Enhanced Protein Footprinting with Single Amino Acid Resolution of HOS using the GenNext AutoFox System & Agilent 6560 Ion Mobility

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Structural Biology the Easy Way

Computational biology and artificial intelligence (AI) are revolutionizing therapeutic development by rapidly generating structural and interaction models for biotherapeutics and small molecules. Despite significant investments and advances in AI, empirical validation remains crucial due to frequent predictive failures in dynamic protein conformations, allosteric changes, and intrinsically disordered regions. Conventional validation techniques (NMR, X-ray crystallography, cryo-EM) are costly, slow, and require extensive sample quantities, hindering timely therapeutic advancements.

GenNext's AutoFox Protein Footprinting System overcomes these challenges, providing rapid empirical validation and high-resolution insights into protein structure and interactions at significantly lower costs and sample requirements.

Fully Automated HRPF Labeling

The AutoFox System uses a proprietary flash oxidation lamp to generate hydroxyl radicals (•OH), rapidly modifying solvent-exposed amino acid side chains. These covalent modifications provide a direct, quantitative measure of solvent accessibility and conformational dynamics, offering critical insights into protein higher order structure. With fully automated and reproducible labeling, the AutoFox enables precise spatial mapping of protein folding, surface topology, and interaction interfaces—supporting high-confidence analysis of protein–protein and protein–ligand interactions.

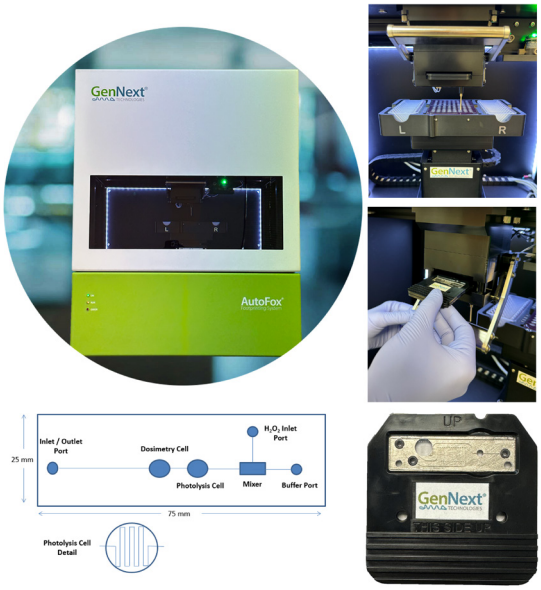


Figure 1: Automated & Reproducible Labeling. *HRPF labeling is performed in an automated fashion using a 96-well plate in the AutoFox System. Sample delivery, reagent mixing, and flashing are performed through a microfluidic chip designed to maximize irradiated volume. Samples are quenched in an adjacent well prior to LC-MS/MS analysis.*

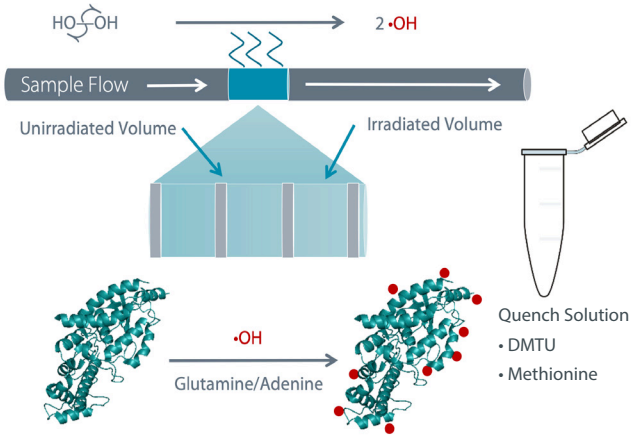


Figure 2: Schematic of the AutoFox Footprinting Method. *With this method, protein is mixed with hydrogen peroxide and flowed passed a flash lamp which photolyzes the hydrogen peroxide into two •OH and modifies solvent exposed amino acids. Following labeling, the sample is deposited into a quench solution of DMTU and methionine.*

Protein Footprinting with the AutoFox System

Precision. Reproducibility. Confidence.

The AutoFox System delivers highly reproducible and accurate hydroxyl radical protein footprinting (HRPF) data—empowering confident decision-making in biopharmaceutical discovery and development.

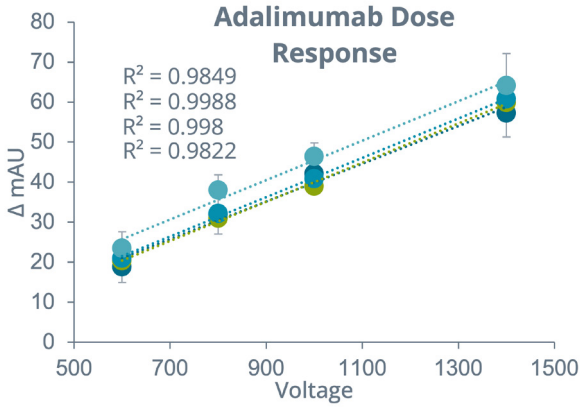


Figure 3: High Reproducibility of Protein Dose Response Curves Using the AutoFox System. *Dose response curves generated by the AutoFox System exhibit strong linear correlation ($R^2 > 0.98$) between hydroxyl radical concentration (Δ mAU) and the voltages applied (V) with excellent relative standard deviations (RSDs) of ~1–11% across three technical replicates. Four independent biological replicates were conducted on different days, using separate chips and operators, demonstrating the system's robust day-to-day reproducibility.*

Epitope Mapping of TNFα-Adalimumab Complex Using Fox® Protein Footprinting

Epitope mapping reveals the specific regions where antibodies bind their antigens, offering critical insight into mechanism of action and supporting intellectual property claims. In this case study, we analyzed the well-characterized interaction between TNFα, a pro-inflammatory cytokine, and adalimumab, a therapeutic monoclonal antibody.

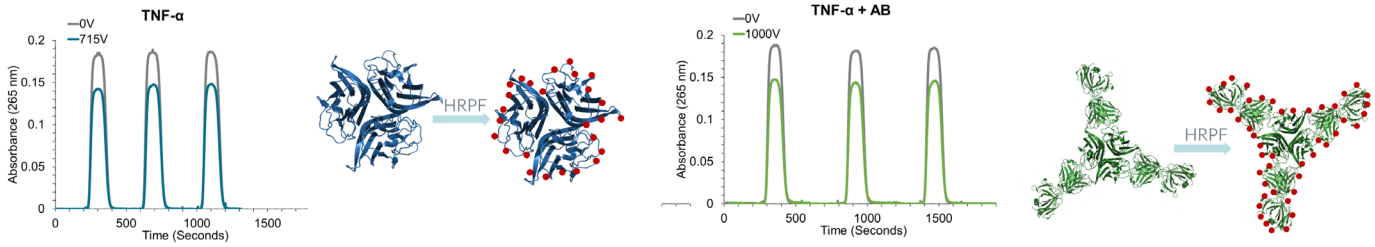


Figure 4: Hydroxyl Radical Dosimetry. *TNFα was first labeled alone, and adenine dosimetry was used to monitor OH radical generation. Gray traces represent adenine absorbance with the photolysis lamp off; upon increasing the lamp voltage to 715 V, a 40 mAU decrease in absorbance confirmed OH radical production. When adalimumab was added, it scavenged a portion of the radicals. To maintain equivalent radical exposure, the lamp voltage was adjusted until the adenine absorbance drop again reached 40 mAU. Immediately after labeling, samples were quenched, digested with trypsin, and analyzed by LC-MS/MS. FoxWare® Software was then used for data processing, enabling identification and quantification of oxidation events.*

Quantitative Mapping of Oxidation to Identify Epitope Regions

The extent of oxidation is calculated by comparing the total chromatographic area of modified peptides to their unmodified counterparts. FoxWare Software automates this quantification, streamlining data processing and ensuring reliable, reproducible results across the protein.

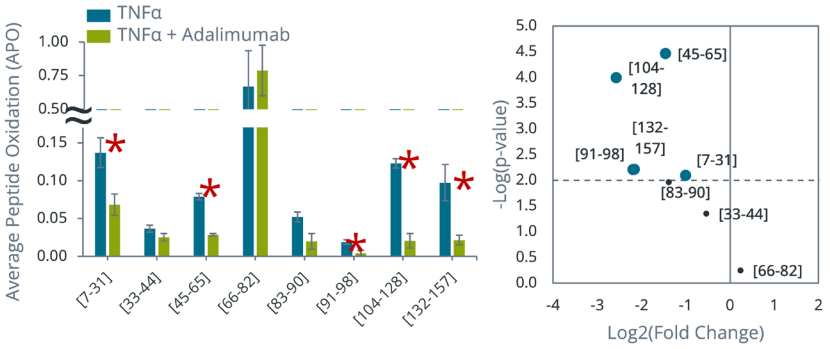


Figure 5: Quantifying and Visualizing Oxidation with FoxWare® Software for Epitope Mapping. *Visualization of oxidation data is shown using both a histogram and a volcano plot. In the histogram, oxidation levels for TNFα alone are shown in blue, and TNFα in complex with adalimumab is shown in green. Red asterisks denote peptides with statistically significant changes in oxidation. These differences are further analyzed in the volcano plot, which displays the log_2 fold change in oxidation versus the $-log_{10}$ p-value. The horizontal dotted line indicates a p-value threshold of 0.01, above which data points are considered statistically significant.*

Due to the size of some peptides, resolution may be limited at the peptide level. To address this, FoxWare Software provides an automated feature that generates targeted peptide lists with suggested retention times based on the unmodified peptide, enabling refined analysis. In this example, only peptides with significant oxidation changes were selected to highlight the most informative regions.

High-Confidence Site Localization and Quantification of Oxidation with Targeted MS and FoxWare Software

Targeted MS acquisition significantly increases peptide-spectrum match (PSM) density across the full retention time of modified peptides, improving confidence in amino acid-level localization. Modified and unmodified species are detected using in-house peak-picking algorithms and a specialized site-localizing PSM search.

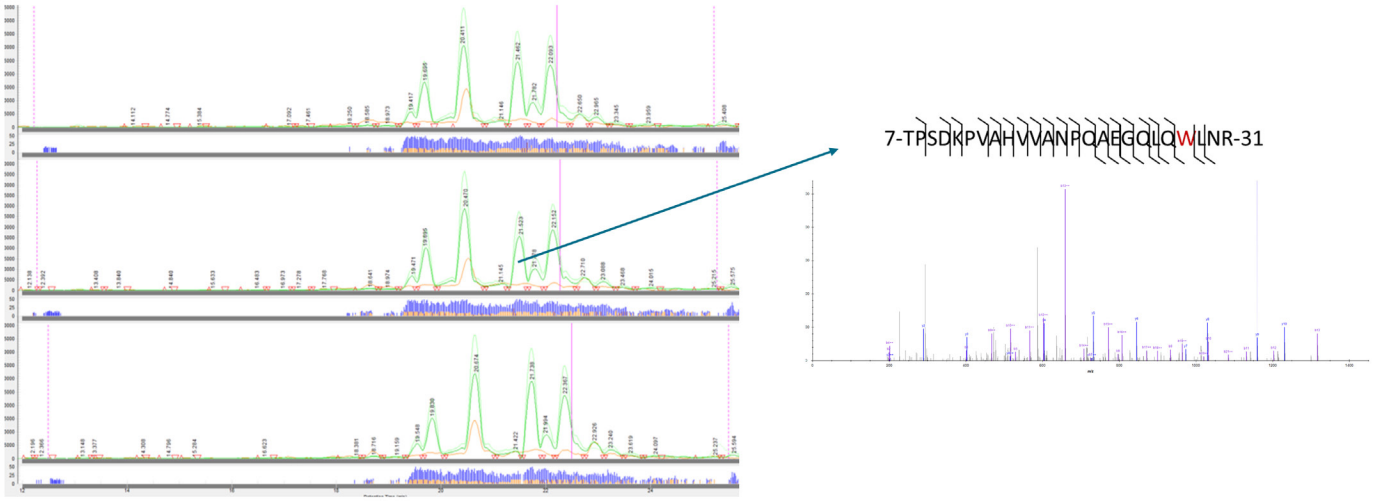
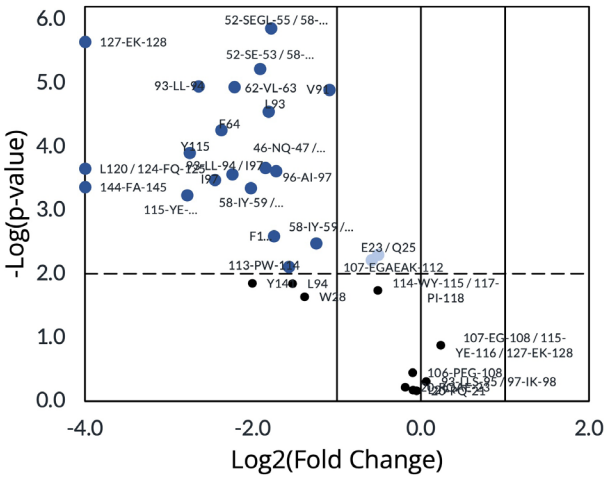
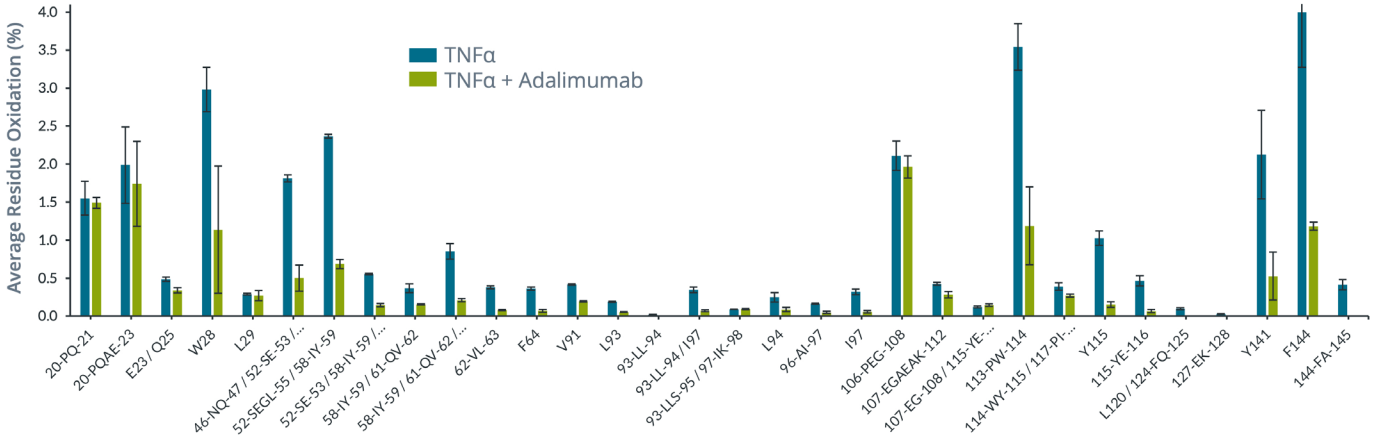


Figure 6: Automated Data Analysis — Localizing Modifications with MS/MS. *Data from multiple replicates are aligned in FoxWare Software using relative native-peptide retention times, shown as magenta lines in the extracted ion chromatograms (XICs). Representative MS/MS spectra (right) demonstrate how unique fragment ions enable precise localization of oxidative modifications. Each PSM is assigned an ambiguity score, indicating the confidence of site assignment. This integrated approach—combining targeted MS, robust retention time alignment, and high-confidence localization—enables accurate quantification of oxidation at the residue level and provides detailed insight into protein structural changes and interactions.*

Residue-Level Mapping of Oxidation for Structural Insight

Residue-level oxidation data are visualized using a histogram and volcano plot. The histogram shows the distribution of oxidative modifications across individual amino acids, while the volcano plot highlights residues with statistically significant changes in oxidation between conditions. This high-resolution analysis enables precise localization of structural changes, allowing researchers to draw clear, actionable conclusions. Together, these visualizations demonstrate the power of the AutoFox System for detailed protein structural analysis at the single-residue level.

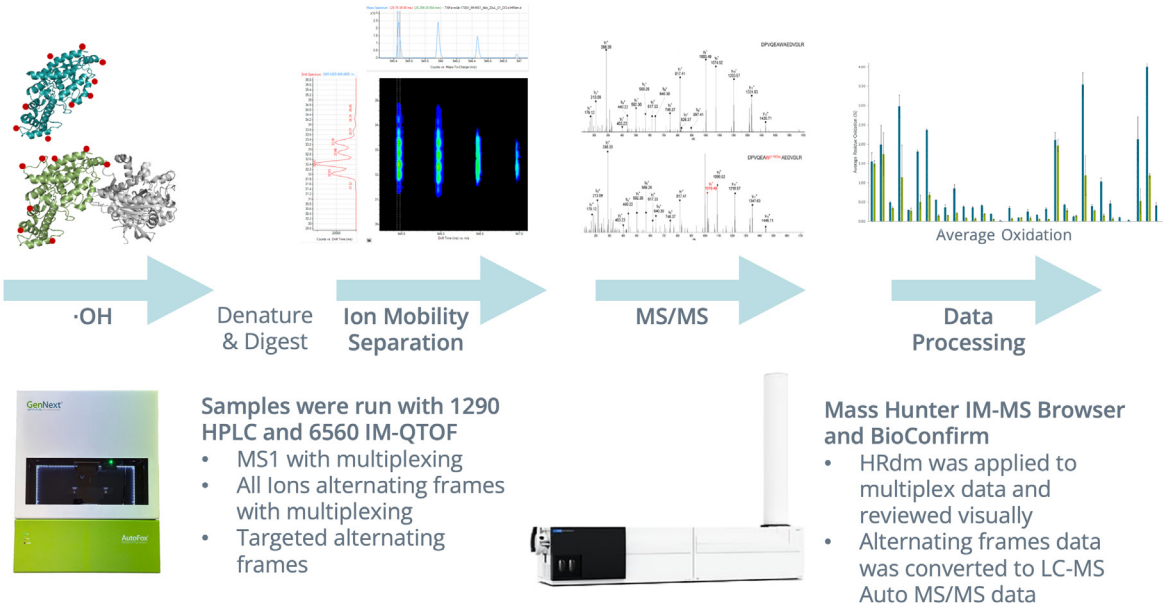




Enhancing HRPF Resolution with Ion Mobility Separation (IMS)

In Hydroxyl Radical Protein Footprinting (HRPF), co-eluting isobaric peptides can lead to ambiguous peak assignments, with oxidation attributed to multiple residues or peptide fragments. This challenge limits confidence in site-specific localization. To address this, ion mobility separation (IMS) was integrated into the workflow. IMS separates isobaric species based on their shape and charge, significantly improving resolution and enabling more accurate assignment of oxidation events to individual residues. Following labeling and digestion, samples were analyzed using Agilent's 1290 HPLC system coupled to the 6560 IM-QTOF. Three acquisition modes were used: MS1 with multiplexing, all-ions alternating frames with multiplexing, and targeted alternating frames.

Multiplexed data were processed using High-Resolution Demultiplexing (HRdm) and visually inspected for accuracy. Alternating frames data were converted to LC-MS Auto MS/MS format for further analysis in BioConfirm. This evolving workflow—developed through close collaboration between GenNext and Agilent—aims to deliver robust, high-resolution, and reproducible HRPF data, even in complex experimental settings.



HRdm Enhances Ion Mobility Resolution of Modified Peptides

Comparison of the collision cross sections (CCS) reveals peak broadening in some modified peptides relative to their unmodified counterparts. Upon applying HRdm, these broadened peaks are resolved into distinct features, revealing additional CCS values not observed in the unmodified form. This indicates the presence of co-eluting isobaric modified peptides. Importantly, these resolution improvements extend beyond the monoisotopic peak and are consistently observed across the full isotope envelope, providing increased confidence in the identification of multiple conformers in the modified peptide. These results highlight the power of HRdm to resolve complex, overlapping features in peptide ion mobility data.

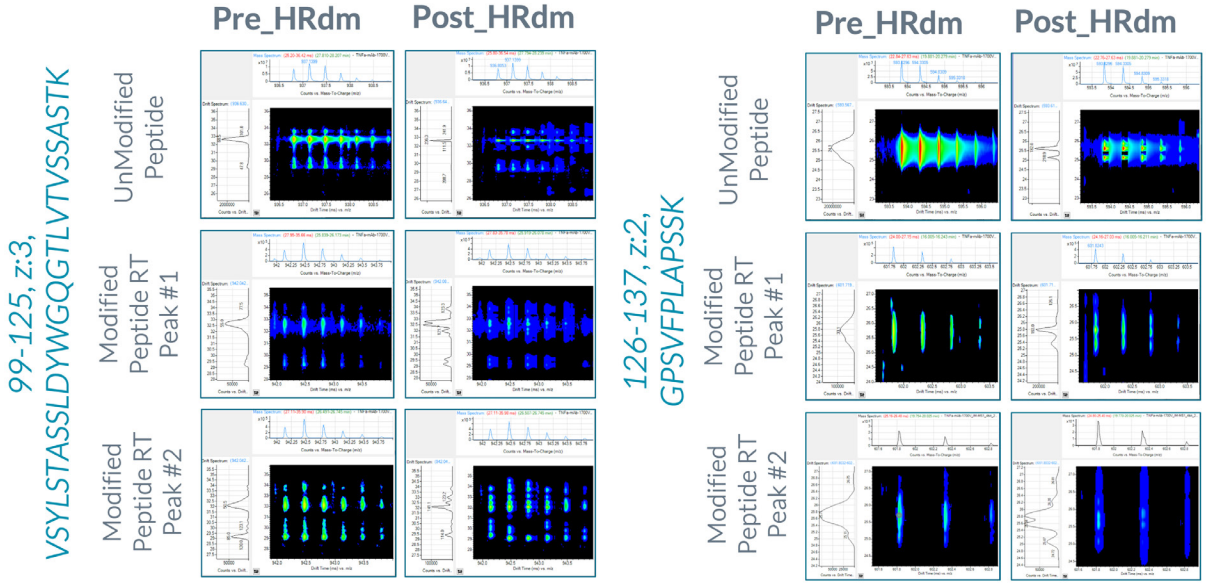


Figure 7: Representative Mobilograms for Selected Targeted Peptides *For each peptide, the unmodified species and two retention time peaks corresponding to modified forms are represented. For each, pre- and post-HRdm data are displayed to demonstrate the enhanced resolution achieved through HRdm processing.*

Ion Mobility Separates Isobaric Peptides

Preliminary fragmentation data suggesting that the modified peptide conformers correspond to multiple oxidation sites. Although the fragmentation coverage is incomplete, we can distinguish the modification localization between two peptide collision cross sections.

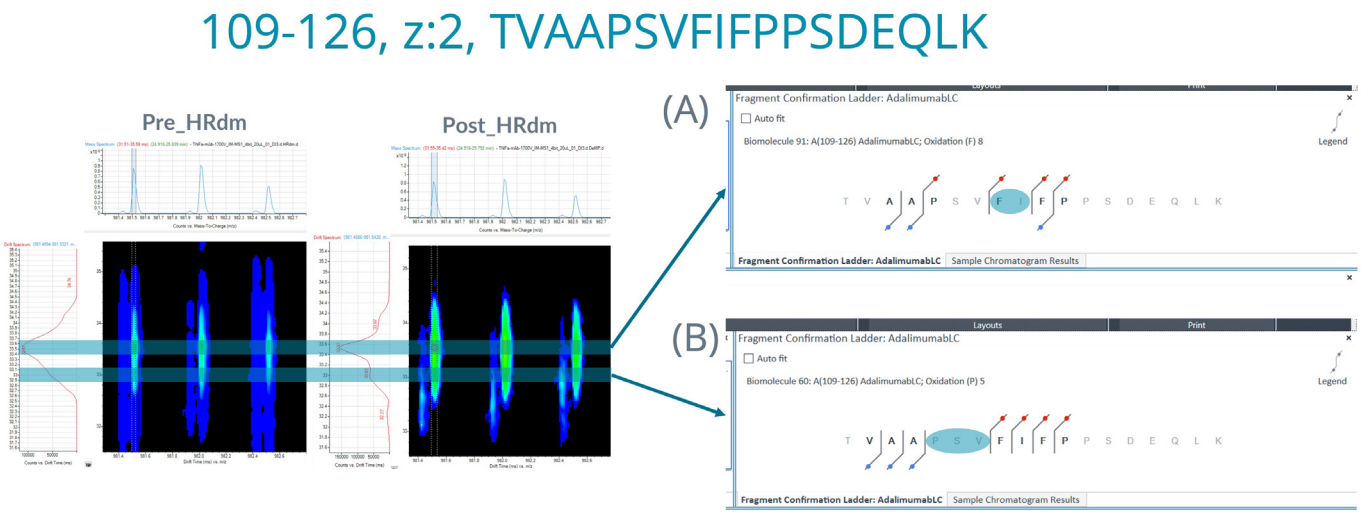


Figure 8: Preliminary Fragmentation Data. *(A) The top highlighted peak shows an MS/MS fragmentation pattern that localizes the oxidation to either phenylalanine or isoleucine. (B) While The bottom peak, has a fragmentation pattern indicating the oxidation is on proline, serine, or valine. While these findings are promising, we still need to dive deeper into the data and further optimize the acquisition process to enhance the identification of oxidized peptides.*

Conclusions

- The AutoFox System provides fully automated, reproducible Hydroxyl Radical Protein Footprinting (HRPF) for precise structural analysis.
- The workflow demonstrates high reproducibility across days, operators, and chips, supporting robust and scalable analysis.
- FoxWare® Software automates data processing, aligning replicates and quantifying oxidation at both the peptide and residue level with high confidence.
- The TNFα–adalimumab case study validated the system's ability to detect known epitope regions and map conformational changes at single-residue resolution.
- Integration with Agilent's 6560 Ion Mobility QTOF enhances resolution, enabling separation of isobaric species and improved site-specific localization.
- Use of HRdm (High-Resolution Demultiplexing) significantly improves detection of co-eluting conformers and enhances confidence across isotope envelopes.