the first 2 hours and add 500 µl complete media (containing the required amount of BFA) after two hours. Other protocols recommend adding BFA two hours before the end of the incubation. You may wish to adjust the respective volumes and times to what is most appropriate for you. Other tubes or culture

dishes may be used as well, for example 96-well plates (an example protocol is provided below). You may develop your own protocols or consult the literature. There is no single best protocol for these assays. The use of polypropylene instead of polyethylene tubes is thought to reduce cell adhesion, in which the use of EDTA (see below) to detach cells prior to staining can be omitted. However, whichever protocol you use, make sure that the DMSO concentration does not exceed 1% (v/v) at any time to avoid toxicity.

5.1.4. Ending the incubation

5. Add 3 ml of ice-cold PBS to each tube to stop incubation.
6. Centrifuge (400xg, 8 min, 4°C) and decant or aspirate supernatant. If decanting the supernatant, it is recommended to blot tubes dry on a paper towel while holding them in the inverted position to remove excess liquid. Never invert tubes more than once to avoid loss of cells.
7. Carefully resuspend cell pellets in the remaining fluid before adding additional volume.
8. If using polyethylene tubes add 3 ml of a 2 mM EDTA solution (in PBS). If tubes were in a slanted position, make sure that the area of the tube wall, which was covered with media and has adherent cells attached to it, is fully covered with EDTA-buffer.
9. Incubate all tubes for 10 min at 37°C (water bath). Close tubes tightly to avoid splashing water into them.
10. Vortex tubes carefully at low speed for 30 seconds.
11. Centrifuge again (400xg, 8 min, 4°C), decant and blot dry or aspirate supernatant.
12. Resuspend the cell pellets in the remaining fluid.
13. Add 1 ml of PBS containing 0.5% (w/v) bovine serum albumin.
14. Centrifuge (400xg, 8 min, 4°C), decant and blot dry or aspirate supernatant.
15. Resuspend pellets in the remaining fluid.
16. Proceed with surface staining, cell permeabilization and/or intracellular staining according to your preferred protocol.

5.2. Cell stimulation in 96-well cell culture plates

The principal procedure is the same as in tubes. However, in order to be able to use 96 well plates, some of the volumes and the concentrations of your working solutions (PepMix™, SEB, and BFA) must be adjusted. The total volume of stimulation in a standard 96-well round-bottom plate is 200 µl. You may stimulate between 0.2 x 10^6 and 0.5 x 10^6 cells in this volume. We recommend you adjust your cell concentration to 2.5 x 10^6 cells/ml so that 0.2 x 10^6 cells can be dispensed in 80 µl of the suspension.

5.2.1. The assay in brief:

- PepMix™, positive and negative stimulation solutions are added to the respective wells of a 96-well plate in a volume of 80µl each.
- BFA working solution is added to each well in a volume of 20µl.
- Cell suspension is added in a volume of 100µl.
- The final assay volume is 200µl.
- The incubation time is 6-16 hours.

Note: All procedures until the end of the incubation time are to be performed under sterile conditions.

5.2.2. Preparing the stimulation assay

1. Prepare PepMix™ working solution in complete media. Dissolve the contents of one PepMix™ vial containing 25 tests in 50 µl of DMSO (stock solution, see section 4.1). If less than 25 tests are required, store unused stock solution in suitable aliquots at -20°C or below. Prepare a PepMix™ working solution by diluting the required amount of PepMix™ stock solution 1:200 with complete media. Add 80 µl of PepMix™ working solution to each well requiring stimulation (the equivalent of 0.2 tests).
2. Prepare negative control solution by diluting DMSO 1:200 in complete media. Add 80 µl of negative control solution to each well designated a negative control.
3. Prepare positive control solution by diluting your positive control in complete media. If you wish to use, for example, staphylococcus enterotoxin B (SEB) at a final concentration of 1 µg/ml and your stock is 1 mg/ml, dilute your SEB stock solution

1:40 in complete media. Add 80 µl of positive control solution to each well designated a positive control.

4. Prepare BFA working solution from BFA stock solution (usually prepared in ethanol and stored at -80°C). We recommend using BFA at 10 µg/ml final concentration. As with negative and positive stimulation controls, you may add BFA stock solution in supplemented media as well. If your BFA stock solution is 5 mg/ml, dilute it 1:50 in complete media. Add 20 µl of BFA solution to each well. Different concentrations of your stock solution require you to adjust the dilution step.

   Note: Brefeldin A working solution should be added later (at least one hour later) if you are performing stimulation with a protein, or a bacterial or viral lysate in parallel to your peptide stimulation, since, unlike peptides, such antigen preparations may require processing by antigen-presenting cells, which would be disrupted by BFA if is added too early. In this case, defer the preparation and addition of BFA working solution until that time and proceed with the next step.

5.2.3. Performing the stimulation assay

4. Add 80 µl of cell suspension to each well and mix by gently pipetting up and down.
5. Incubate in a standard incubator (37°C, H_2O-saturated 5% CO_2-atmosphere). Cells can be stimulated for 6 to 16 hours depending on your scientific question. Consider the time required for upregulation of your activation marker of interest.
6. If BFA was not added immediately, freshly prepare BFA working solution and add 20 µl of BFA working solution to each well at the desired time.
7. **Always use the same conditions to ensure comparability between assays.**

5.2.3. Ending the incubation

1. After the desired stimulation time centrifuge the plate for 6 min at 300xg and 4°C. Carefully discard the supernatant by swiftly but gently inverting the plate and blotting the edges dry.
2. Resuspend the cell pellets in 200µl PBS containing 0.5% (w/v) bovine serum albumin.
3. Centrifuge the plate again and discard the supernatant as described in step 4.
4. Proceed with surface staining, cell permeabilization and intracellular staining according to your preferred protocol.

5.3 Data acquisition and analysis

We do not recommend any particular acquisition or analysis protocols as most laboratories will have their own standard approach. However, it is important to consider that activated T-cells will downregulate the T-cell receptor to varying degrees. This must be taken into account when setting gates. Gates must be designed to include events with lower expression of CD3, CD4 or CD8 than would normally be tolerated in the gating of non-activated samples. During data acquisition it is important to avoid live storage gates that may exclude such activated events. We recommend acquiring as many T-lymphocytes as possible (ideally 200.000 or more) in order not to miss small populations of activated cells.

5.3 ELISpot Assay

This protocol is designed for PBMCs. You may need to adjust it for use with other materials such as isolated CD4 or CD8 cells. PBMCs can be isolated freshly from whole blood or buffy coats. Alternatively, cryopreserved PBMCs samples may be used. In that case a resting step allowing the cells to recover may be useful (see below). Further information on Elispot assays can be found in the literature (see references at the end).

5.3.1. The assay in brief:

- Elispot plates are coated on day 1 (unless previously coated plates are used)
- PepMix™, positive and negative stimulation solutions are added to the respective wells in a volume of 100 µl each.
- Cell suspension is added to each well in a volume of 100µl.
- The final assay volume is 200µl.
- The usual incubation time is 18-24 hours.

Note: All procedures until the end of the incubation time are to be performed under sterile conditions.

5.3.2. Coating ELISpot plate(s) and thawing cryopreserved cells (if applicable, day 1)

If you are working with pre-coated ELISpot plates and fresh PBMC, start with section 5.3.4.

1. After diluting the capture antibody of your choice (*e.g.* anti-human IFN-γ Mab 1-DK1, Mabtech) with DPBS (10 µg/ml) pipette 100µl of the dilution into each well of a suitable ELISpot plate* (i.e. 1µg/well). Seal the plate and incubate in a horizontal positon at 4°C overnight (coated plates should be used within one week when stored at 4°C).
2. If applicable, thaw cryopreserved cells for your experiment using a validated protocol providing maximum cell viability (up to 24h prior to performing stimulation). Thawed cells can be suspended in OpTmizer media (1-2x10^6/ml) and placed in sterile, 50 ml conical polypropylene tubes in a standard 37°C incubator with a humidified 5% CO_2 atmosphere.

5.3.3. Blocking the ELISpot plate

1. Wash the ELISpot plate coated on day 1 several times with DPBS to remove unbound antibody.
2. Add suitable blocking media (*e.g.* containing 2% HSA) to each well and incubate for at least 1 hour at 37°C in a standard incubator (37°C, humidified 5% CO_2 atmosphere).
3. Empty the plate by inverting it to discarding the media.

5.3.4. Performing the stimulation assay (day 2)

4. Wash thawed, overnight-rested PBMCs with ELISpot cell culture media to remove cell debris.
5. Count viable cells and adjust your cell suspension to 1-3 x 106 cells/ml.
6. Add 100 µl of stimulant working solution to each well. We recommend setting up three replicates for each condition and six replicates for the negative control.
7. Add 100 µl of cell suspension to each well.
8. Incubate the ELISpot plate for 18-24 hours in a standard incubator.

Note: An incubation time of 16 hours works well for IFN-γ secretion. Different activation markers may require different incubation times.

5.3.5. Ending incubation and preparing the ELISpot plate for analysis

1. Add 200µl of PBS containing 0.05% Tween 20. Flip plate to remove liquid.
2. Add 200µl of de-ionized water. Wait 1 min, then flip to remove liquid.
3. Add 200µl of PBS containing 0.05% Tween 20. Flip plate to remove liquid. Repeat five times.
4. Add the detection antibody of your choice (e.g. biotinylated anti human IFN-γ) diluted in DPBS containing 5% BSA to the recommended concentration and incubate for 2 hours at 37°C. **Note:** enzyme-coupled detection antibodies can be used in this step. The subsequent color reaction will depend on the enzyme/substrate used.
5. Add 200µl of PBS containing 0.05% Tween 20. Flip plate to remove liquid. Repeat five times.
6. Apply your detection system for bound antibody. Biotinylated antibodies can be detected by applying a streptavidin-coupled enzyme (e.g. ExtraAvidin-Alkaline

Phosphatase) followed by adding the appropriate substrate. For Alkaline Phosphatase conjugates, BCIP/NBT substrate can be used. If an enzyme-coupled antibody was used under step 4. a substrate can be added directly.

7. Add tap water to stop the enzymatic reaction after 3 to 10 minutes.

*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 Ethanol.

6. References/Further reading:

T-cell stimulation/ICS

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