POSTER NOTE

High-Throughput Epitope Mapping of PCSK9-Evolocumab Biosimilar Interactions Using the AutoFox® System with Agilent's 6545XT Q-TOF

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KEY TAKEAWAYS

Automated Reproducibility | **Solution-State Epitope** | **Long-Range Allosteric Effects** | **Cross-Platform Transferability** | **Empirical Structural Validation**

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.

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.*

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.

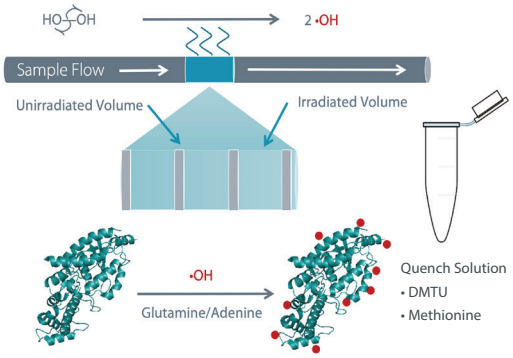


Figure 2: Schematic of the AutoFox Footprinting Method. *With this method, protein is mixed with hydrogen peroxide and flowed passed a flash lamp which photolyzes the hydrogen peroxide into two •OH and modifies solvent exposed amino acids. Following labeling, the sample is deposited into a quench solution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

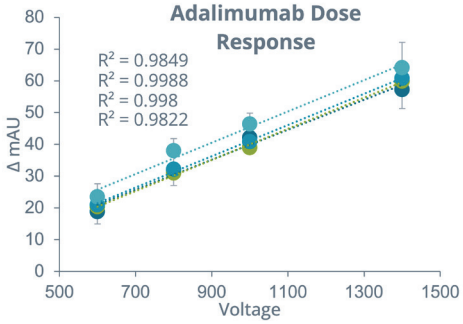


Figure 3: High Reproducibility of Protein Dose Response Curves Using the AutoFox System. *Dose response curves generated by the AutoFox System exhibit strong linear correlation (R^2 > 0.98) between hydroxyl radical concentration (Δ mAU) and the voltages applied (V) with excellent relative standard deviations (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 PCSK9 Against an Evolocumab Biosimilar

PCSK9 regulates LDL receptor (LDLR) recycling and plays a central role in cholesterol homeostasis. Unbalanced PCSK9 activity reduces LDL clearance from circulation and is associated with elevated cardiovascular disease risk. As a result, PCSK9 is a major therapeutic target for monoclonal antibody inhibitors such as Evolocumab. Structural characterization of PCSK9 binding interactions is important for understanding therapeutic binding mechanisms and for evaluating whether biosimilar candidates maintain comparable target engagement and epitope recognition relative to the reference therapeutic.

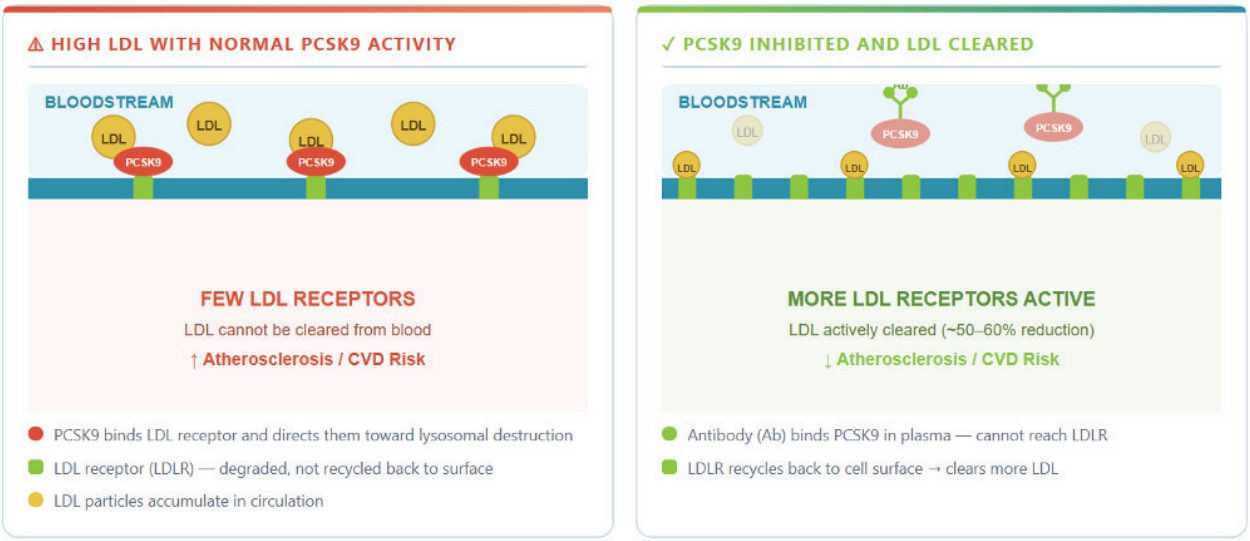


Figure 4: Role of PCSK9 in LDL Receptor Regulation. *PCSK9 promotes LDL receptor (LDLR) degradation, reducing receptor recycling and limiting LDL clearance from circulation. Therapeutic inhibition of PCSK9 prevents LDLR degradation, increasing receptor availability at the cell surface and enhancing LDL clearance*

HRPF Workflow for PCSK9 Epitope Mapping

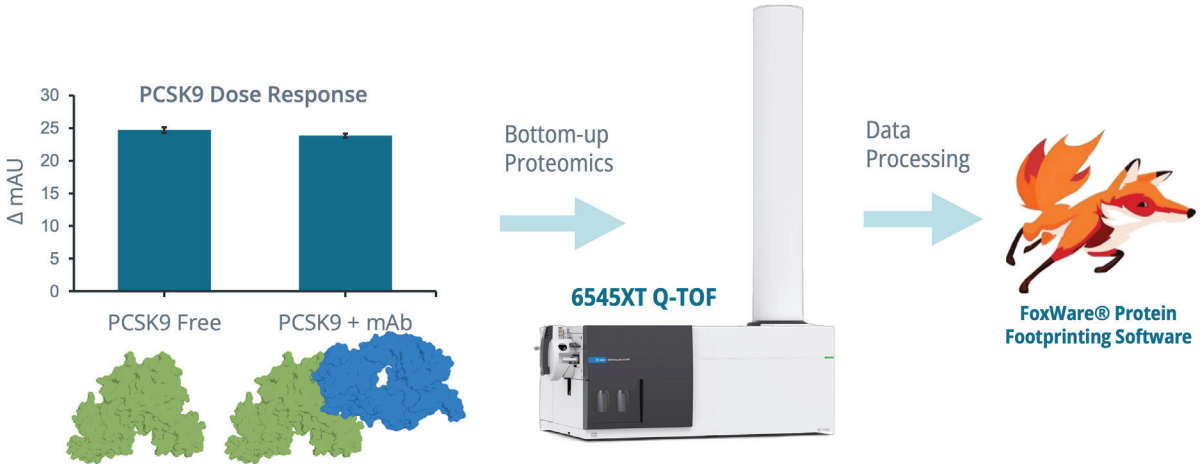


Figure 5: HRPF Workflow. *HRPF was used to characterize solvent accessibility changes in PCSK9 upon binding to an evolocumab biosimilar. Apo PCSK9 and PCSK9–mAb complex samples were labeled using the AutoFox system with real-time dosimetry to ensure equivalent effective radical exposure between conditions. Lamp voltage was automatically adjusted to achieve matched radical dose prior to downstream comparison. Following labeling, samples were digested using trypsin and analyzed by LC-MS using an Agilent 6545XT Q-TOF coupled to an Evosep LC system. A secondary LC-MS platform was also evaluated for cross-platform comparison. Peptide identification and oxidation quantitation were performed using FoxWare® Protein Footprinting Software to determine peptide-level oxidation differences associated with antibody binding.*

Extracted Ion Chromatogram Examples of Modified Peptides

HRPF oxidation products were detected and quantified at the peptide level using extracted ion chromatograms (XICs). Unmodified peptides eluted as a single dominant chromatographic peak, while oxidized peptides exhibited retention time shifts resulting from changes in peptide hydrophobicity following oxidation. Multiple oxidized isomers were observed for individual peptides due to oxidation occurring at different amino acid residues, each producing slightly distinct chromatographic behavior. MS/MS fragmentation can enable assignment of oxidation to specific residues for downstream residue-level analysis (not shown here).

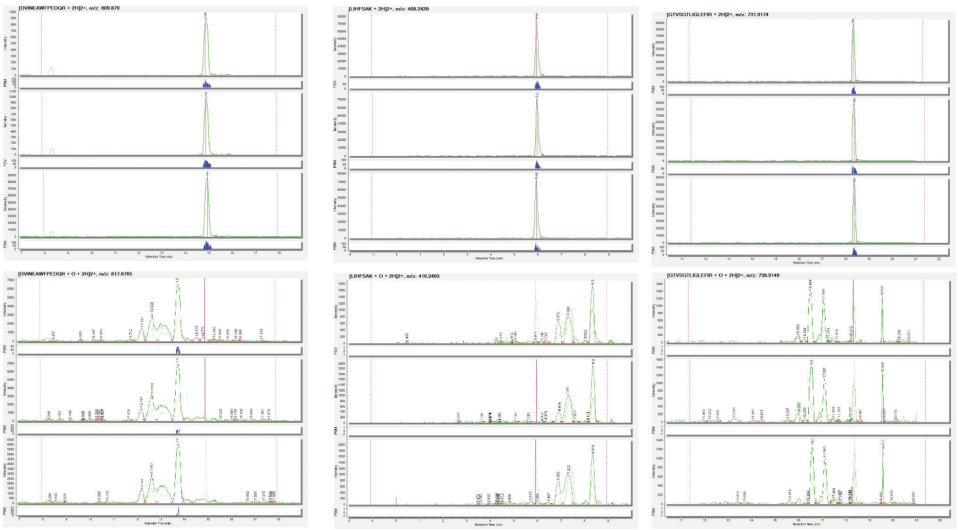


Figure 5: XICs of HRPF Oxidation Products. *Representative XICs showing unmodified (top) and oxidized (bottom) PCSK9 peptides following HRPF labeling. Oxidized peptides exhibit retention time shifts and multiple chromatographic peaks corresponding to site-specific oxidation isomers. Representative peptides shown include residues 422–434 (left), 415–421 (middle), and 259–272 (right).*

Peptide-Level HRPF Oxidation Analysis (Histograms)

Peptide-level oxidation data are visualized as histograms showing the average oxidation measured for individual peptides across replicate HRPF experiments. Comparison of oxidation levels between apo PCSK9 and the antibody-bound complex identifies peptides exhibiting protection upon mAb binding, consistent with reduced solvent accessibility at or near the binding interface. Comparable oxidation trends were observed between the Agilent 6545XT and the secondary LC-MS platform, demonstrating strong cross-platform agreement.

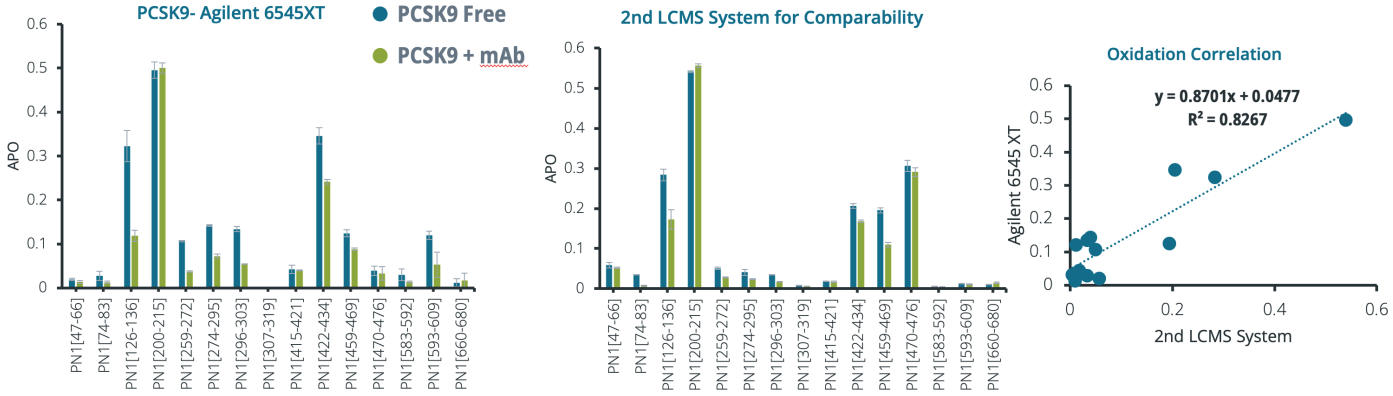


Figure 6: Peptide-Level HRPF Oxidation Comparison. *Average peptide oxidation levels for apo PCSK9 and PCSK9 bound to an evolocumab biosimilar were quantified across three replicate HRPF experiments. Error bars represent standard deviation between replicates. Peptides exhibiting reduced oxidation in the antibody-bound condition indicate decreased solvent accessibility associated with mAb binding. Results obtained using the Agilent 6545XT platform (left) showed similar oxidation trends to those generated using a secondary LC-MS system (right). Correlation analysis between platforms demonstrated strong agreement in peptide-level oxidation measurements, with each point representing an individual peptide.*

Statistical Identification of Protected PCSK9 Peptides

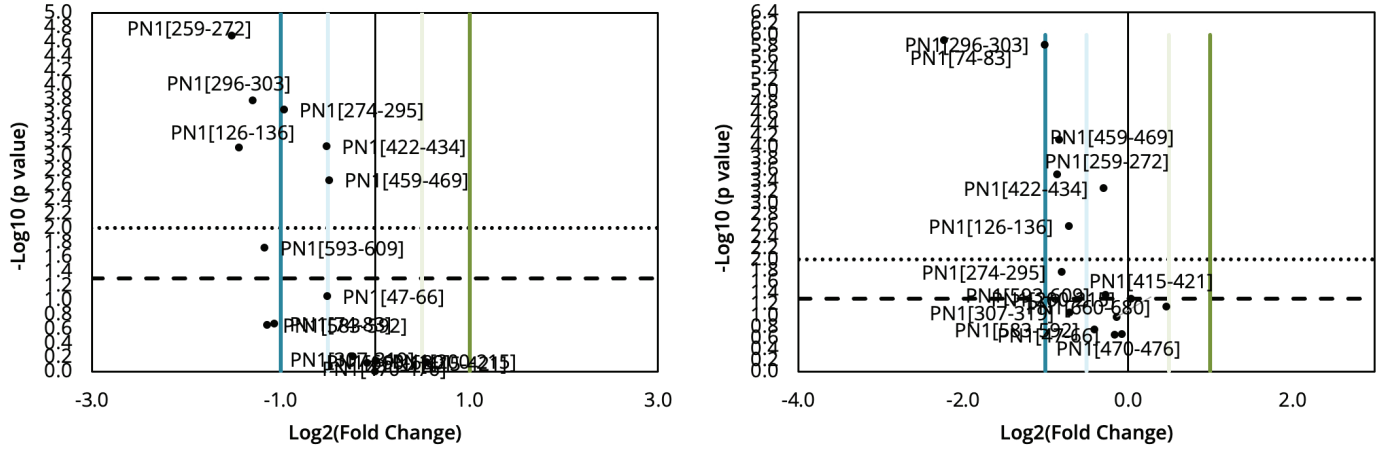


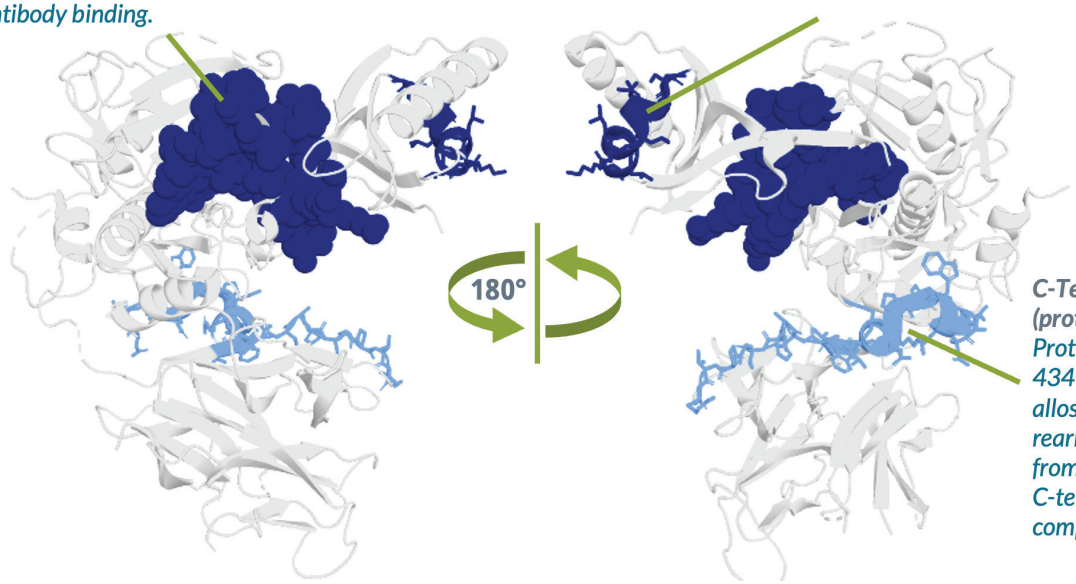
Figure 7: Volcano Plot Analysis of Peptide-Level Oxidation Changes. *Volcano plots were used to visualize peptide-level oxidation differences between apo PCSK9 and PCSK9 bound to an evolocumab biosimilar. Each point represents an individual peptide, with fold-change in oxidation plotted against statistical significance across replicate measurements. Peptides exhibiting decreased oxidation in the antibody-bound condition indicate reduced solvent accessibility consistent with protection upon mAb binding. Visualization of the data in volcano plot format enables rapid identification of statistically significant oxidation changes while simultaneously evaluating the magnitude of protection, improving confidence in identifying peptides associated with the antibody binding interface. Comparison between the Agilent 6545XT and secondary LC-MS platform demonstrated strong agreement, with all but one peptide showing consistent significance trends between systems.*

HRPF Reveals the Epitope and Allosteric Structural Response Upon Evolocumab Biosimilar Binding to PCSK9

Peptide-level oxidation changes mapped onto the PCSK9 crystal structure (PDB: 2QTW) identify a protected epitope consistent with the known Evolocumab binding interface. Additional protection in the prodomain suggests localized stabilization transmitted from the primary binding interface. Distal protection further supports antibody-induced structural rearrangements extending beyond the direct epitope, consistent with long-range allosteric effects across PCSK9.

Primary epitope (protected)
Peptides 259–272, 274–295, and 296–303 exhibit the strongest decreases in oxidation, corresponding to the known Evolocumab binding interface on the catalytic domain and reflecting reduced solvent accessibility upon antibody binding.

Prodomain (protected)
Protection at peptide 126–136 suggests steric occlusion or conformational stabilization of the prodomain transmitted from the primary binding interface.



C-Terminal Domain (protected)
Protection at peptides 422–434 and 459–469 reflects allosteric conformational rearrangement propagating from the epitope to the C-terminal domain upon complex formation.

Conclusions

- The AutoFox System delivers fully automated, reproducible HRPF with $R^2 > 0.98$ dose-response linearity and RSDs of ~1–11% across independent replicates, operators, and days, demonstrating the robustness required for biopharmaceutical structural characterization workflows.
- HRPF confirmed that the evolocumab biosimilar engages PCSK9 at the expected binding interface in solution, providing direct structural evidence of target engagement without requiring crystallization or large sample quantities.
- Protection extending into the prodomain and C-terminal domain revealed antibody-induced conformational changes propagating beyond the primary epitope, demonstrating the ability of HRPF to detect long-range allosteric structural responses.
- Consistent oxidation trends across two independent LC-MS platforms demonstrated that the observed HRPF measurements reflect true structural changes rather than instrument-specific artifacts, supporting the transferability and comparability of the workflow.
- The dynamic conformational and allosteric effects identified by HRPF represent precisely the class of structural behavior that remains difficult to accurately capture using computational prediction alone, highlighting the importance of empirical structural validation for therapeutic development and biosimilar characterization.

The AutoFox System and the 6545XT Q-TOF deliver automated, reproducible HRPF that not only confirms where a biosimilar antibody binds its target in solution — without crystallization or large sample quantities — but also reveals long-range allosteric structural effects that computational prediction alone cannot reliably capture, making empirical structural validation indispensable for biosimilar development.