Validation of a New Recombinant Human anti-c-Myc Tag Antibody

Introduction

Antibodies are essential tools for therapeutic, diagnostic and research purposes. However, antibodies are often poorly characterized in terms of specificity and cross-reactivity. The recent Nature feature “Reproducibility crisis: Blame it on the antibodies” highlighted a number of serious issues caused by such poorly characterized antibodies.[1] For instance, researchers at Mount Sinai Hospital in Toronto, Canada, had been chasing a protein called CUZD1, a supposed diagnostic marker protein for pancreatic cancer. They bought a protein-detection kit comprising a CUZD1 specific antibody and wasted two years, $500,000 and thousands of patient samples before they realized that the antibody actually recognized a different cancer protein, CA125, but did not bind to CUZD1 at all.

A high cross-reactivity or frequent off-target effects are often caused by less characterized antibodies with very short epitopes, epitopes with poor sequence conservation, non-specific protein binding or complex polyclonal responses. This raises not only the urgent need for new and highly specific antibodies, but also for high quality solutions for in-depth antibody validation and cross-reactivity testing. Only well characterized and highly specific antibodies offer the guarantee to avoid misleading results and a waste of time, money and samples.

The comprehensive **PEPperPRINT toolbox for antibody characterization** is ideally suited for in-depth analysis of epitopes and antibody cross-reactions:[2] Our high resolution **PEPperMAP® Epitope Mapping** allows identification of linear and conformational epitopes of mono- and polyclonal antibodies at single amino acid resolution in a cost-effective and straightforward approach. A multiplexed PEPperMAP® Epitope Mapping against several antigens at once further enables the identification of cross-reactions in a single assay. Based on a known wild-type epitope, **PEPperMAP® Epitope Substitution Scans** enables to unambiguously determine conserved and variable amino acid positions, the degree of sequence conservation of each amino acid, the actual epitope lengths and epitope variants. In contrast to a typical alanine scan, a PEPperMAP® Epitope Substitution Scan covers 20 times more data points by exchanging all amino acid positions with the 20 physiological amino acids. That way it also accounts for structural and/or functional similarities of amino acid side chains.

This application note describes the in-depth characterization and validation of the recombinant human monoclonal anti-c-myc antibody "Hypermyc" in comparison with the well-known anti-c-myc tag monoclonal antibody 9E10 antibody by PEPperMAP® Epitope Mapping and PEPperMAP® Epitope Substitution Scan using PEPperPRINT’s high density peptide microarray platform.

[1] M. Baker, *Reproducibility crisis: Blame it on the antibodies*. Nature 2015, **521**, 274.
[2] http://www.pepperprint.com/applications/epitope-and-antibody-analysis/

Human Monoclonal anti-c-Myc Tag Antibody (Hypermyc)

By using recombinant *in vitro* antibody phage display, a recombinant human monoclonal anti-c-myc antibody named Hypermyc was generated.[3] The affinity of this scFv-Fc antibody was analyzed in solution by microscale thermophoresis, and its stability was characterized by differential scanning fluorometry. It was shown that the Hypermyc clone not only exhibited a significantly higher stability, but also higher affinity compared to the ubiquitous monoclonal anti-c-myc tag antibody 9E10 (Fig. 1).[4]

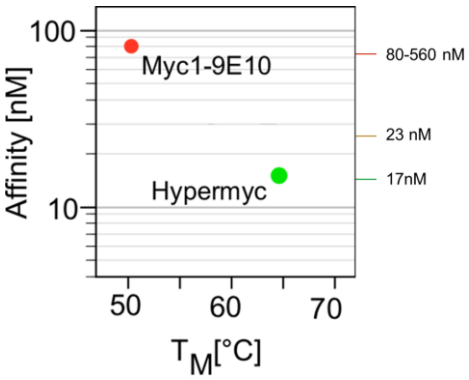

Fig. 1: Thermal stability and target affinity of the known 9E10 antibody (red) in comparison to the new recombinant Hypermyc antibody (green).

Results & Discussion

For the high resolution **PEPperMAP® Epitope Mapping** of both antibodies, human Myc proto-oncogene protein (UniProt ID P01106) was translated into linear 15 amino acid peptides with a maximum peptide-peptide overlap of 14 amino acids. The resulting c-myc peptide microarrays contained 439 different peptides printed in duplicate (878 peptide spots) and were further framed by additional 82 HA (YPYDVPDYAG) control peptides.

The c-myc peptide microarrays were incubated with the recombinant anti-c-myc Hypermyc antibody and a chimeric version of the monoclonal anti-c-myc tag antibody 9E10 at concentrations of 0.1 µg/ml and 1.0 µg/ml in incubation buffer. After washing, the microarrays were stained with secondary antibody goat anti-human IgG (H+L) DyLight680 and with control antibody anti-HA (12CA5) DyLight800. Microarray read-out was done with a LI-COR Odyssey Imaging System; quantification of microarray spot intensities, data analysis and peptide annotation were carried out with PepSlide® Analyzer.[5]

Both antibodies showed a main response against peptides with the expected C-terminal consensus motif QKLISEEDL with high spot intensities and signal-to-noise ratios (see Fig. 2). From the epitope mapping data, it could not be unambiguously determined whether the N-terminal QK and the C-terminal DL actually contributed to antibody binding or not. In addition to the main signal for the epitope of both antibodies, we further observed a weak second response against peptides with the consensus motif AKLVSE that hinted at a cross-reaction within the same antigen.

[3] Yumab GmbH, http://yumab.com/

[4] W. Schiweck et al., *Sequence analysis and bacterial production of the anti-c-myc antibody 9E10: the V(H) domain has an extended CDR-H3 and exhibits unusual solubility*. FEBS Lett. 1997, **414**, 33.

[5] http://www.sicasys.de/pepslide/

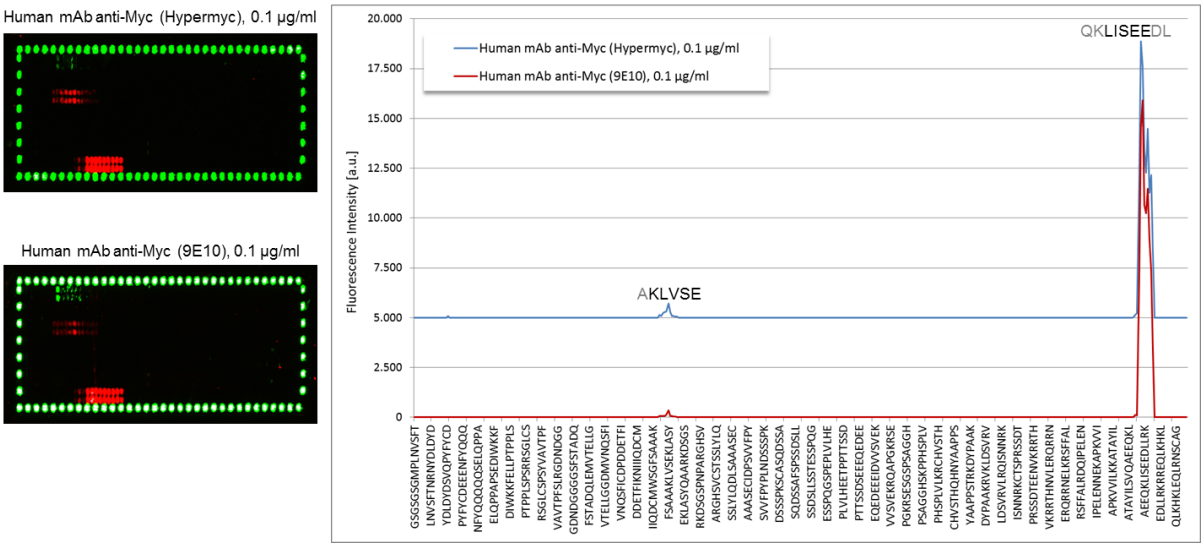

Fig. 2: High resolution PEPperMAP® Epitope Mapping of human monoclonal antibodies Hypermyc and 9E10 with the microarray raw scans (left) and the intensity plot against the antigen sequence from the N- to the C-terminus (right); we observed a clear monoclonal response of both antibodies against peptides with the C-terminal consensus motif QKLISEEDL as well as a weaker cross-reaction against peptides with the consensus motif AKLVSE. For a better data overview, the baselines of the intensity plots were leveled.

Both anti-c-myc clones exhibited a nearly identical response against the proposed human Myc proto-oncogene protein epitope QKLISEEDL. However, the additional cross-reaction against peptides with the consensus motif AKLVSE showed slightly higher spot intensities with the monoclonal Hypermyc antibody, what hinted at a slightly higher cross-reactivity compared to the well-known monoclonal 9E10 antibody (Fig. 3). The relative intensity of the cross-reaction increased with antibody concentrations, and reached an amount of 18% of the main signal with the Hypermyc clone compared to only 6% of the main signal of the 9E10 clone.

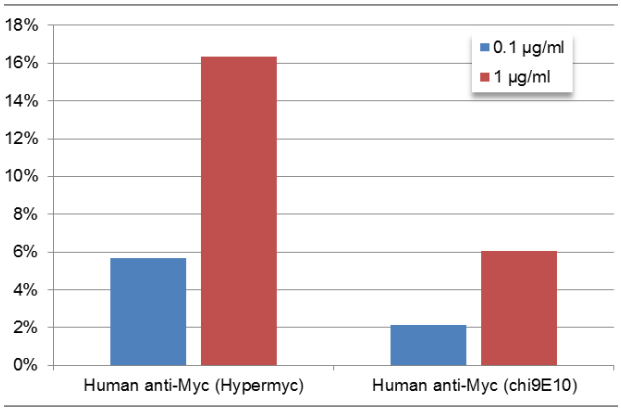

Fig. 3: Relative intensity of the intramolecular cross-reactions at concentrations of 0.1 µg/ml and 1.0 µg/ml compared to the intensity of the main response against epitope QKLISEEDL.

For an in-depth analysis of the observed cross-reactions and the epitopes of both anti-c-myc antibodies, we performed additional **PEPperMAP® Epitope Substitution Scans** of wild type peptide ^{1}EQKLISEEDL^{10} with an exchange of all amino acid positions by the 20 main amino acids. Similar to an alanine scan, a PEPperMAP® Epitope Substitution Scan enables the identification of essential and variable amino acid positions of an epitope, but with 20 times more data points that also highlight the degree of sequence conservation of each amino acid position as well as preferred and less preferred amino acid exchanges.

Based on wild type peptide ^{1}EQKLISEEDL^{10}, the exchange of all amino acid position by the 20 main amino acids resulted in peptide microarrays with 200 peptides with a length of 10 amino acids, printed

in triplicate. The peptide microarrays were further framed by 76 additional HA control peptides, and incubated with the recombinant Hypermyc antibody and the chimeric 9E10 antibody at a concentration of 0.1 µg/ml followed by staining with secondary and control antibodies (Fig. 4).

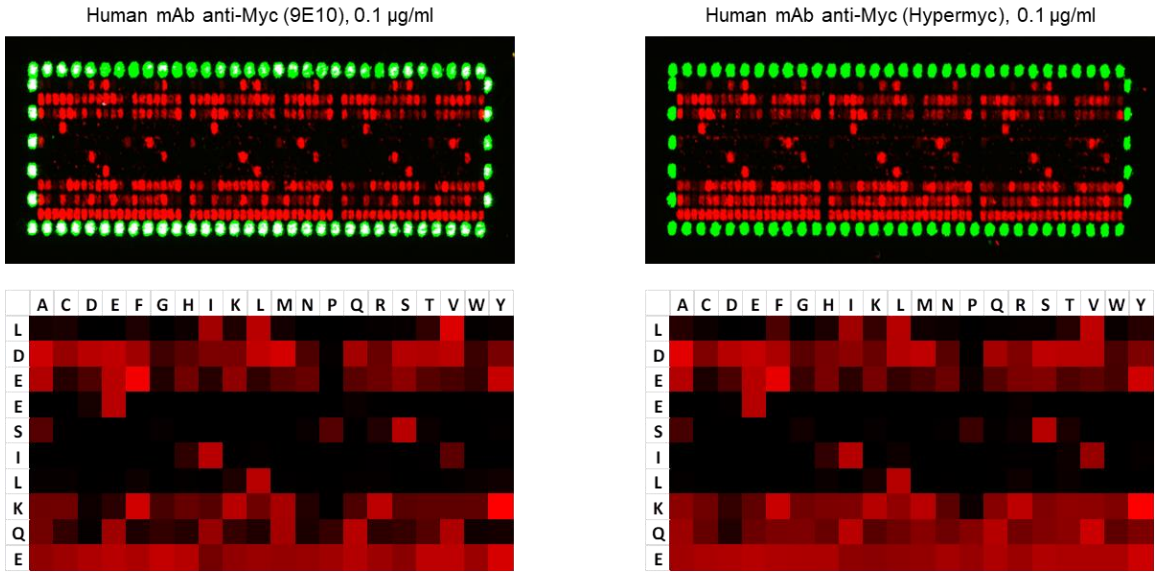

Fig. 4: PEPperMAP® Epitope Substitution Scans of monoclonal antibodies 9E10 (left) and Hypermyc (right) against wild type peptide ^{1}EQKLISEEDL^{10} with the raw microarray scans on top and the resulting heat maps on bottom. We observed very similar epitope substitution patterns for both anti-Myc antibodies with conserved (few spots in a row) and variable (continuous row of spots) amino acid positions.

To investigate the degree of sequence conservation and preferred and less preferred amino acid exchanges in a quantitative manner, we generated so-called amino acid plots of both epitope substitution scans. The amino acid plots are calculated by dividing the spot intensity of a given amino acid exchange (e.g. ^{7}E by Y in ^{1}EQKLIS<u>Y</u>EDL^{10}) by the spot intensity of the wild type peptide (^{1}EQKLISEEDL^{10}) normalized to 100%. The resulting amino acid plots visualize the preference for a certain amino acid relative to the amino acid of the wild type epitope (Fig. 5).

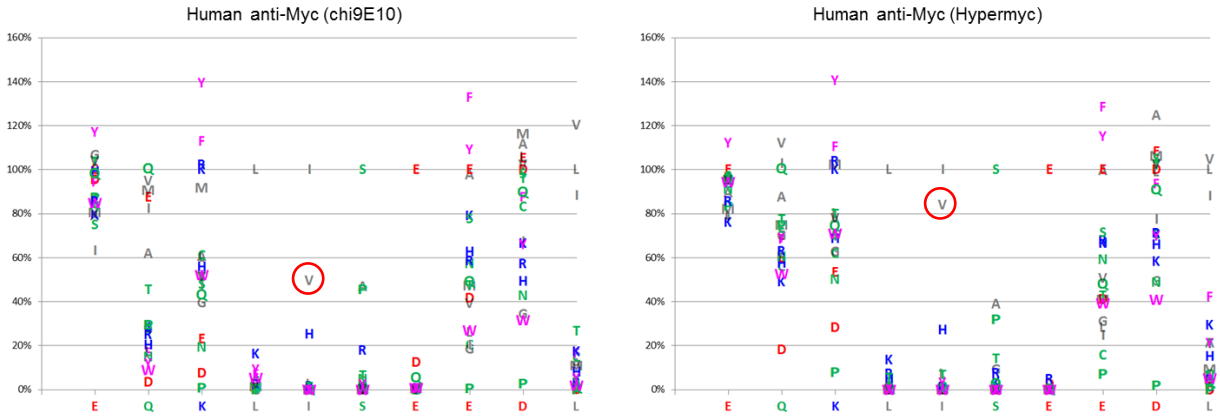

Fig. 5: Amino acid plots of monoclonal antibodies 9E10 (left) and Hypermyc (right) with the intensity of wild type peptide ^{1}EQKLISEEDL^{10} set to 100%. Both antibodies exhibited a highly conserved core motif ^{4}LISE^{7}, whereas only position ^{1}E was unambiguously variable. All other amino acid positions showed varying degrees of sequence conservation with at least a certain preference for the wild type amino acid. The main difference in position ^{5}I was highlighted by red circles.

Amino acid positions [4]L and [7]E showed the highest degree of sequence conservation with more than 80% to a nearly complete loss of antibody binding upon substitution by any other amino acid. Amino acid positions [5]I and [6]S were also well-conserved with a >50% decrease of spot intensities upon exchange by H ([5]I) or A and P ([6]S). The main difference between monoclonal antibodies 9E10 and Hypermyc, however, was found in the tolerance of position [5]I for a conservative exchange by V: Whereas 9E10 binding was reduced by ca. 50%, Hypermyc binding was significantly less affected with only 16% lower spot intensities.

Comparison of the epitope mapping cross-reaction with peptides with the consensus motif AKLVSE with the actual epitope QKLISEEDL highlighted a pronounced similarity of the highly conserved core motif LISE with the LVSE stretch of the cross-reaction. According to the epitope substitution scans, however, the recombinant Hypermyc antibody exhibited a higher tolerance for conserved exchange of I by V, and hence for a cross-reaction with peptides and antigens with the LVSE stretch. **While the AKLVSE/LVSE motif KLVSE is not part of the typical c-myc tag, knowledge of this potential cross-reactivity allows the user to check for the occurrence of this motif and its potential implications upon application of this antibody.** Compared to the new recombinant Hypermyc antibody, the well-known chimeric 9E10 antibody exhibited a lower tolerance for this cross-reaction, and hence a slightly higher target specificity.

Another cross-reactivity profiling on a much broader scale with the PEPperCHIP® Human Epitome Microarray covering 29,128 different epitopes of the human proteome was done with the chimeric 9E10 antibody. Interestingly, this alternative one-step approach also highlighted the LVSE core motif.[6]

Summary

The combination of high resolution PEPperMAP® Epitope Mapping and a subsequent PEPperMAP® Epitope Substitution Scan enabled a very detailed assessment of antibodies cross-reactivities and a detailed understanding of epitope requirements of the new recombinant human monoclonal anti-Myc antibody Hypermyc in comparison to the chimeric monoclonal antibody 9E10: Both antibodies recognize the very same epitope QKLISEEDL in human Myc proto-oncogene protein, but also showed a single cross-reaction with peptides with the consensus motif AKLVSE, containing the KLISE core motif with a conservative exchange of I to V. The epitope mapping data further indicated that the cross-reaction of the recombinant Hypermyc antibody was slightly stronger than with the 9E10 clone. This higher tolerance for the conservative exchange of I by V was unambiguously identified as the source for this higher cross-reactivity - what would not have been possible by a standard alanine scan.

Based on combination of high resolution PEPperMAP® Epitope Mappings and PEPperMAP® Epitope Substitution Scans, this case study demonstrates the in-depth characterization of antibody epitopes and a detailed view on antibody cross-reactions and off-target effects with a specificity on the amino

[6] http://www.pepperprint.com/applications/antibody-cross-reactivity-analysis/

acid level. Our peptide microarray-based approach thus enables the most precise antibody epitope validation possible, and is available for both linear and conformational epitopes.[7]

[7] T.J. Gardner et al., *Functional screening for anti-CMV biologics identifies a broadly neutralizing epitope of an essential envelope protein.* Nat. Commun. 2016; **7**, 13627.