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APPLICATION NOTE

High-Resolution Epitope Mapping of Phosphospecific Anti-Ubiquitin Antibodies

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Abstract

Post-translational modifications (PTMs) such as phosphorylation or glycosylation play a critical role in regulating protein function and cellular signaling pathways. Mapping antibody specificity towards PTM-containing epitopes is essential for understanding immune responses and advancing therapeutic antibody development, but remains technically challenging.

In this study, we used high-density PEPperCHIP® cLIFT Peptide Microarrays for the epitope mapping of two monoclonal anti-ubiquitin antibodies against phosphorylated and non-phosphorylated ubiquitin peptides. The ubiquitin sequence was converted into overlapping 15 amino acid peptides with a peptide overlap of 14 amino acids for high-resolution epitope data. Peptides included site-specific phosphorylation variants through precise post-synthetic modification of selected serine, threonine and tyrosine residues. This enabled the simultaneous analysis of native and phosphorylated peptide variants in a single assay.

Incubation of the ubiquitin peptide microarrays with the antibody samples resulted in strong and distinct binding patterns: the phosphospecific rabbit IgG showed strong specificity for phosphorylated ubiquitin motifs with an essential phosphorylated amino acid, while the mouse IgG without given phosphospecificity preferentially recognized non-phosphorylated peptides with limited reactivity to phosphorylated variants.

In addition to validating the phosphospecificty of one of the anti-ubiquitin antibodies, our results also demonstrate the capability of the cLIFT platform to incorporate PTMs with high precision, thus enabling detailed epitope mapping of PTM-specific antibodies. The cLIFT technology significantly expands the possibilities of high-density peptide microarrays for immunological research, biomarker identification, and the development of therapeutic antibodies targeting post-translationally modified epitopes.

Introduction

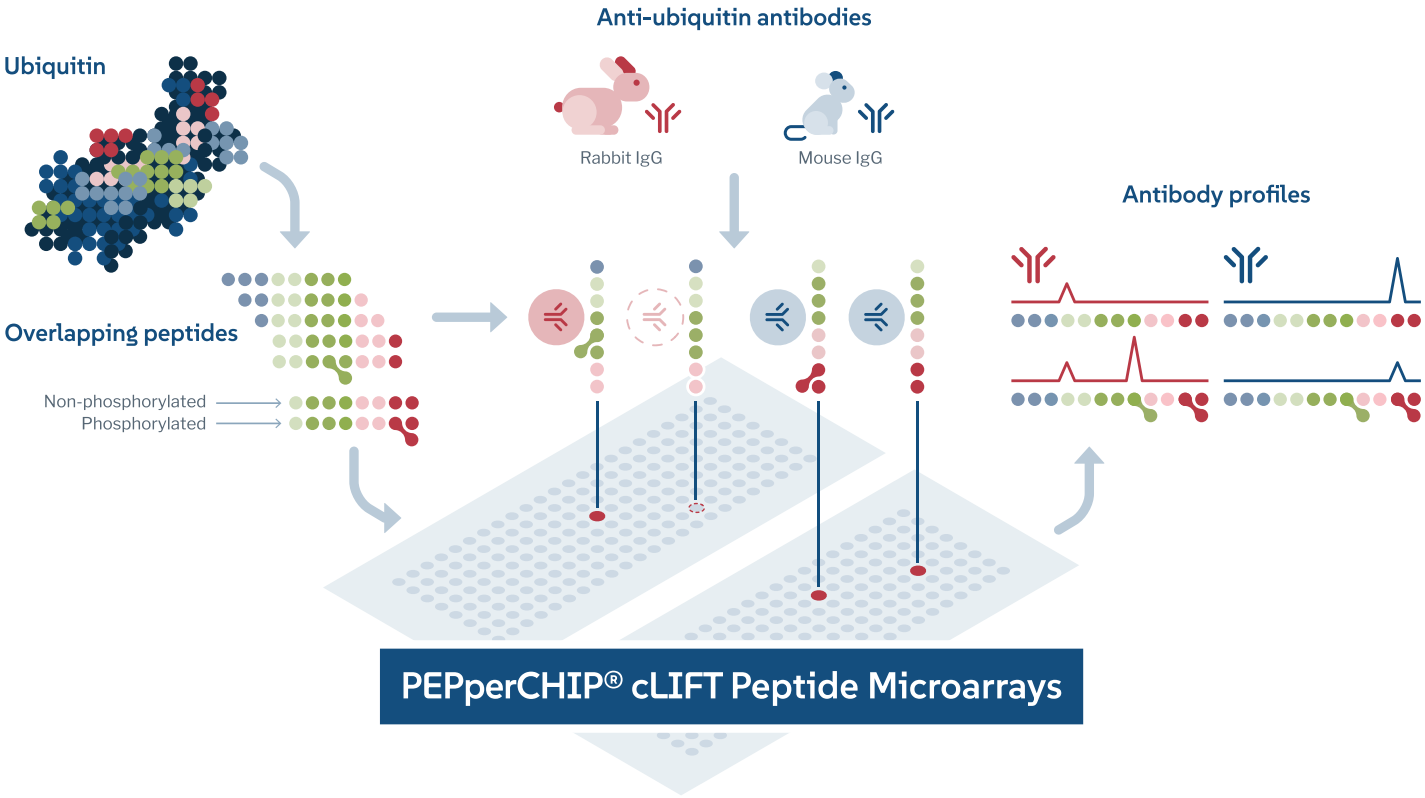
Post-translational modifications (PTMs) are key regulators of protein function, contributing to the dynamic complexity of cellular signaling networks. By altering protein activity, localization, stability, and interaction potential, PTMs such as phosphorylation, glycosylation, ubiquitination, methylation, or acetylation enable cells to respond rapidly to internal and external stimuli. Among these, phosphorylation is particularly well-characterized and plays a central role in regulating

immune responses, including antigen recognition, receptor signaling, and cytokine production (1, 2).

In recent years, the interplay between phosphorylation and ubiquitination has emerged as a critical axis in cellular homeostasis, especially in the context of immunity, inflammation, and neurodegeneration. Ubiquitin, a small 76 amino acid protein, is best known for targeting proteins for degradation via the proteasome. However, ubiquitin itself can be modified, which significantly alters its signaling capacity (3).

Given the expanding biological relevance of phosphorylated epitopes, there is a growing demand for tools that can accurately characterize antibody binding to PTM-specific sequences. Antibodies that differentiate between modified and unmodified peptides are widely used in research, diagnostics, and therapeutic development, but validating their specificity remains challenging due to subtle structural differences introduced by phosphorylation.

To address this, we developed the cLIFT technology, a



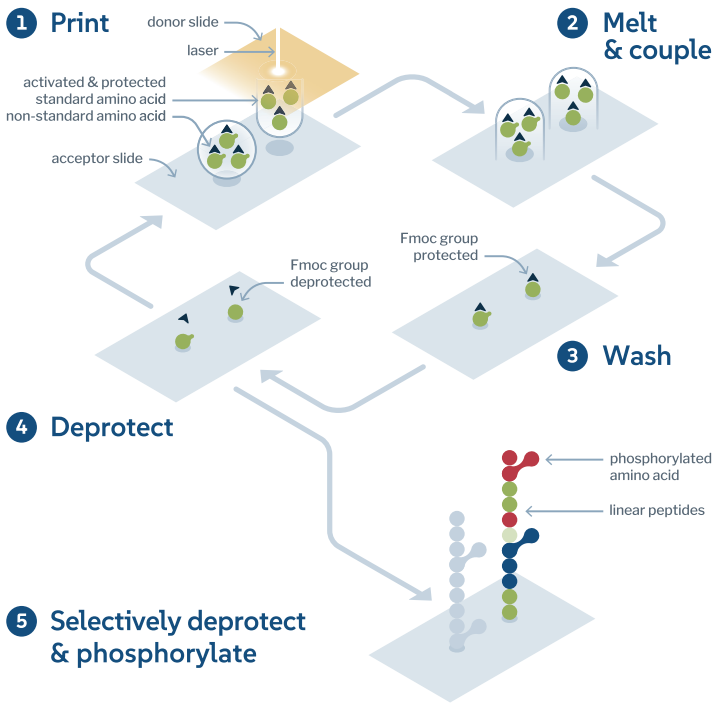


Figure 1. Epitope mapping of monoclonal antibodies against ubiquitin. (Top) The sequence of ubiquitin is converted into overlapping peptides with and without phosphorylated amino acids and printed onto glass slides using the PEPperCHIP® cLIFT Peptide Microarray platform. Anti-ubiquitin antibodies are incubated onto the microarray, and specific binding profiles are identified following a fluorescence-based immunoassay. (Left) Peptide microarray synthesis and site-specific phosphorylation of amino acids is achieved following a multi-step process.

new peptide microarray platform capable of incorporating multiple PTMs, enabling applications such as high-resolution epitope mapping with site-specific phosphorylations (4). In this application note, we demonstrate this capability by the fine epitope mapping of an anti-ubiquitin (phospho S65) antibody [30H3/30K1] from Abcam (cat. # ab309155) and a ubiquitin (P4D1) mouse monoclonal antibody from Cell Signaling Technology (cat. # 23982) using microarrays that included both native and phosphorylated peptides derived from human ubiquitin. Our analysis confirmed

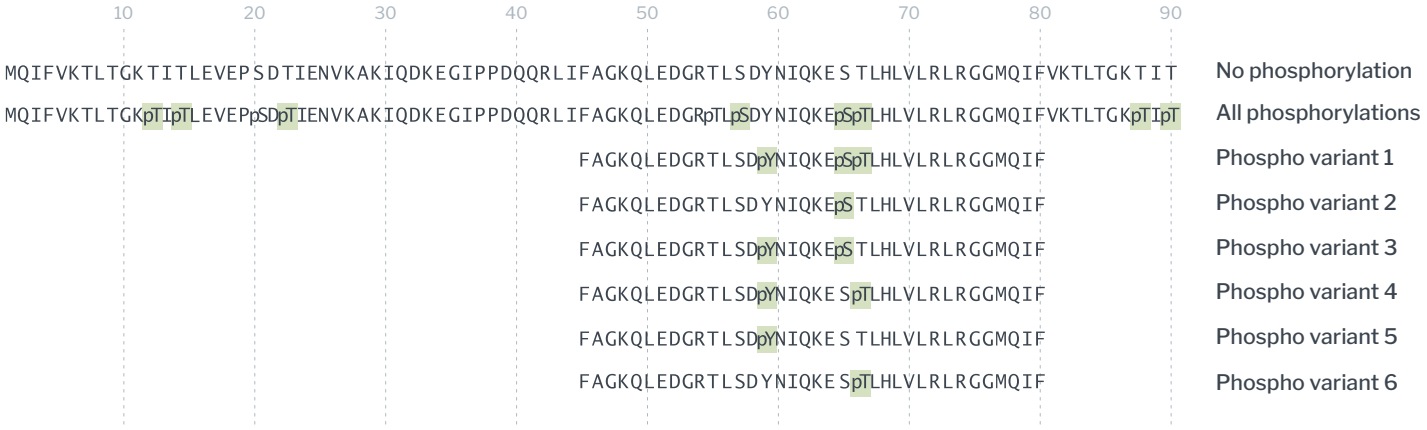


Figure 2. Ubiquitin sequences and phosphorylation sites. The epitope mapping was based on phosphorylated and non-phosphorylated ubiquitin sequences with phosphorylated amino acids coded as pT, pS and pY, highlighted in green on the corresponding peptides.

that site-specific phosphorylation was essential for binding of the phosphospecific Abcam antibody, while the Cell Signaling Technology was not phosphospecific. This validated both the expected antibody specificity and the robustness of the cLIFT platform for PTM-focused antibody profiling.

Results & Discussion

In this study, we analyzed the binding specificity of two different monoclonal anti-ubiquitin antibodies with next-generation PTM-enabled PEPperCHIP® cLIFT Peptide Microarrays (Fig. 1 Top). The sequence of human ubiquitin (UniProt ID: P0CG47) was converted into linear non-phosphorylated and phosphorylated 15 amino acid peptides with a peptide overlap of 14 amino acids for high-resolution epitope data. Site-specific phosphorylation was based on the phosphorylation sites given in the UniProt entry, along with an additional six variants with partial phosphorylation of a small section of ubiquitin (Fig. 2).

Selective phosphorylation of peptides was achieved by adding serine, threonine and tyrosine building blocks with orthogonal side chain protecting groups (5). Upon completion of the combinatorial peptide microarray synthesis, the corresponding building blocks were selectively deprotected and phosphorylated following a 2-step phosphinylation and oxidation reaction (Fig. 1 Left). After cleavage of all other side chain protecting groups, the resulting ubiquitin peptide microarrays contained 396 different peptides printed in triplicates (1,188 spots), and were framed by additional hemagglutinin (HA)-derived control peptides.

The anti-ubiquitin (phospho S65) antibody [30H3/30K1] and the ubiquitin (P4D1) mouse monoclonal antibody were diluted to a final concentration of 1 µg/ml in PBS with 0.05% Tween 20 and 10% Rockland blocking buffer MB-070 and incubated overnight at 4 °C. After washing, staining was done with fluorescently labeled secondary goat anti-rabbit or anti-mouse IgG (Fc) DyLight680 antibodies. Fluorescence readout was performed with an Innopsys InnoScan® 710-IR Microarray Scanner. Quantification of spot intensities was done with PepSlide® Analyzer based on the scanner 16-bit gray-scale images.

The ubiquitin peptide microarrays showed distinct epitope binding patterns for both antibodies (Fig. 3A). The assay with the anti-ubiquitin (phospho S65) antibody [30H3/30K1] resulted in very strong responses against epitope-like spot patterns formed by adjacent fully phosphorylated ubiquitin peptides and three of the phosphorylated variants. The responses were based on peptides with the common motifs ^{57}pSDYNIQKEpS^{65}, ^{57}SDpYNIQKEpS^{65}, ^{57}pSDYNIQKEpS^{65} and ^{57}SDpYNIQKEpS^{65}, sharing a conserved consensus motif ^{60}NIQKEpS^{65} as epitope of the rabbit IgG antibody. Other non-phosphorylated peptides and phosphorylation variants 4 to 6 without phosphorylated ^{65}S did not show any binding at all. As expected from the supplier specifications, this underlined the essential character of phosphorylated ^{65}S for antibody binding, while all other adjacent phosphorylation sites (^{55}T, ^{57}S, ^{59}Y, ^{66}T) exhibited a variable character (Fig. 3B).

In contrast, the ubiquitin (P4D1) mouse monoclonal antibody exhibited a moderate to strong response against native ubiquitin peptides with the non-phosphorylated epitope ^{3}IFVKTLTGKTITL^{15} and a clearly weaker response to the phosphorylated variant ^{3}IFVKTLTGKpTIpTL^{15}, indicating the antibody's preferential binding to the non-phosphorylated epitope (Fig. 3B). Furthermore, an additional weaker interaction was found for peptides with the very similar common

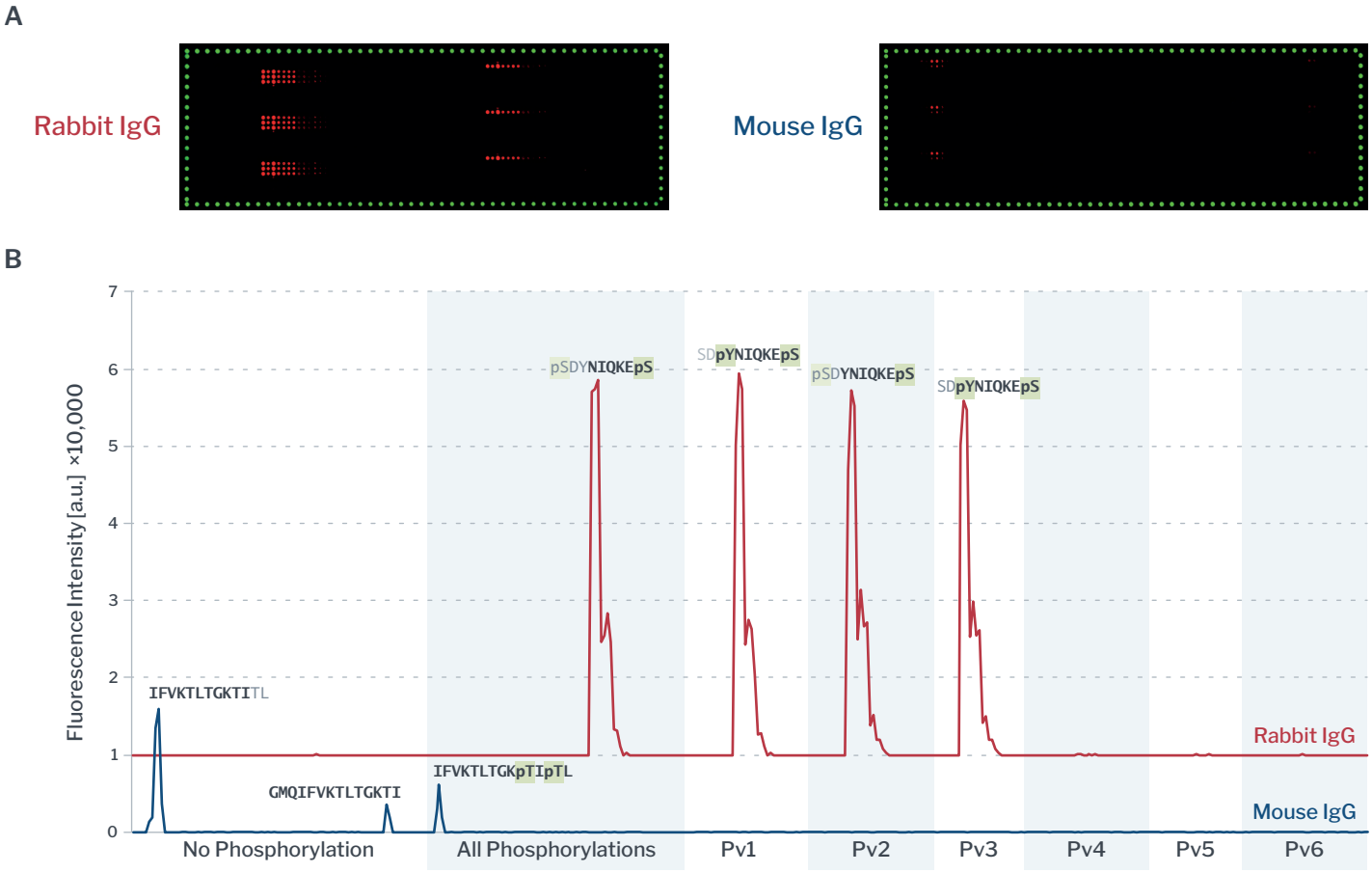


Figure 3. Epitope mapping of anti-ubiquitin antibodies with next-generation PEPperCHIP® cLIFT Peptide Microarrays. Microarray scans (A) show distinct epitope stretches (red spots) which were quantified and plotted from the N- to the C-terminus against the different ubiquitin sequences (B). Binding motifs are annotated next to the corresponding peaks in the intensity plot; baselines are shifted up for data visualization clarity. Pv = Phospho variant.

motif ^{76}GMQIFVKTLTGKTI^{89}. Here, the missing binding of adjacent peptides MQIFVKTLTGKTITG and QIFVKTLTGKTITGS presumably resulted from disadvantageous folding effects induced by the C-terminal glycine.

Conclusion

This study successfully demonstrates the power of next-generation PEPperCHIP® cLIFT Peptide Microarrays for high-resolution antibody profiling against post-translationally modified epitopes. By analyzing both phosphorylated and non-phosphorylated ubiquitin peptides simultaneously in a single assay, we were able to clearly differentiate the binding specificities of two anti-ubiquitin antibodies, and validate the strict phosphospecificity of the anti-ubiquitin (phospho S65) antibody [30H3/30K1] from Abcam to phosphorylated ^{65}S.

These findings not only confirm the distinct binding behaviors of the tested antibodies, but also validate the robustness of our next-generation cLIFT peptide microarrays for PTM-specific epitope mapping with up to 25,000 different peptides per microarray. The ability to synthesize and print defined phosphorylation sites expands the scope of microarray-based antibody characterization, opening new possibilities for the study of post-translational modifications in immunological research, biomarker discovery, and therapeutic antibody development.

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