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Application Note

Antibody Validation with a Combination of Protein and Peptide Microarrays

ABSTRACT

In this application note, we tested a mouse monoclonal antibody alongside a rabbit polyclonal antibody directed against human TGM2 using a new antibody validation workflow: A three-step approach based on a protein array screening for protein hit discovery, a multiplexed epitope mapping for hit validation and epitope identification, and a final epitope substitution scan for an in-depth analysis of conserved and variable amino acid positions. The goal was to compare validation methods and to analyze off-target binding of two different research antibodies.

The rabbit polyclonal antibody showed a strong cross-reactivity on the protein microarray. The multiplexed epitope mapping on the peptide microarray identified strong polyclonal epitopes in the expected region of TGM2, together with similar interaction motifs in the cross-reactive targets.

For the mouse monoclonal antibody, a higher target specificity with only few off-target interactions was found on the protein array. Multiplexed epitope mapping showed only a single response corresponding to the epitope of the antibody, and the concluding epitope substitution scan identified seven well conserved amino acid positions - a prerequisite for a low cross-reactivity and rare off-target binding.

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 validation of antibody specificity 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.

The HuProt™ Human Proteome Microarray v4.0 (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 converted into overlapping peptides and printed onto PEPperCHIP® Peptide Microarrays. These allow the validation of the hit proteins and the precise identification of the underlying epitopes at single amino acid resolution. The resulting epitopes are further characterized by PEPperMAP® Epitope Substitution Scans: Each amino acid position of the wild type peptide is exchanged by each of the other 20 standard amino acids, resulting in all possible peptide variants with single point mutations. A full epitope substitution scan enables the

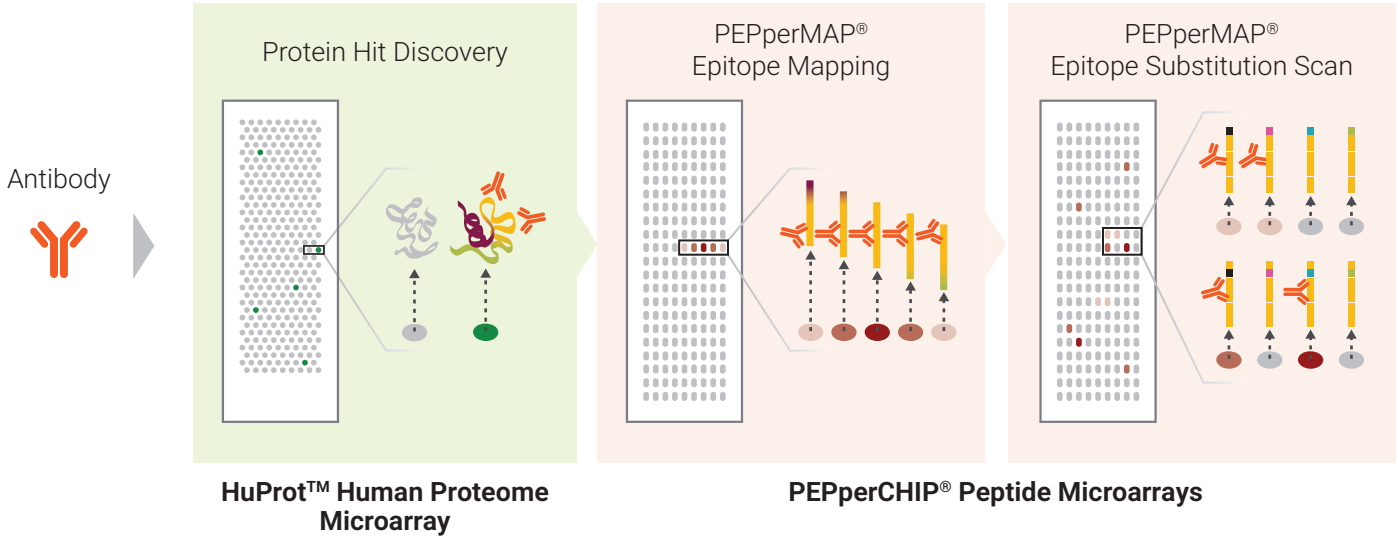

Figure 1. Antibody validation workflow. Selected antibodies are initially screened with a HuProt™ Human Proteome Microarray v3.1 covering ~20,000 full-length human proteins. Positive hit proteins are selected and printed as overlapping peptides on a PEPperCHIP® Peptide Microarray to perform a multiplexed 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 completes the antibody validation process.

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 raised against a C-terminal fragment of TGM2 by using the validation workflow as described above. 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 functional methods, including IHC, Western blot and immunofluorescence in cell lines.

RESULTS AND DISCUSSION

Both anti-TGM2 antibodies were initially incubated on a HuProt™ Human Proteome Microarray v3.1. The mouse anti-TGM2 mAb showed a main response against TGM2 and weaker cross-reactions with proteins CMIP and PRM1 (Fig. 2).

In contrast, the rabbit anti-TGM2 pAb exhibited a strong cross-reactivity on the protein level. The target TGM2, represented on the HuProt™ array as isoform 2 (UniProt P21980-2) was not recognized, in line with the fact that isoform 2 is missing the C-terminus of the canonical protein, against which the pAb was raised.

The top hit proteins of the HuProt™ Human Proteome Microarray analyses were converted into 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 motif RFPLRDAV. The cross-reactions with proteins CMIP and PRM1 could not be validated on the peptide level and presumably resulted from non-specific binding to the full-length proteins. The rabbit anti-TGM2 pAb showed a very strong polyclonal response against various C-terminal TGM2 epitopes based on peptides with the motifs KIRILGE, PLPVALE, TVEGA and DPVE, consistent with the pAb being raised against a C-terminal immunogen. We additionally observed substantial cross-reactions to other hit proteins such as SRBS1 (QPEVIV), IST1 (GVETDL), UBA5 (PVDLPEG) and F117A (VQTDQPETVI). All cross-reactive motifs exhibited sequence similarities to the actual TGM2 epitopes.

The epitope RFPLRDAV of the mouse anti-TGM2 mAb was further characterized by a PEPperMAP® Epitope Substitution Scan. Each amino acid position of wild type peptide TKA**RFPLRDAV**EEGD was exchanged by each of the other 20 standard amino acids. The resulting peptide microarray contained 300 peptide variants of the wild type peptide and was incubated with the mouse anti-TGM2 mAb. The results showed 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

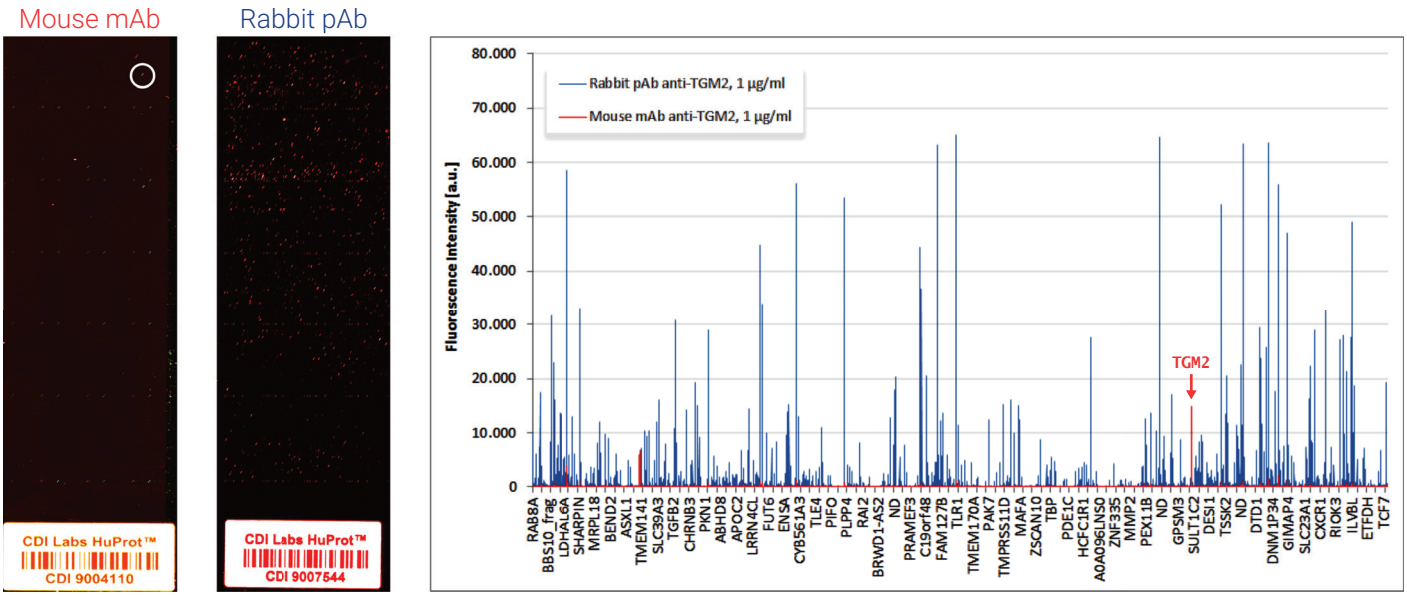

Figure 2. Left: Raw scans of the HuProt™ Human Proteome Microarrays. Two protein microarrays were incubated with the mouse anti-TGM2 mAb (red) and with the rabbit anti-TGM2 pAb (blue). A white circle indicates the location of TGM2 protein. **Right: Fluorescence intensity profiles on the protein arrays.** The mouse anti-TGM2 mAb (red / arrow) showed a main response against TGM2. The rabbit anti-TGM2 pAb (blue) exhibited numerous cross-reactions against a wide range of proteins.

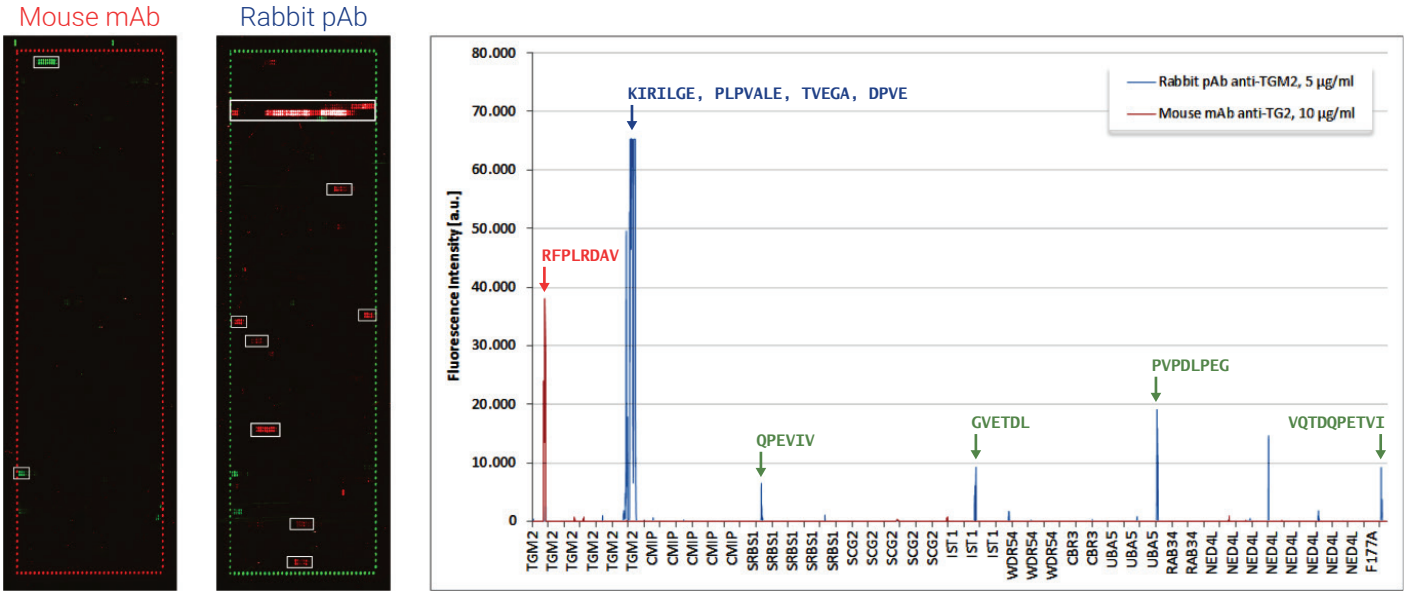

Figure 3. Multiplexed high-resolution epitope mapping of the top hit proteins selected of the initial HuProt™ Human Proteome Microarray screening. Peptide microarray scans (left) and a combined intensity plot of the mouse anti-TGM2 mAb and rabbit anti-TGM2 pAb response profiles (right). The mouse anti-TGM2 mAb showed a single response against the Nterminal 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 similarity to the actual TGM2 epitopes (green arrows).

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 this comparably high number of highly conserved amino acid positions, off-target binding of mouse anti-TGM2 mAb due to sequence similarities is unlikely, in line with the low cross-reactivity observed against the comprehensive HuProt™ content. For the rabbit anti-TGM2 pAb, a similar in-depth analysis of the C-terminal TGM2 epitopes was omitted, due to the high cross-reactivity observed on the HuProt™ array and confirmed in the multiplexed epitope mapping.

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

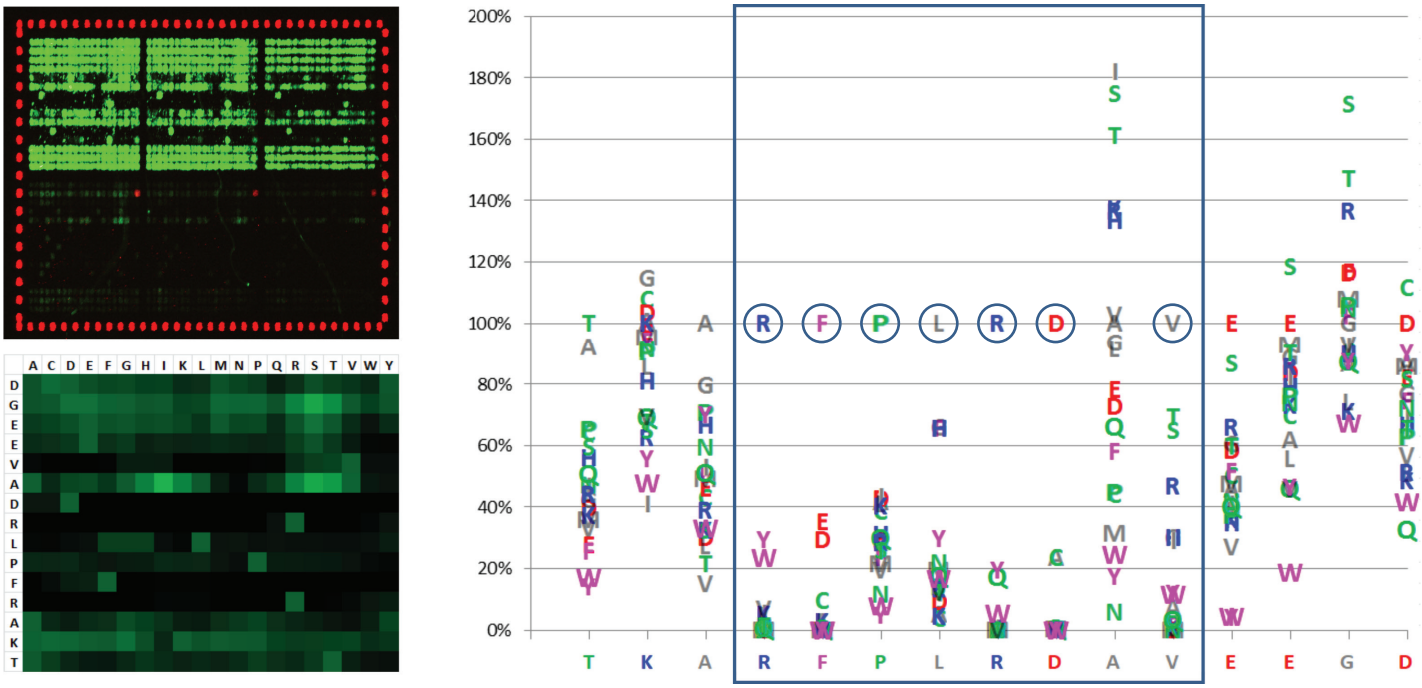

Figure 4. PEPperMAP® Epitope Substitution Scan of wild type peptide TKARFPLRDAVEEGD. The microarray scan, the heat map and amino acid substitution plot of the mouse anti-TGM2 mAb validated the epitope RFPLRDAV and highlighted seven highly conserved amino acid positions (encircled).

PEPperCHIP® Peptide Microarrays. Further 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 HuProt™ microarray. TGM2, represented as isoform 2 lacking the C-terminus of the canonical protein, was not recognized, consistent with use of a C-terminal immunogen. The subsequent peptide microarray analysis identified strong polyclonal epitopes located in the C-terminal part of TGM2. We were also able to validate most of the top cross-reactions observed on HuProt™ array, which displayed sequence similarities to the actual TGM2 epitopes. This is likely to result in off-target binding in real-world applications of the rabbit anti-TGM2 pAb. This possible issue was not identified by previous purely functional validation methods.

The mouse anti-TGM2 mAb showed high target specificity with a main response against TGM2 and only few weaker cross-reactions on the HuProt™ Human Proteome Microarray. The subsequent epitope mapping identified a single monoclonal response against TGM2 epitope RFPLRDAV. The concluding epitope substitution scan identified seven well conserved amino acid positions within the epitope - a prerequisite for a low cross-reactivity and next to no off-target binding, consistent with the initial observation on the HuProt™ array.

The outcome of this study demonstrates 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. Complementing the classical 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.

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