Antibody Validation with a Combination of Protein and Peptide Microarrays

Introduction

Antibodies are essential tools in life sciences and are applied in many research areas such as antibody-based proteomics, cellular biology or diagnostic test development. However, the specificity of mono- and polyclonal antibodies and hence their ability to generate reliable and robust data is under scrutiny in the scientific community. Non-specific binding or cross-reactions with non-target antigens may lead to inconclusive data or misleading results, and thus to a waste of time, money and samples. Highly specific antibodies are a prerequisite to generate reliable results, emphasizing the urgent need for appropriate validation methods in order to determine antibody specificity and cross-reactivity. Frequently used validation methods are often based on a purely functional approach (e.g. Western blot, IHC), but cover only a limited scope and - more importantly - lack a detailed understanding of antibody binding.

This application note describes a novel experimental workflow for the unambiguous validation of antibodies by combining protein and peptide microarray screening (Fig. 1). In contrast to functional validation methods, this approach provides an in-depth view on antibody binding from the protein down to the epitope and amino acid level.

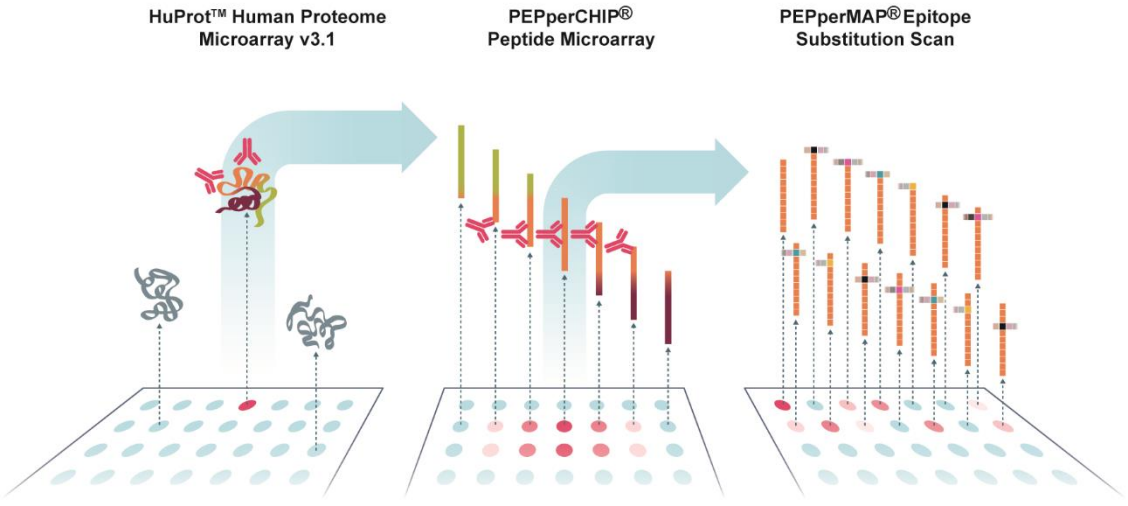

Figure 1: Antibody validation workflow. Selected antibodies are initially screened with a HuProt® Human Proteome Microarray v3.1 covering ~20,000 full-length human proteins. Positive hit proteins are selected and printed as overlapping peptides on a PEPperCHIP® Peptide Microarray to perform a multiplexed epitope mapping with the same set of antibodies. In-depth analysis of the identified epitope(s) with regard to essential, conserved and variable amino acid positions by a PEPperMAP® Epitope Substitution Scan complete the antibody validation process.

The HuProt™ Human Proteome Microarray v3.1 (CDI Laboratories) comprises approximately 81% of the canonical proteome defined by the Human Protein Atlas and is used for the initial screening of the antibody in order to examine cross-reactivities on the protein level. The top hits of the protein array are then translated into overlapping peptides printed onto PEPperCHIP® Peptide Microarrays. These allow the validation of the hit proteins and the precise identification of the underlying epitopes with amino acid resolution. The resulting epitopes are further characterized by PEPperMAP® Epitope Substitution Scans. Thereby, each amino acid position of the wild type peptide is exchanged in a stepwise manner by the other 20 natural amino acids, resulting in all possible peptide variants with single point mutations. A full epitope substitution scan enables the unambiguous identification of essential conserved and variable amino acid positions of the epitope, tolerated amino acid exchanges, the precise motif length and new epitope variants.

This study compares two different commercial research antibodies targeting human transglutaminase-2 (TGM2), a mouse monoclonal anti-TGM2 antibody (beta Sheet Domain, clone XTG11) from ZEDIRA GmbH, Darmstadt, Germany and a rabbit polyclonal anti-TGM2 antibody (HPA029518) from Atlas Antibodies, Stockholm, Sweden by using the validation workflow as described above. While the mouse monoclonal anti-TGM2 antibody was characterized by a multiplexed epitope mapping against various transglutaminases, the rabbit polyclonal anti-TGM2 antibody was validated by IHC, Western blot and immunofluorescence in cell lines.

Results & Discussion

Both anti-TGM2 antibodies were initially incubated on the HuProt™ Human Proteome Microarray v3.1 covering ~20.000 unique proteins. The mouse anti-TGM2 mAb showed a main response against TGM2 and weaker cross-reactions with proteins CMIP and JHU07836.P082A01 (Fig. 2).

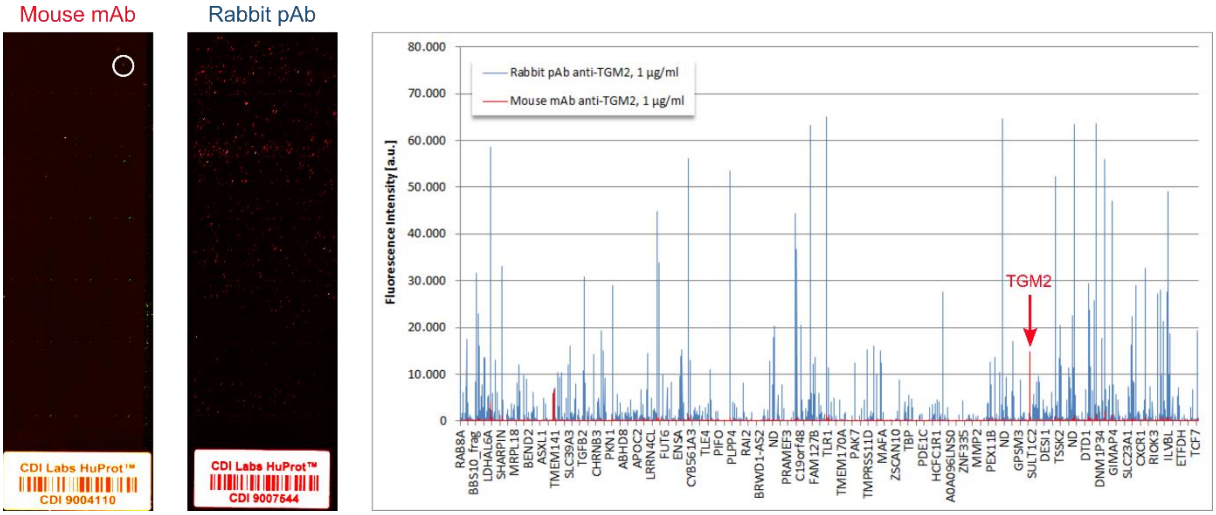

Figure 2: **Left: Raw scans of the HuProt™ Human Proteome Microarrays v3.1:** Two protein microarrays were incubated with the mouse anti-TGM2 mAb (red) and with the rabbit anti-TGM2 pAb (blue). A white circle indicates the location of TGM2 protein. **Right: Fluorescence intensity profiles or the protein array assays**: The mouse anti-TGM2 mAb (red / arrow) shows a main response against TGM2. The rabbit anti-TGM2 pAb (blue) exhibited a strong cross-reactivity on the protein level, but no response against TGM2 protein.

In contrast, the rabbit anti-TGM2 pAb exhibited a strong cross-reactivity on the protein level, but no response against its actual target antigen TGM2.

The top hit proteins of the HuProt™ Human Proteome Microarray analyses were subsequently translated into overlapping 15 amino acid peptides with a maximum peptide-peptide overlap of 14 amino acids printed in duplicate on PEPperCHIP® Peptide Microarrays. Both antibodies were assayed on these peptide microarrays (Fig. 3). The mouse anti-TGM2 mAb exhibited a single response against an N-terminal TGM2 epitope based on peptides with the consensus motif RFPLRDAV and no off-target binding. The cross-reactions with proteins CMIP and JHU07836.P082A01 could not be validated and presumably resulted from less specific or non-specific binding to the full-length proteins. In contrast to the HuProt™ Human Proteome Microarray analysis, the rabbit anti-TGM2 pAb showed a very strong polyclonal response against various C-terminal TGM2 epitopes based on peptides with the consensus motifs KIRILGE, PLPVALE, TVEGA and DPVE. We additionally observed substantial cross-reactions to other hit proteins such as SRBS1 (QPEVIV), IST1 (GVETDL), UBA5 (PVDLPEG) and F117A (VQTDQPETVI). All cross-reactive peptides exhibited sequence similarities to the actual TGM2 epitopes.

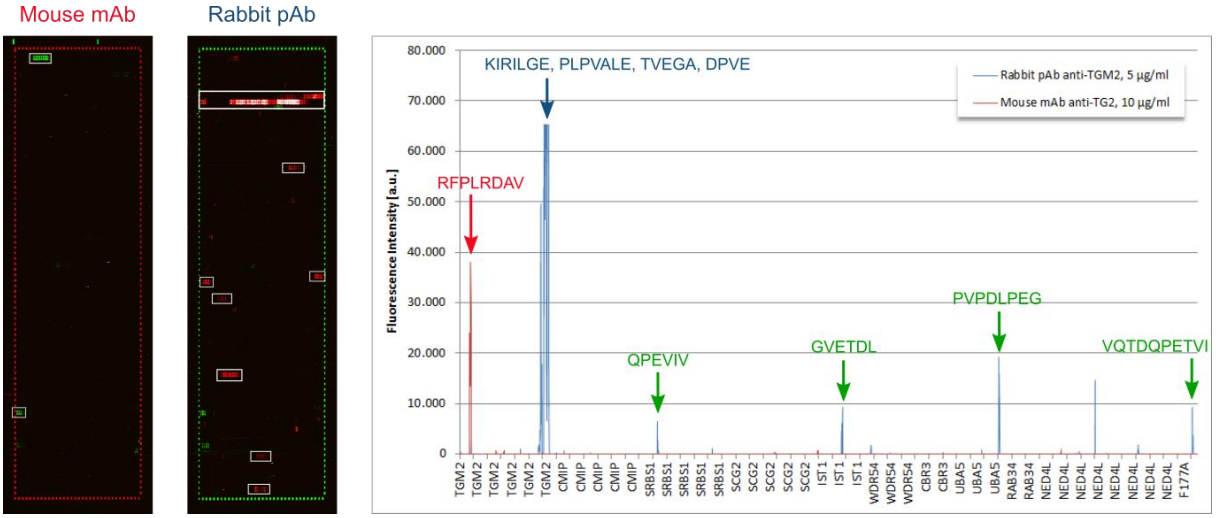

Figure 3: Multiplexed high-resolution epitope mapping of the top hit proteins selected of the initial HuProt™ Human Proteome Microarray screening. Peptide microarray scans (left) and a combined intensity plot of the mouse anti-TGM2 mAb and rabbit anti-TGM2 pAb response profiles (right). The mouse anti-TGM2 mAb showed a single response against the N-terminal TGM2 epitope RFPLRDAV (red arrow). The rabbit anti-TGM2 pAb exhibited a strong polyclonal response against various C-terminal TGM2 epitopes (blue arrow), but also a number of cross-reactions against peptides with a sequence homology to the actual epitopes (green arrows).

The epitope RFPLRDAV of the mouse anti-TGM2 mAb was further characterized by a PEPperMAP® Epitope Substitution Scan. Thereby, each amino acid position of wild type peptide TKA**RFPLRDAV**EEGD was gradually exchanged by the other 20 main amino acids. The resulting peptide microarray contained 300 peptide variants of the wild type peptide and was incubated with the mouse anti-TGM2 mAb and highlighted a typical epitope substitution pattern with conserved (few or single spots in a row) and variable (continuous row of spots) amino acid positions (Fig. 4, microarray scan and heat map on the left). In order to investigate antibody binding on a quantitative scale, an

amino acid substitution plot was generated in which the intensity of an amino acid exchange was referenced to the wild type peptide set to 100%. Thus, the position of an amino acid exchange in the intensity plot reflected an increase or decrease of antibody binding compared to the wild type peptide. The amino acid plot validated the proposed epitope RFPLRDAV and highlighted seven highly conserved amino acid positions **RFPLRD**A**V**. Due to the comparably high number of highly conserved amino acid positions, off-target binding of mouse anti-TGM2 mAb due to sequence homologies is rather unlikely. Since the cross-reactivity of the previously validated rabbit anti-TGM2 pAb was already validated by the multiplexed epitope mapping, an in-depth analysis of the C-terminal TGM2 epitopes was omitted.

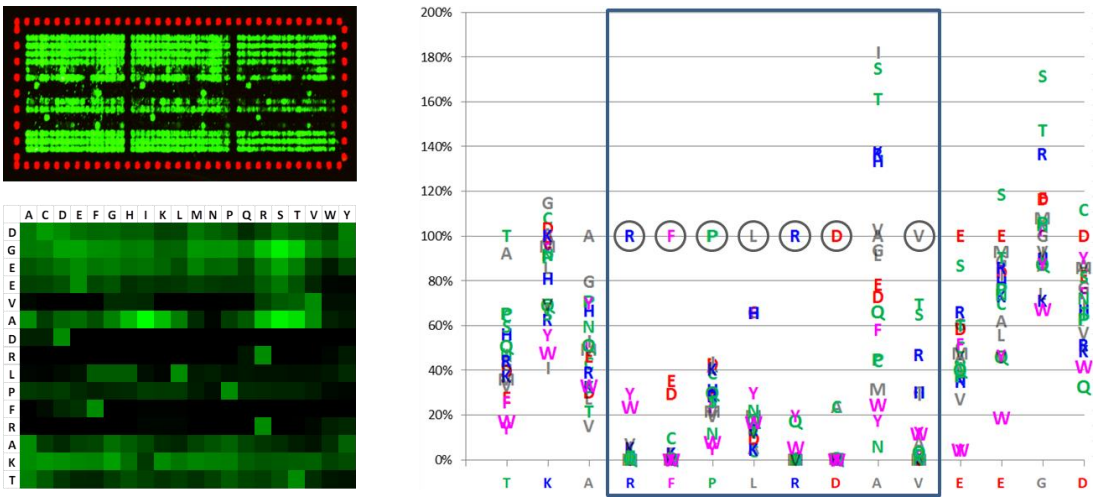

Figure 4: PEPperMAP® Epitope substitution scan of wild type peptide TKA<u>RFPLRDAV</u>EEGD: The microarray scan, the heat map and amino acid substitution plot of the mouse anti-TGM2 mAb validated the epitope RFPLRDAV and highlighted seven highly conserved amino acid positions (encircled).

Summary

A mouse mAb and a rabbit pAb directed against human TGM2 were analyzed by a novel antibody validation workflow with a focus on the detailed understanding of antibody binding. First, both antibodies were screened on a HuProt™ Human Proteome Microarray followed by a multiplexed epitope mapping of the top protein hits using PEPperCHIP® Peptide Microarrays. An additional PEPperMAP® Epitope Substitution Scan analysis was performed for epitope validation and the identification of essential, conserved and variable amino acid positions of the identified epitope to assess antibody off-target binding.

The rabbit anti-TGM2 pAb showed multiple strong cross-reactions on the protein microarray and no binding to TGM2. The latter could be explained by a spotted TGM2 isoform without the C-terminal part with the epitopes. The subsequent peptide microarray analysis revealed that the antibody epitopes were located in the C-terminal part of TGM2. We were also able to validate most of the top cross-reactions due to sequence homologies to the actual epitopes, what likely leads to off-target binding in

real applications of the rabbit anti-TGM2 pAb. This possible issue was obviously not identified by previous purely functional validations methods

The mouse anti-TGM2 mAb exhibited higher target specificity with a main response against TGM2 and some weaker cross-reactions on the protein array. The subsequent epitope mapping highlighted only a single monoclonal response corresponding to the epitope of the antibody, but could not validate the cross-reactive proteins of the HuProt™ Human Proteome Microarray. The final epitope substitution scan highlighted seven well conserved amino acid positions - a prerequisite for a low cross-reactivity and no off-target binding.

The outcome of this study shows that the combined protein and peptide microarray screening is not only an ideal tool for antibody validation, but also for the identification and validation of unknown antigens. In contrast to purely functional validation methods, such a comprehensive analysis of antibody binding from the protein to the epitope and amino acid level helps to increase the reliability of antibodies as tools in therapeutics, diagnostics and basic research.