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 µg/ml), polyclonal anti-M13 LL-DyLight680 antibody (4 µg/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 µm

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 µg/ml (monoclonal anti-HA (12CA5)-LL-DyLight680) and 4 µg/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 µm before (Fig. 1, left) and after staining of HA and Flag control peptides (Fig 1, right).

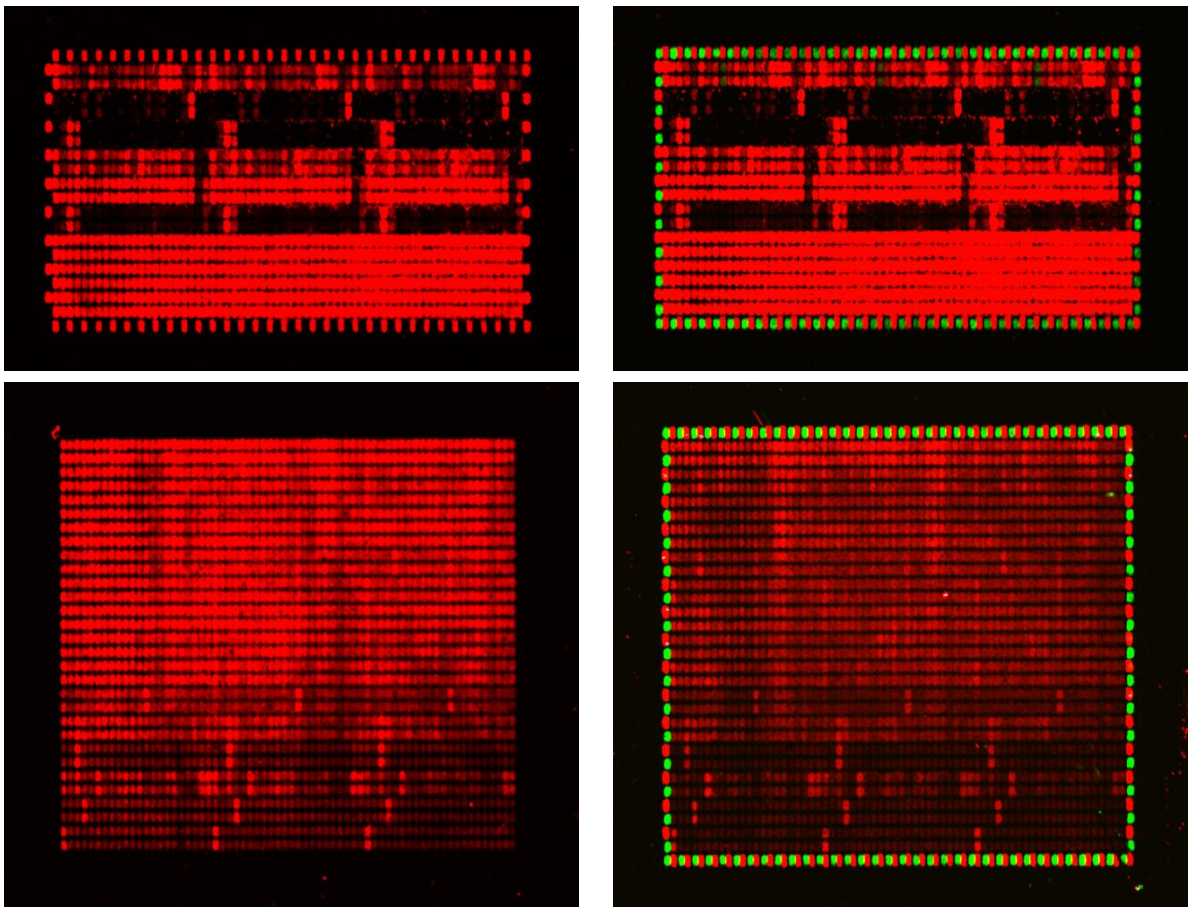

Fig. 1: Incubation of peptide microarrays with anti-HA (left on top) and anti-M13 (left on bottom) antibodies was followed by staining of HA and Flag control peptides (right) and read-out at scanning intensities of 5/5 (HA) and 7/7 (M13). All epitope permutations were printed as triplicates in three 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. ^{1}YPYDVQDYA^{9}) by the averaged spot intensities of all 20 peptides that were permuted at the same position (^{1}YPYDVXDYA^{9}).

The amino acid plot was calculated by dividing the spot intensity of a given peptide (e.g. ^{1}YPYDVQDYA^{9}) by the spot intensity of the starting epitope (^{1}YPYDVPDYA^{9}). 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 ^{1}YPYDVPDYA^{9}

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 ^{7}DYA^{9} and amino acid ^{4}D were highly conserved, while motif ^{5}VP^{4} showed a certain tolerance for replacement by other amino acids. The N-terminal motif ^{1}YPY^{3}, 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 ^{7}DY^{8} and ^{4}D as well as a clear but less defined differentiation at positions ^{9}A and ^{5}VP^{4} (Fig. 2, left on bottom).

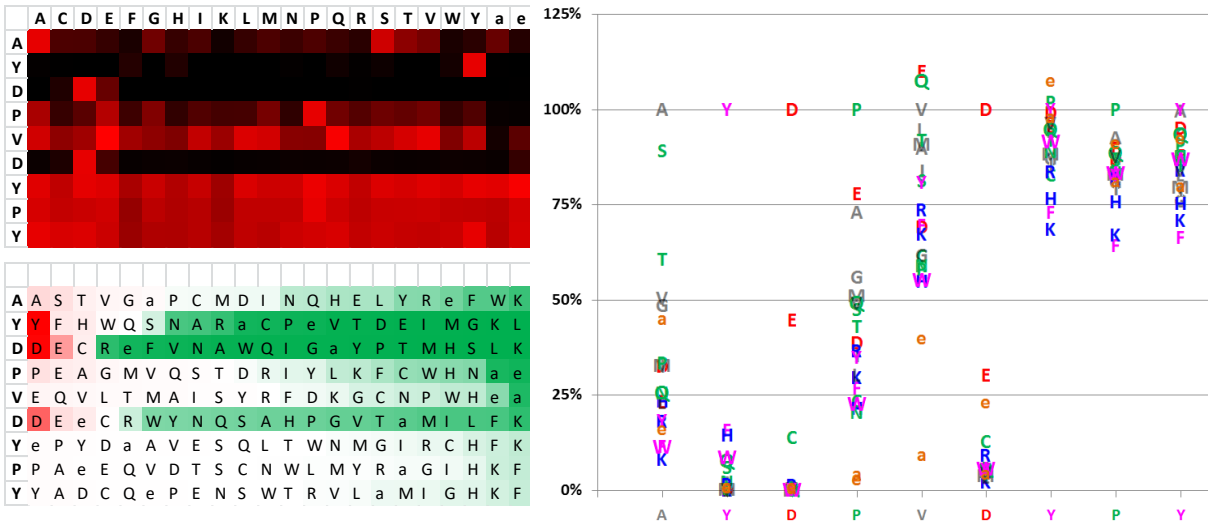

Fig. 1: The heatmap of the HA permutation visualized the intensity pattern of the microarray scans (left on top); the substitution matrix (left on bottom) highlighted the preference for a given amino acid at each position of the HA epitope by color codes, while the amino acid plot (right) reflected the preference of a given amino acid relative to the amino acid of the starting epitope.

The amino acid plot reflected the influence of an amino acid exchange on spot intensity and thus antibody binding normalized to the starting epitope (Fig. 2, right). It was shown that native amino acids ^{2}P, ^{4}D, ^{6}P, ^{7}D, ^{8}Y and ^{9}A actually represented the best amino acid at a given position, although the variability of ^{2}P enabled straightforward substitution by all other amino acids without a remarkable loss in binding. Amino acid ^{9}A could be readily replaced by S and to some extent also by T; other amino acids caused a decrease in spot intensities by 50% or higher. Amino acids ^{8}Y did not tolerate any exchange without significant loss of HA binding, while ^{7}D tolerated to some extent conservative exchange by E. ^{6}P was less conserved and could be substituted by E and A with a loss of ~25% binding. Other amino acids caused a drop in spot intensities by 50-75%, while D-amino acids a and e were not accepted at all due to conformational effects. A similar effect was found at poorly conserved ^{5}V with a limited tolerance for D-amino acids e and no preference for a. Highly conserved ^{4}D accepted only conservative exchange by E and e, albeit along with a significant loss in HA binding. Substitution of variable amino acids ^{1}Y, ^{2}P and ^{3}Y had only a minor influence on spot intensities, although bulky tryptophan and basic amino acids R, K and H were less preferred.

Permutation Scan of M13 Epitope ^{1}AEGDDPAKAAFSDLQ^{15}

Taking the higher scanning intensity and antibody concentration compared to the HA assay into account, staining of the M13 peptide microarray with polyclonal anti-M13 antibody also gave rise to a well-defined spot pattern reflecting the three subarrays at excellent signal-to-noise ratios (Fig, 1, left on bottom). The spot pattern was translated into a heatmap that visualized dependency of amino acid substitutions on the spot intensity of the corresponding peptide (Fig. 3, left on top). The less clear intensity pattern reflected the polyclonal nature of the anti-M13 antibody with the N-terminal stretch ^{1}AEGDDP^{6} as conserved and the C-terminal ^{7}AKAAFSDLQ^{15} as variable motif.

This finding was supported by the substitution matrix with a clearer red/green offset in the ^{1}AEGDDP^{6} motif, while the differentiation was less defined in the variable part terminal ^{7}AKAAFSDLQ^{15} (Fig. 3, left on bottom). The substitution matrix further indicated that the native amino acids given in the starting epitope also were the preferred amino acids in each position.

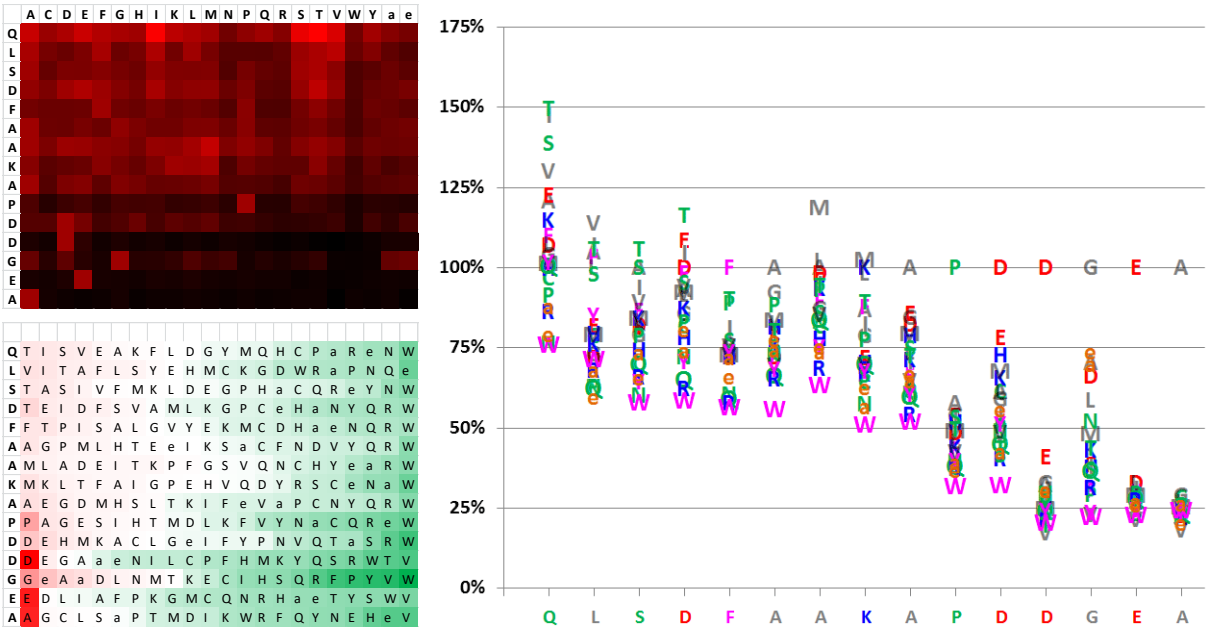

Fig. 3: The heatmap of the M13 permutation visualized the intensity pattern of the microarray scans (left on top); the substitution matrix (left on bottom) highlighted the preference for a given amino acid at each position of the HA epitope by color codes, while the amino acid plot (right) reflected 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 ^{7}A, however, indicated a certain contribution of this variable amino acid to antibody binding. In the variable motif ^{7}AKAAFSDLQ^{15} 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 ^{1}A, ^{2}E and ^{4}D that did not tolerate any substitution without significant loss in binding. Only ^{4}D showed a limited susceptibility for conservative exchange by E. Amino acid ^{6}P 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 ^{3}G and ^{5}D 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 ^{3}G and for S, R and W in position ^{5}D. Exchange by amino acids like E, H and M in position ^{5}D as well as by G, A and D-amino acids a and e in position ^{3}G resulted in a spot intensity decrease of approx. 25%.

Conclusion

The permutation scans of HA and M13 epitopes ^{1}YPYDVPDYA^{9} and ^{1}AEGDDPAKAAFSDLQ^{15} 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 ^{4}DVPDYA^{9} with a high sequence conservation of amino acids ^{4}D, ^{7}D and ^{8}Y, a less pronounced conservation of amino acids ^{6}P and ^{9}A as well as the variable ^{5}V 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 ^{7}AKAAFSDLQ^{15} at the C-terminus and a conserved core motif ^{1}AEGDDP^{6} 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 ^{1}A and ^{2}E. Amino acids ^{4}D and ^{6}P also exhibited a limited tolerance for exchange by other amino acids, while ^{3}G 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 ^{7}A 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.