Innovative Peptide Solutions

Protocol

PepMaster

for Antigen-Specific Stimulation of T-Lymphocytes

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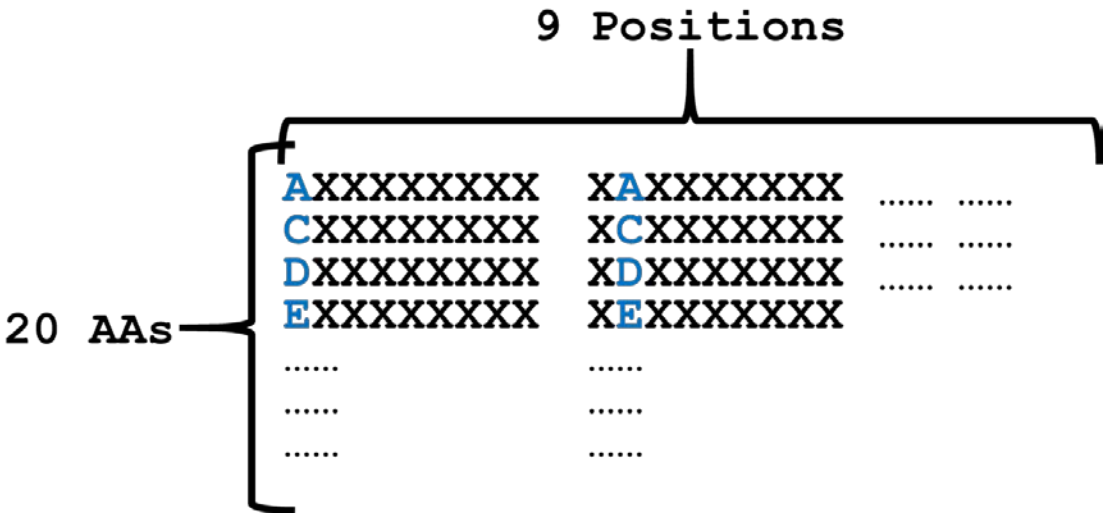
1. Introduction

PepMaster reagents are designed for the stimulation of T-lymphocytes ('T-cells') with a view to collecting information about T-cell-specificity. This type of library is often referred to as 'positional scanning peptide library' (see chapter 4 for references). Each library contains a very large number of peptides that are identical with respect to one single, fixed amino acid position but are variable with respect to all other positions. The manufacturing protocol for these libraries ensures that all amino acids occur with equal probability at each variable position. This creates peptides, for example, of the format 'A_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9', 'X_1-A_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9', 'X_1-X_2-A_3-X_4-X_5-X_6-X_7-X_8-X_9', etc., where 'A' represents alanine, and 'X' represents any amino acid except cysteine. The position of amino acids in the peptide are counted from the N-terminus to the C-terminus. Cysteine is avoided at the random positions in order to prevent oligomerization or cyclization of peptides. The ability of a specific T-cell receptor (TCR) to recognise, for example, the 'X_1-**A_2-**X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}' library depends on the preference of this TCR for 'A' in position 2 of the peptide chain.



PepMaster libraries can be used, for example, to identify candidate T-cell stimulating peptides when the specificity of a T-cell clone or line is unknown. When T-cells isolated from tissue are cloned, **PepMaster** may help identify the TCR-specificity of such a clone. It is likely that a number of potentially recognized peptides may be derived from the patterns of preferred amino acids in each position. The sequences of such peptides may then be screened against a protein database in order to identify identical or very homologous peptides that occur in tissues or organisms of interest. Individual peptides representing such identified sequences can then be tested in the next step. **PepMaster** reagents are a research tool and may be used for other purposes than described here.

PepMaster peptide libraries are used like **PepMix™** peptide pools, however, the ideal concentration of the reagents has to be determined by the researcher and will depend on the purpose and design of the experiments.

2. Reagents

PepMaster reagents contain peptides of fixed, uniform length (see specifications on the delivery note and/or vials/packaging). Each vial contains the same amount of total peptide (see specifications on the delivery note and/or vials/packaging). All **PepMaster** libraries are chemically synthesized, but as a result of the huge diversity of peptides in each library cannot be purified.

3. Protocols

No specific protocol or specific reagent concentrations are recommended. Please refer to the **PepMix™** Protocol for general information on the antigen-specific stimulation of T-Lymphocytes (available at www.jpt.com). Please find a modification of this protocol below that was used for testing **PepMaster reagents in the JPT laboratory. It may serve as a guidance, however researchers should establish their own optimized protocol depending on the intended use of the reagents.**

Cryopreserved PBMC's from a healthy donor with known T-cell reactivity to the CMV-pp65 protein-derived peptide, VFPTKDVAL, were thawed and rested overnight. Prior to the T-cell stimulation assay they were adjusted to a cell concentration of 3 x 10^6 lymphocytes/ml (twice the final cell concentration).

PepMaster libraries were carefully resuspended in assay-media at 37°C at an overall peptide concentration of 1 mg/ml or 0.2 mg/ml (two different concentrations, twice the final assay concentration).

Co-incubation of the PBMCs and the **PepMaster** suspension was done in two biological replicates containing 3 x 10^6 lymphocytes and 1 mg of overall peptide library in a final incubation volume of 2 ml in a 24-well plate with lid.

A positive control (VFPTKDVAL peptide at 1 µg/ml) and a negative control (no peptide) were run in parallel.

After 2 hours of incubation Brefeldin A working solution in assay medium was added to a final assay concentration of 10 µg/ml. Incubation was then continued at 37°C in a 5% CO_2 humidified atmosphere for another 14 hours.

After the stimulation period, plates were incubated for 10 min at 37 °C with a final concentration of 3 mM EDTA to detach adherent cells. Subsequently, cells were transferred to 2 ml Eppendorf tubes and washed twice with flow cytometry buffer at 300 x g for 6 min at 4°C.

Cells were then fixed and permeabilized using 150 µl per tube of cytofix/cytoperm for 20 min at 4°C followed by two wash steps with 2 ml of perm/wash buffer. Extracellular and intracellular staining was performed in one step in a volume of 150 µl for 30 min at 4° C in the dark with a cocktail of staining antibodies containing anti-human CD3-APC (UCHT1), CD4-PE-Cy7 (SK3), CD8-FITC (SK1), and IFN-γ-PE (B27). Following the staining procedure, cells were washed once with perm/wash buffer, followed by a washing step with flow cytometry buffer, and finally resuspended in 400 µl of flow cytometry buffer for acquisition on a flow-cytometer.

Reagents:

Reagent	**Manufacturer**	**Order No.**
X-Vivo 15	Lonza	BE 02-060Q
CTLwash	C.T.L.	CTLW-010
DNase I	PanReac	A3778.0010
OpTmizer Basic media	Gibco	A10221-01
OpTmizer supplement T-cell expansion	Gibco	A10484-02
L-Glutamine 200mM	Gibco	25030-24
EDTA 0.5 M	Sigma-Aldrich	03690-100ml
RPMI 1640 GlutaMAX™ Media Sterile	ThermoFisher Scientific	61870-044
Sodium Pyruvate Solution (100mM)	ThermoFisher Scientific	11360-039
MEM Non-Essential Amino Acids Solution (100x)	Life Technologies GmbH	11140-035
AB-Serum 100%	Sigma-Aldrich	H4522-100ML
Brefeldin A	Sigma	B6542
BD Cytofix/Cytoperm™	BD Biosciences	554722 or 554714 (Kit)
BD Perm/Wash™	BD Biosciences	554723 or 554714 (Kit)
DPBS w/o Ca, Mg	ThermoFisher Scientific	14190169
Albumin bovine fraction V	Serva	11930

Media:

Assay medium: RPMI 1640 Medium GlutaMAX™ supplemented with 5% AB-Serum, 1% MEM Non-Essential Amino Acids Solution (100x), 1mM Sodium Pyruvate Solution

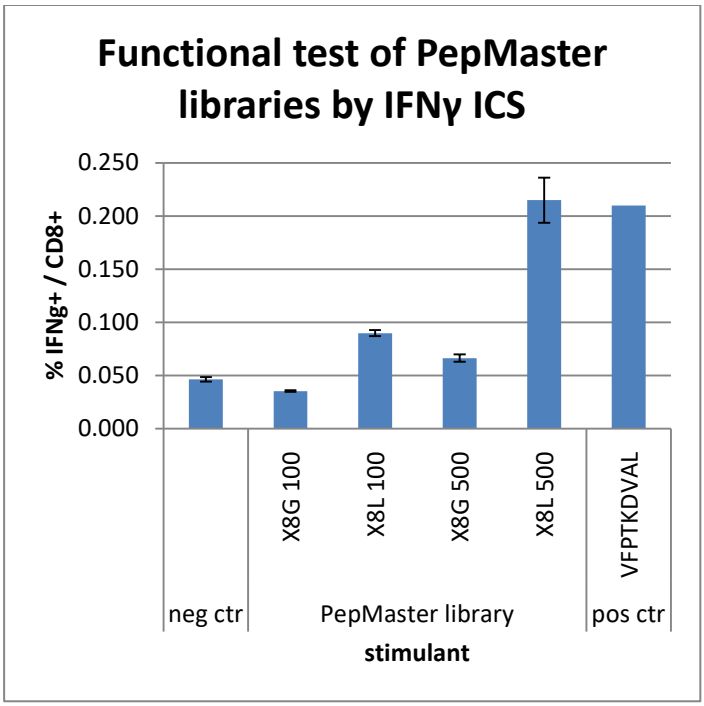
Thaw medium: X-Vivo 15, 10% CTLwash, 1µg/ml DNase

Resting medium: OpTmizer, 2.6% T-cell expansion supplement, 2 mM L-Glutamine

Flow cytometry buffer: D-PBS 0.5% m/v bovine albumine fraction V

Results:

Figure 1 shows the results that were obtained for the described functional test of **PepMaster** libraries using T-cell stimulation followed by intracellular cytokine staining (ICS) with Interferon-γ.



	stimulant	% IFN-γ+ / CD8+	SD
neg ctr		0.047	0.002
PepMaster library	X8G 100	0.036	0.001
	X8L 100	0.090	0.003
	X8G 500	0.067	0.004
	X8L 500	0.215	0.021
pos ctr	VFPTKDVAL	0.210	0.000

Figure 1: Functional test of PepMaster libraries by using IFN-γ intracellular cytokine staining. 3 x 10^6 lymphocytes were stimulated with 100 µg/ml or 500 µg/ml **PepMaster** peptide libraries. Values are given as frequency of IFN-γ positive events in percent of CD8 positive T-lymphocytes (mean of two biological replicates). 'X8G' stands for X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-G_9' and ‚X8L' for h X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-L_9'.

The experiment shows that:

- The library X8L, which contained all 9-mer peptides with the fixed anchor L (Leu) in position nine, led to a higher frequency of IFN-γ positive events compared to the respective X8G library
- The known stimulating peptide VFPTKDVAL could successfully be used as positive control peptide and also has L in position 9.

This example illustrates that PepMaster libraries are suitable for use in T-cell stimulation experiments with concentrations ranging from 100 to 500 µg/ml for PBMC´s, however, depending on the assay conditions and the cell population analyzed the optimum concentration may vary. Preference for L in position 9 is in line with the presence of HLA-A*24:02 and HLA-C*08:02 on the tested PBMC, which were predicted to be binding the positive control peptide. Leu is known to be a preferred anchor amino acid in position 9 for both alleles [IEDB database].

4. Positional scanning peptide libraries in the scientific literature (selection)

The use of positional scanning combinatorial (PCL) libraries for T-cell epitope identification is described in the following publications. Please refer to the experimental details for assay conditions.

Paper	**Assay Principle**
Hemmer, *J. Exp. Med.* **1997**, *185(9)*, 1651	Cytotoxic T Lymphocyte assay with radiolabeled target cells
La Rosa, *Blood* **2001**, *97(6)*, 1776	*In vitro* binding of peptide-loaded HLA-dimers to T-cell clones
Maynard, *Immunity* **2005**, *22*, 81	T-cell hybridomas are cultured with peptide libraries and antigen presenting cells (APCs). IL-2 responses are monitored by adding the supernatant to IL-2 addicted HT2. Responses were determined by the ^{3}H-thymidine proliferation assay.
Jüse, *Hum. Immunol.* **2010**, *71*, 475	Peptide competition assay with fluorescently or radio-labeled indicator peptide
Rasmussen, *J. Immunol,* **2014**, *193(10)*, 4790	Proximity-based dissociation assay: Radiolabeled b2microglobulin is incubated with peptide library and biotinylated MHC-I chain on Streptavidin coated plates