



Permutation Scan of HA and M13 Epitopes by PEPperCHIP® Peptide Microarrays

Introduction

PEPperCHIP® peptide microarrays are a uniquely flexible tool for the cost-effective high throughput screening of peptide-ligand and particularly antibody-epitope interactions. To determine or discover the epitope of a mono- or polyclonal antibody, one or multiple antigens can be translated into overlapping peptides (e.g. 15 aa peptides with 14 aa peptide-peptide overlap), and the corresponding peptide microarray assayed with the antibody sample. If the epitope of an antibody or serum sample is already known, the PEPperMAP® Type 3 Epitope Mapping enables the unambiguous determination of conserved and variable amino acid positions, the actual motif length as well as the identification of new epitope variants. This in-depth epitope analysis is based on a peptide microarray in which each amino acid position of the starting peptide is gradually exchanged by the 19 other main amino acids as well as optional uncommon building blocks like e.g. D-amino acids, citrulline or ornithine. In this application note we demonstrate the potential of PEPperPRINT's peptide microarray platform for the in-depth analysis of two known antibody epitopes derived from Influenza hemagglutinin (HA) and bacteriophage M13 coat protein based on full permutation scans.

Material and Methods

Microarray Content: Two different peptide microarrays covered full permutation scans of epitopes YPYDVPDYA (HA) and AEGDDPAKAAFSDLQ (M13), in which all amino acid positions were substituted with by the 19 other main amino acids as well as D-amino acids D-alanine ("a") and D-glutamic acid ("e"). Peptides were synthesized as duplicates in three blocks side by side on chip from the C- to the N-terminus and attached to the PEPperCHIP® glass slide coatings via poly(ethylene glycol) linkers and a three amino acid spacer based on two β -alanines and aspartic acid. Each array was framed by FLAG (DYKDDDDKGG) and HA (YPYDVPDYAG) control peptides.

Immunoassay Protocol: Standard buffer: PBS, pH 7.4 + 0.05 % Tween20
Blocking buffer: Rockland blocking buffer MB-070
Incubation buffer: Standard buffer with 10 % blocking buffer
Samples: Monoclonal anti-HA (12CA5)-LL-DyLight680 antibody (1 μ g/ml), polyclonal anti-M13 LL-DyLight680 antibody (4 μ g/ml); incubation for 1 h at RT and shaking at 500 rpm
Post staining: Monoclonal anti-HA (12CA5)-LL-DyLight680, monoclonal anti-Flag(M2)-LL-DyLight800; staining in incubation buffer for 1 h at RT and a dilution of 1:1000



Scanner: Odyssey Imaging System, LI-COR Biosciences; scanning intensities of 5/5 (HA) and 6/6 (M13) in red/green at a resolution of 21 μ m

Microarray Data Files: MicroarrayData_HA-PermutationScan.xlsx
MicroarrayData_M13-PermutationScan.xlsx (see enclosed)

Results

After 10 min pre-swelling in standard buffer and 60 min in blocking buffer, each peptide array was incubated with the respective labeled antibody at concentrations of 1 μ g/ml (monoclonal anti-HA (12CA5)-LL-DyLight680) and 4 μ g/ml (polyclonal anti-M13 LL-DyLight680). Afterwards, the peptide microarrays were washed 3x1 min with standard buffer, rinsed with Millipore[®] water and dried in a stream of air. Read-out was done with an Odyssey Imaging System at a resolution of 21 μ m before (Fig. 1, left) and after staining of HA and Flag control peptides (Fig 1, right).

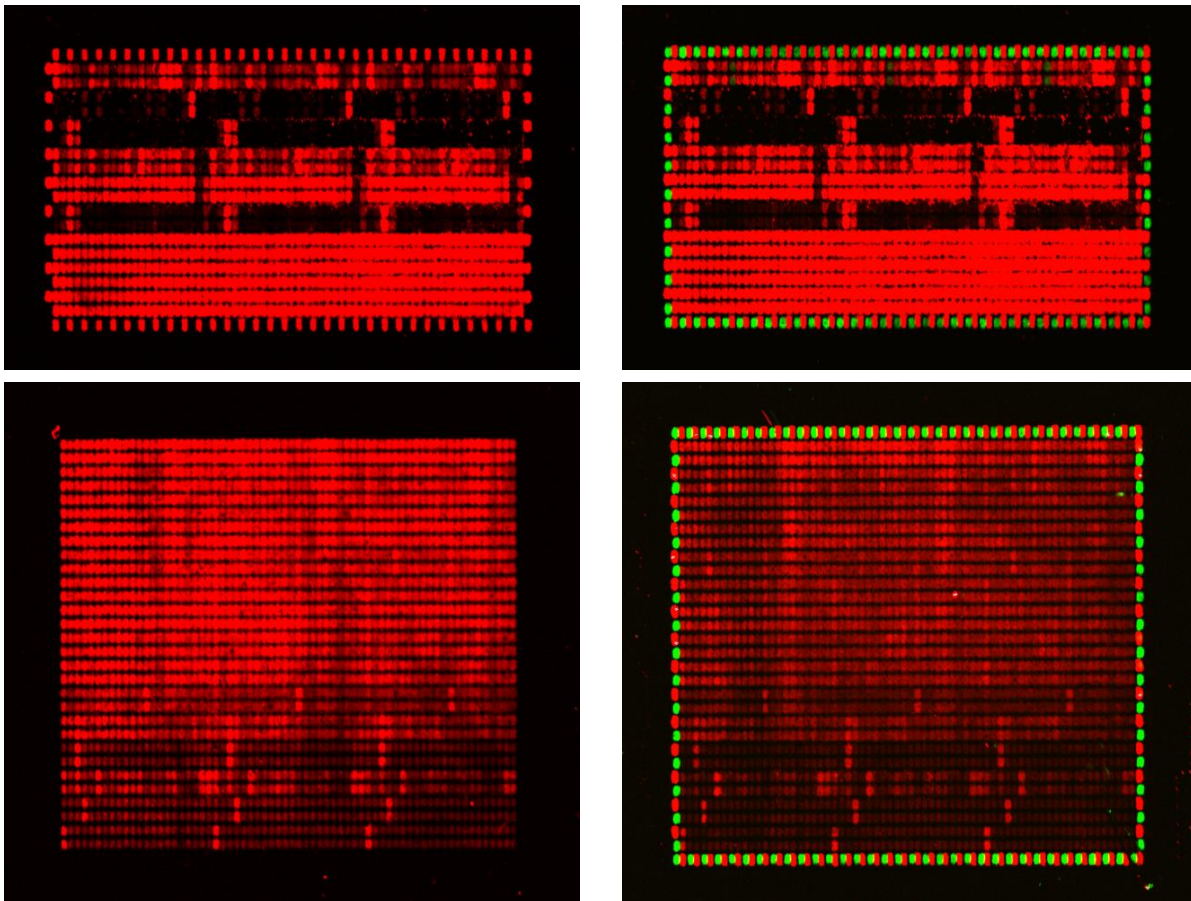


Fig. 1: Incubation of peptide microarrays with anti-HA (left on top) and anti-M13 (left on bottom) antibodies was followed by staining of HA and Flag control peptides (right) and read-out at scanning intensities of 5/5 (HA) and 7/7 (M13). All epitope permutations were printed as triplicates in three blocks side by side.

Staining with the labeled monoclonal anti-HA and the polyclonal anti-M13 antibodies gave rise to clear and well-defined spot patterns with peptide duplicates in each of the three blocks, i.e. each peptide was represented at least six times on the microarrays. Subsequent staining of HA and Flag control peptides was done with high and homogeneous spot intensities.



Quantification of spot intensities and peptide annotation were done with PepSlide® Analyzer and summarized in the Excel files Peptide_Map_HA-PermutationScan.xlsx and Peptide_Map_M13-PermutationScan.xlsx. A software algorithm breaks down fluorescence intensities of each spot into raw, foreground and background signal (see “Mapping Raw Data” tabs), and calculates the standard deviation of foreground median intensities (see “Mapping Summary” tab). Based on averaged foreground median intensities, intensity maps were generated and binders in the peptide map highlighted by an intensity color code with red for high and white for low spot intensities.

To provide an in-depth view on the permutation scans, we further generated heatmaps of the permutation scans as well as substitution matrices and amino acid plots reflecting the amino acid preferences at a given position. The data sets were correlated with peptide and intensity maps as well as visual inspection of the microarray scans to analyze the permutation patterns in consideration of sequence conservation and possible amino acid exchanges.

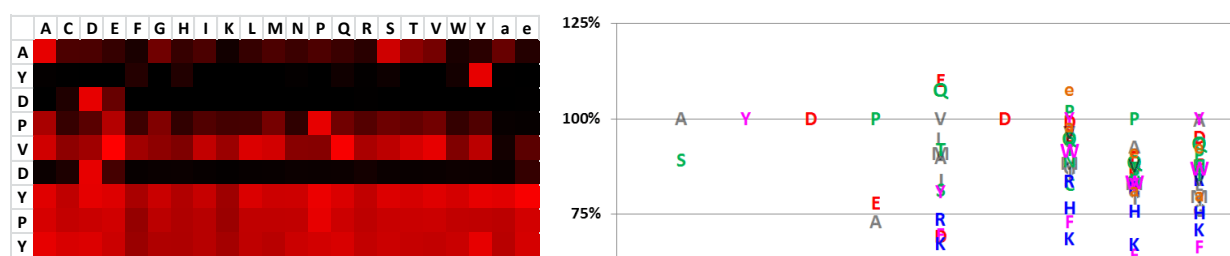
The substitution matrices highlighted the preference for a given amino acid by color codes (red: preferred amino acids; green: less preferred amino acids) and were calculated by dividing the spot intensity of a given peptide (e.g. ¹YPYDVQDYA⁹) by the averaged spot intensities of all 20 peptides that were permuted at the same position (¹YPYDVX⁹).

The amino acid plot was calculated by dividing the spot intensity of a given peptide (e.g. ¹YPYDVQDYA⁹) by the spot intensity of the starting epitope (¹YPYDV⁹PDYA⁹). The position of an amino acid at a given position thus reflected the intensity ratio compared to the amino acid of the native epitope at the same position.

Permutation Scan of HA Epitope ¹YPYDV⁹PDYA⁹

Staining of the HA peptide microarray with monoclonal anti-HA antibody gave rise to a well-defined spot pattern reflecting the three subarrays at excellent signal-to-noise ratios (Fig. 1, left on top). The spot pattern was translated into a heatmap that visualized dependency of amino acid substitutions on the spot intensity of the corresponding peptide (Fig. 2, left on top). According to the heatmap, motif ⁷DYA⁹ and amino acid ⁴D were highly conserved, while motif ⁵VP⁴ showed a certain tolerance for replacement by other amino acids. The N-terminal motif ¹YPY³, however, was highly variable without any preference for a certain amino acid.

This finding was supported by the substitution matrix with a pronounced red/green offset at positions ⁷DY⁸ and ⁴D as well as a clear but less defined differentiation at positions ⁹A and ⁵VP⁴ (Fig. 2, left on bottom).



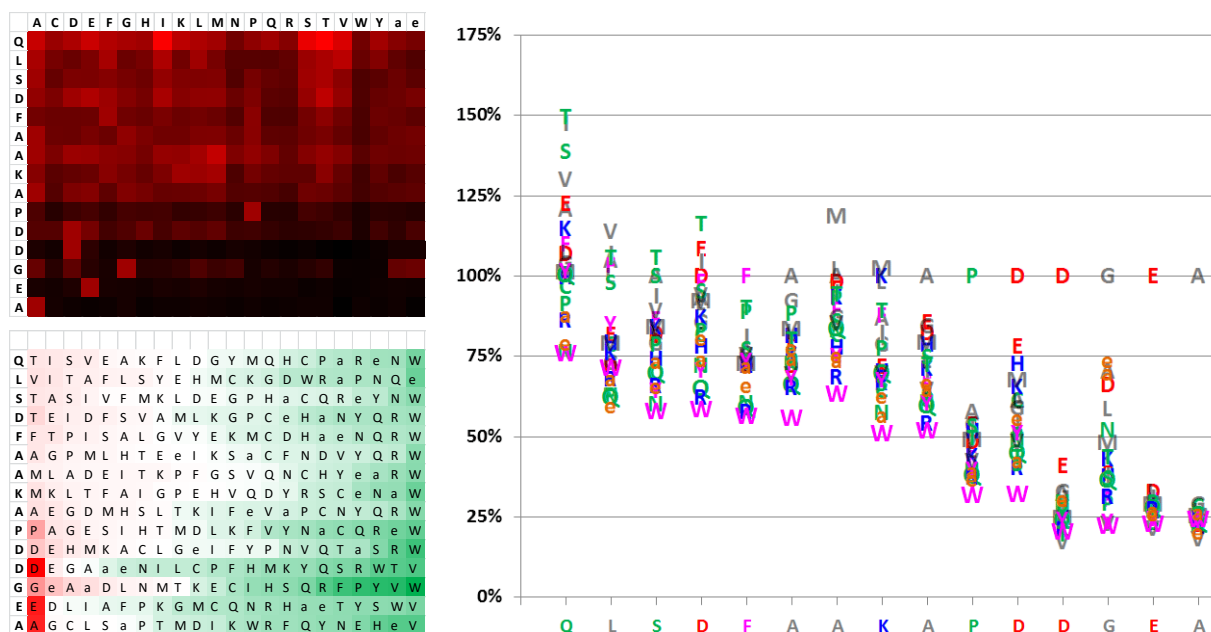


Fig. 3: The heatmap of the M13 permutation visualized the intensity pattern of the microarray scans (left on top); the substitution matrix (left on bottom) highlighted the preference for a given amino acid at each position of the HA epitope by color codes, while the amino acid plot (right) reflected the preference of a given amino acid relative to the amino acid of the starting epitope.

The amino acid plot also underlined the finding of the heatmap and the substitution matrix with the variable C-terminal and the conserved N-terminal motif (Fig. 3, right). The less pronounced but clear preference for A in position ⁷A, however, indicated a certain contribution of this variable amino acid to antibody binding. In the variable motif ⁷AKAAFSDLQ¹⁵ we further observed a weak preference for S and T as well as for hydrophobic amino acids V and I, while W, Q, N and R were generally less preferred. The highest degree of sequence conservation was found for amino acids ¹A, ²E and ⁴D that did not tolerate any substitution without significant loss in binding. Only ⁴D showed a limited susceptibility for conservative exchange by E. Amino acid ⁶P was also well conserved with approx. 50% loss in spot intensity and thus antibody binding by replacement with any other amino acid. The smaller drop in spot intensities compared to the HA permutation scan further underlined the polyclonal nature of the anti-M13 antibody. Amino acids ³G and ⁵D were less conserved with a clear preference for the starting amino acids and a poor tolerance for bulky amino acids Y, F, W and V in position ³G and for S, R and W in position ⁵D. Exchange by amino acids like E, H and M in position ⁵D as well as by G, A and D-amino acids a and e in position ³G resulted in a spot intensity decrease of approx. 25%.

Conclusion

The permutation scans of HA and M13 epitopes ¹YPYDVPDYA⁹ and ¹AEGDDPAKAAFSDLQ¹⁵ were done with labeled monoclonal anti-HA and polyclonal anti-M13 antibodies at concentrations of 1 µg/ml (HA) and 4 µg/ml (M13). Due to the availability of labeled antibodies, staining with a suited secondary antibody was not required. Read out was done with a LI-COR Odyssey Imaging System followed by quantification of spot intensities and peptide annotation with PepSlide® Analyzer.



The permutation scan of the HA epitope revealed a clear core motif ⁴DVPDYA⁹ with a high sequence conservation of amino acids ⁴D, ⁷D and ⁸Y, a less pronounced conservation of amino acids ⁶P and ⁹A as well as the variable ⁵V that could be exchanged by a variety of other amino acids. Within this core motif any incorporation of D-amino acids a and e was not tolerated.

The permutation scan of the M13 epitope gave rise to a variable stretch ⁷AKAAFSDLQ¹⁵ at the C-terminus and a conserved core motif ¹AEGDDP⁶ at the N-terminus. The less clear heatmap as well as the lower drop of spot intensities upon replacement of highly conserved amino acids reflected the polyclonal nature of the antibody. Within the core motif the amino acids of the starting epitope also represented the preferred amino acids with the highest degree of conservation at positions ¹A and ²E. Amino acids ⁴D and ⁶P also exhibited a limited tolerance for exchange by other amino acids, while ³G was less conserved. Interestingly the preferred amino acids for replacement of 3G were A as well as D-amino acids a and e. Such epitope variant with non-natural amino acids may exhibit higher in vivo stabilities for peptide immunizations than the corresponding natural peptide. The slight preference for A in position ⁷A further indicated a contribution of this variable position to anti-M13 binding.

The application note based on full permutation scans of two known epitopes demonstrated the potential of PEPperCHIP[®] Peptide Microarrays for the in-depth analysis of epitopes with respect to sequence conservation, the actual motif length and the possible contribution of variable positions to antibody binding. Despite the high conservation of the core motifs of both epitopes, the PEPperCHIP[®] Peptide Microarrays further enable the identification of new epitope variants as well as the incorporation of non-natural amino acids for e.g. higher in-vivo stabilities for peptide-based immunizations and vaccinations.