Antibody Validation and Antigen Discovery by combining Protein and Peptide Microarrays

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Antibody sensitivity and specificity are paramount when it comes to the design and development of therapeutic and diagnostic antibodies. However, the specificity of antibodies and hence their ability to generate reliable data is under scrutiny in the scientific community. Highly specific antibodies are a prerequisite to generate reliable and reproducible data, going along with the urgent need for appropriate validation methods to determine antibody specificity and cross-reactivity.

Here we present a straightforward approach for the unambiguous validation of antibodies by combining protein and peptide microarray screening. We compared two different commercial antibodies targeting human transglutaminase 2 (TGM2): a mouse monoclonal (mAb) from ZEDIRA GmbH and a rabbit polyclonal (pAb) from Atlas Antibodies. Surprisingly, we observed a high cross-reactivity of the highly validated rabbit polyclonal antibody, albeit also some discrepancies between the protein and the peptide microarray analyses. This outcome raises questions about the reliability of some validation methods such as immunohistochemistry, Western blot or RNA sequencing.

Our approach uses PEPperCHIP® Peptide Microarrays, which are generated by digital laser printing of amino acid toner particles on standard glass slides using a custom peptide laser printer with 24 amino acid toner cartridges. This allows for high peptide spot density, maximum content flexibility, and quicker production times.

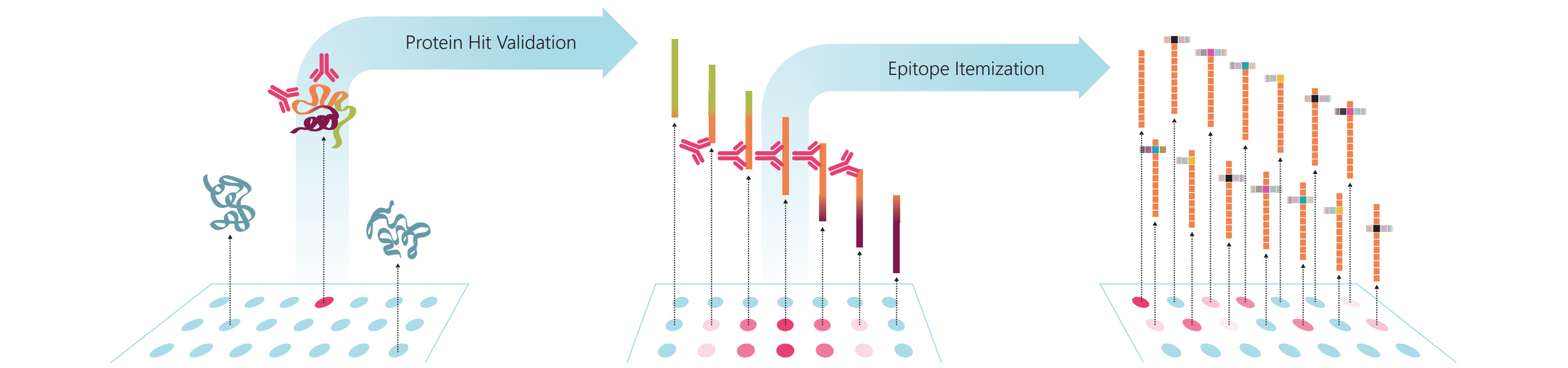
The combination of protein and peptide microarray-based screening allows for the validation of therapeutic antibodies not only through identification of their protein targets and off-target binding, but also through itemization of their epitopes though the detailed analyses of conserved, variable, and essential amino acid positions.

SUMMARY & KEY FINDINGS

We tested a mouse monoclonal along with an "enhanced validated" rabbit polyclonal antibody directed against human TGM2 by a new antibody validation workflow: The three-step approach was 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 of off-target binding of two different types of research antibodies.

While the rabbit polyclonal antibody showed a strong cross-reactivity on the protein microarray and no target-binding, it turned out to be highly target specific and less cross-reactive in the multiplexed epitope mapping on the peptide microarray, in accordance with the expectations. A higher target specificity and less off-target binding was found for the mouse monoclonal antibody. Multiplexed epitope mapping showed only a single response corresponding to the epitope of the antibody, and the final epitope substitution scan highlighted 7 well conserved amino acid positions---a prerequisite for a low cross-reactivity and no off-target binding.

ANTIBODY VALIDATION WORKFLOW



HuProt™ Human Proteome Microarray v3.1

- 20,000 human proteins
- broad cross-reactivity profiling
- antigen discovery

PEPperCHIP® Peptide Microarray

- 11,000 custom peptides
- multiplexed linear or conformational epitope mapping
- protein hit validation

PEPperMAP® Epitope Substitution Scan

- exchange of all amino acid positions by all amino acids of an epitope
- identificaiton of conserved and variable amino acid positions

RESULTS

HuProt™ Human Proteome Microarray v3.1

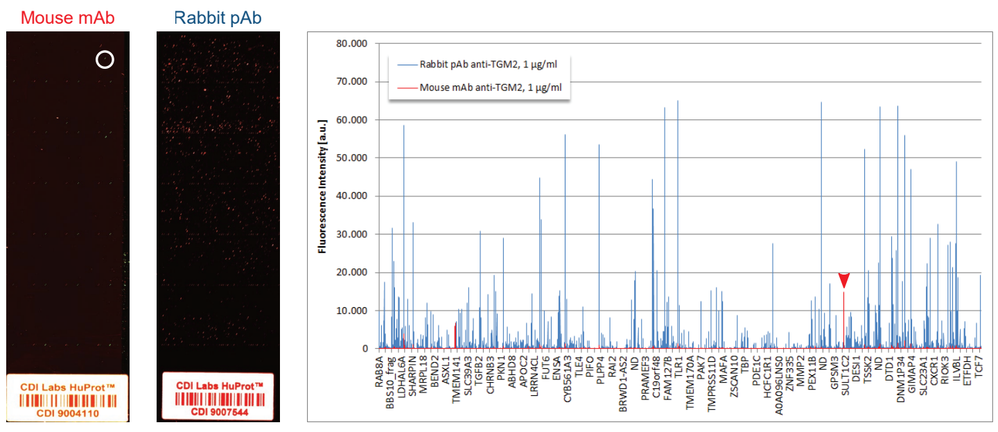


Figure 1. HuProt™ Human Proteome Microarray v3.1, microarray scans and combined intensity plots of the mouse mAb and the rabbit pAb against TGM2.
The mouse anti-TGM2 mAb (red) showed a main response against TGM2 (circled / red arrow) and weaker cross-reactions with the proteins CMIP and JHU07836.P082A01. The highly validated rabbit anti-TGM2 pAb (blue) exhibited a strong cross-reactivity on the protein level, but surprisingly no response against TGM2.

PEPperCHIP® Peptide Microarray

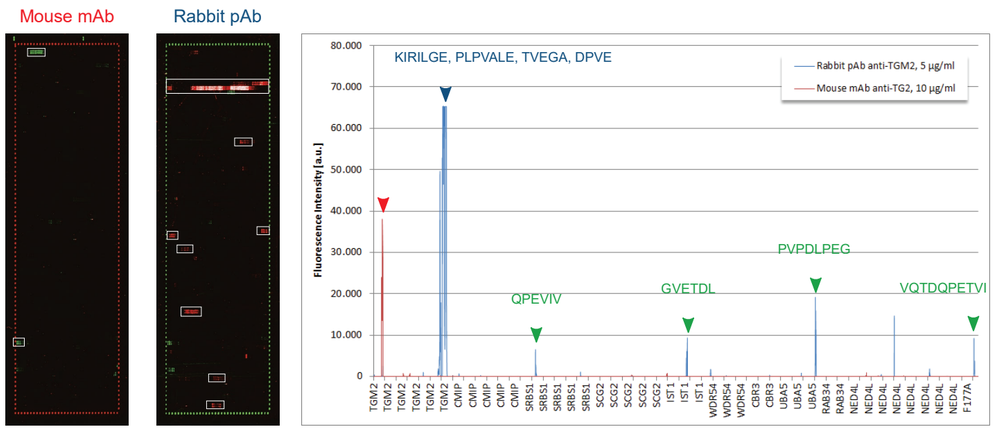


Figure 2. Multiplexed epitope mapping of top hit proteins, microarray scans and combined intensity plots of the mouse mAb and the rabbit pAb against TGM2.
The mouse mAb exhibited the N-terminal TGM2 epitope RFPLRDAV (red arrow) and no off-target binding; the rabbit pAb showed a strong polyclonal response against various C-terminal TGM2 epitopes (blue arrow), but also some cross-reactions with peptides with sequence similarities to the TGM2 epitopes (green arrows). The off-target binding was less strong compared to the protein array.

PEPperMAP® Epitope Substitution Scan

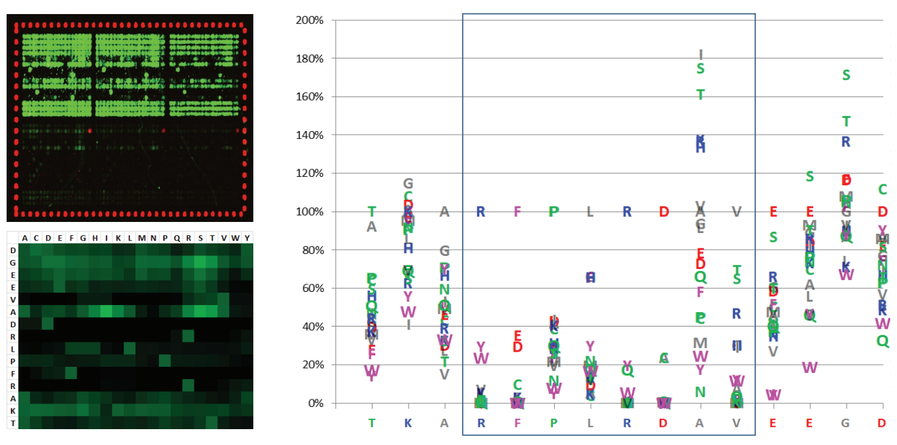


Figure 3. Epitope substitution scan of wild type peptide TKARFPLRDAVEEGD, microarray scan, heat map and amino acid substitution plot of the mouse mAb against TGM2.
The amino acid substitution plot with the wild type peptide at 100% validated the proposed epitope RFPLRDAV and highlighted 7 highly conserved or well-conserved amino acid positions. Such high numbers of conserved amino acids reduce the probability of off-target binding.

The mouse anti-TGM2 mAb (Zedria GmbH) showed a clear response against TGM2 and few weak cross-reactions on the protein array. In the multiplexed epitope mapping, it exhibited no cross-reaction, but a clear epitope with 7 conserved amino acid positons. In contrast, the highly validated rabbit antiTGM2 pAb (Atlas Antibodies) showed a very strong cross-reactivity on the protein array and no target binding, while we observed a strong target binding in multiplexed epitope mapping with multiple weaker off-target effects.

PEPperCHIP® PEPTIDE MICROARRAY PLATFORM TECHNOLOGY

High-density PEPperCHIP® Peptide Microarrays are generated by digital laser printing of amino acid toner particles on standard glass slides by using a custom peptide laser printer with 24 amino acid toner cartridges.

Figure 4. The PEPperCHIP® Peptide Microarray printing process.

The benefits of this technique are an *unique flexibility* in terms of peptide content, *fast production times*, a *high spot density* with up to 11,000 features per chip, and *low material consumption* enabling the generation of customized peptide array at reasonable costs.

Microarray format	DISCOVERY	STANDARD	MAPPING	MULTIPLEXED
Array copies per chip	1	1-3	4-5	16
Peptide range	< 75,460 per chip (up to ~100 proteins)	< 11,288 per array copy	< 2,040 per array copy	< 273 per array copy

Table 1. Examples of different PEPperCHIP® Peptide Microarray formats.

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