POSTER NOTE

Integrated HRPF Workflows Combining Flash Oxidation with End-to-End Data Processing for Structural MS

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KEY TAKEAWAYS

Scalable Data Processing | **Isomer-Resolved Quantification** | **Enterprise-Scale Reproducibility** | **Residue-Level Precision** | **Turnkey Structural Insight**

Structural Biology the Easy Way

Hydroxyl Radical Protein Footprinting (HRPF) is a mass spectrometry-based technique that probes protein higher-order structure by measuring changes in solvent accessibility at the residue level. Hydroxyl radicals rapidly and covalently modify solvent-exposed amino acid side chains, enabling quantitative assessment of protein conformation, dynamics, and interaction interfaces under native-like conditions.

The AutoFox® Protein Footprinting System enables automated and reproducible HRPF through controlled flash oxidation and real-time radical dosimetry. By monitoring and adjusting effective •OH exposure across samples, AutoFox ensures consistent labeling conditions, improving reproducibility across replicates and experimental states. This level of control supports high-confidence structural comparisons in complex protein systems.

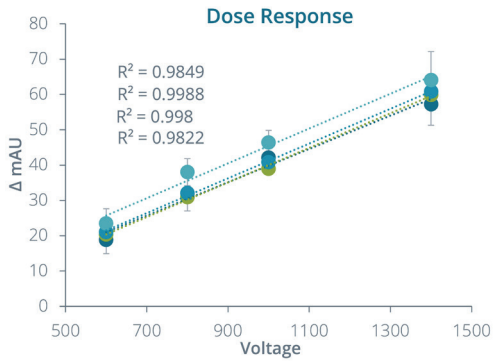


Figure 1: High Reproducibility of Protein Dose Response Curves Using the AutoFox System. *Dose response curves generated by the AutoFox System exhibit strong linear correlation ($R^2 > 0.98$) between hydroxyl radical concentration (Δ mAU) and the voltages applied (V) with excellent relative standard 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.*

Genedata Expressionist for Automated FPOP Workflows

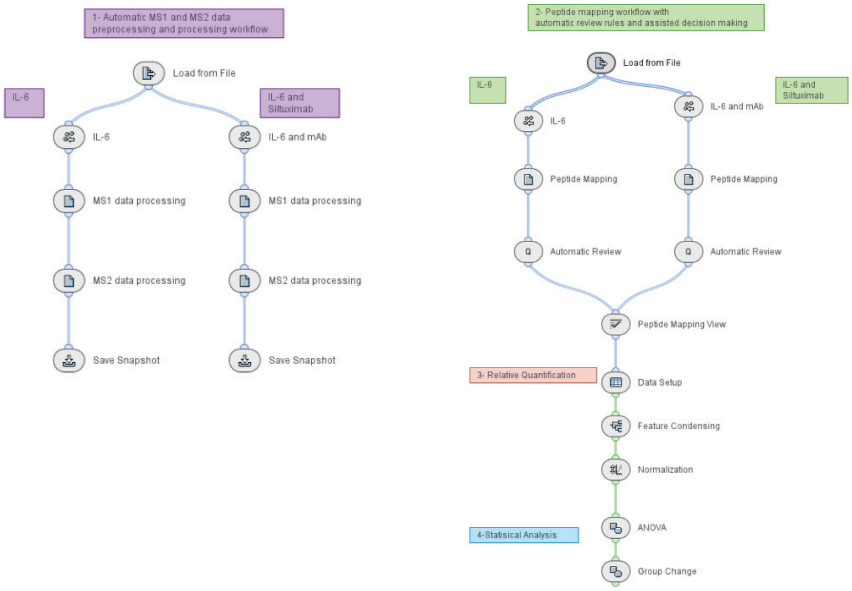
Genedata Expressionist® is an enterprise-scale platform that automates, standardizes, and scales mass spectrometry data analysis. For FPOP-based HRPF experiments, Genedata Expressionist enables fully automated end-to-end workflows covering preprocessing and alignment, streamlined quantification of oxidation events, statistical evaluation, and reporting.

Its workflow-driven architecture ensures reproducibility and consistency across large datasets while minimizing manual intervention. By integrating multiple processing steps into a single, configurable pipeline, Genedata Expressionist ensures robust and high-throughput interpretation of FPOP data, enabling reliable epitope mapping and comparative structural analysis in biosimilar and biologics development.

Figure 2: End-to-end HRPF Data processing with Genedata Expressionist.

(A) Automated data preprocessing and processing workflow.
(B) Oxidation levels at both peptide and residue levels followed by statistical analysis.

- ✓ *Vendor agnostic*
- ✓ *Multiple oxidations*
- ✓ *Alternative labeling agents*
- ✓ *Carbamidomethylation*
- ✓ *Customizable PTMs (TMT, ...)*



Optimized Data Processing with Genedata Expressionist

To address analytical challenges in HRPF, an integrated workflow combining the AutoFox® Protein Footprinting System with Genedata Expressionist was developed to enable rapid, reproducible, and scalable data analysis.

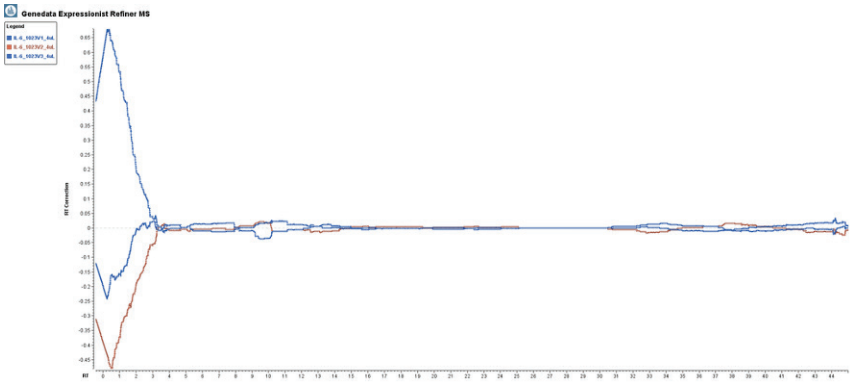


Figure 3: RT variability across runs. *The original RT variation across replicates is shown on the graph below as a function of time. This information is used to better understand chromatography variability and to align retention times for consistent peak detection and quantification.*

Figure 4: Multiple and custom modifications can be searched. *A combination of 44 possible HRPF modifications was included in the search space.*

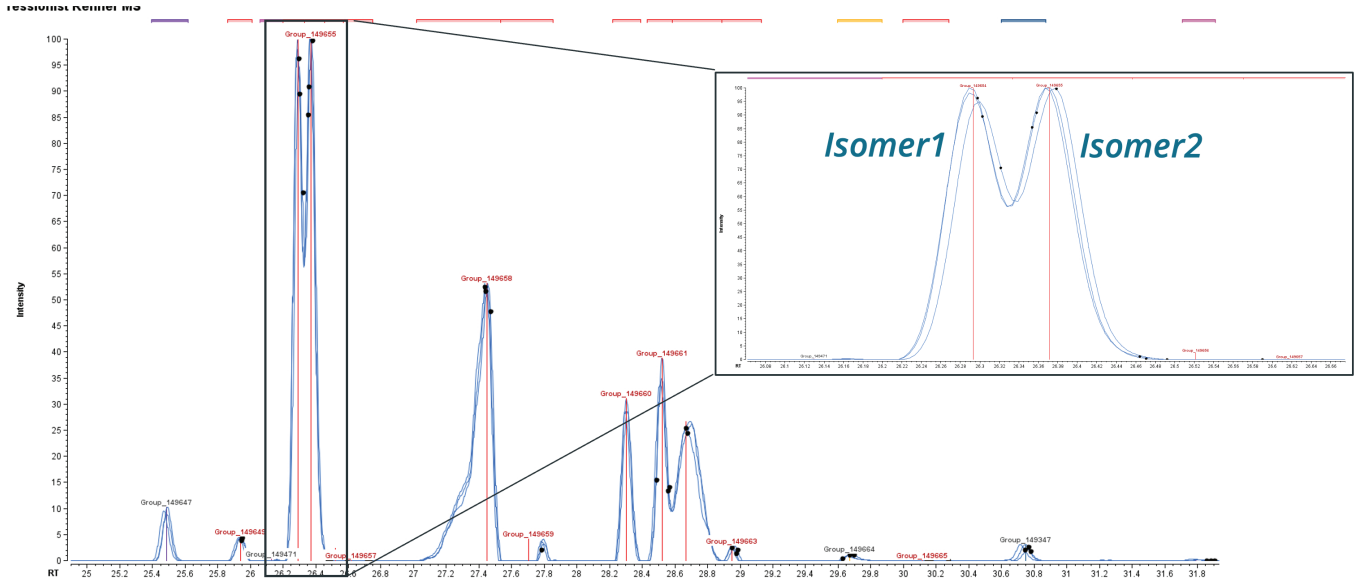
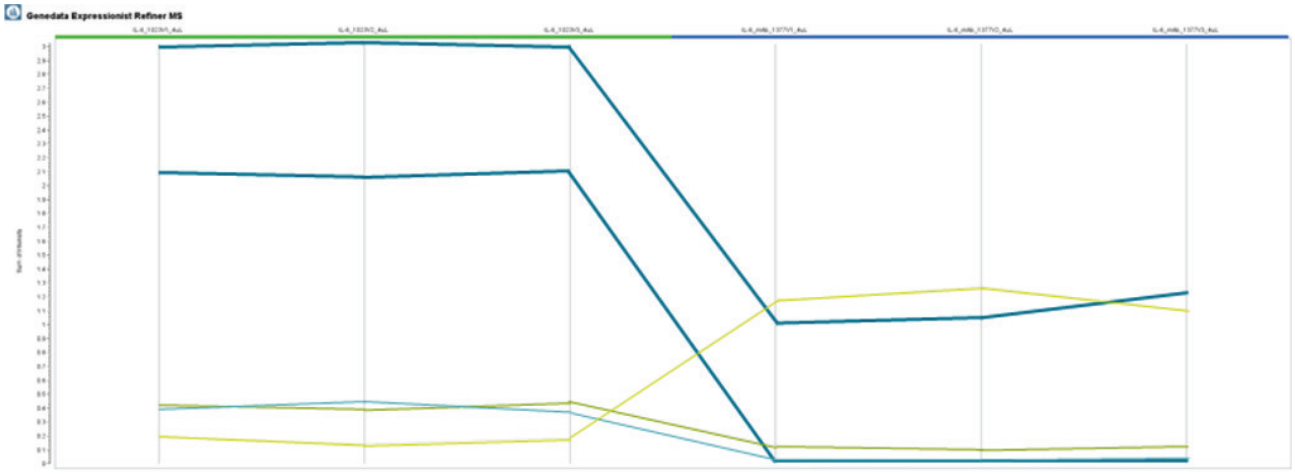


Figure 5: Feature Detection on Isomeric Peptides. *Oxidized peptide 179-196 is automatically identified with multiple isomeric forms. Note peaks labeled Isomer1 and Isomer2 are not fully chromatographically resolved and still detected, quantified and annotated consistently through groups and replicates.*



Name	IL-6_1023V1_4uL	IL-6_1023V2_4uL	IL-6_1023V3_4uL	IL-6_mAb_1377V1_4uL	IL-6_mAb_1377V2_4uL	IL-6_mAb_1377V3_4uL
149-156	96.61	96.53	96.65	98.97	98.92	98.75
149-156 | Oxidation [F153]	2.996	3.022	2.989	1.002	1.051	1.21
149-156 | Oxidation [L154]	0.3894	0.4497	0.3648	0.03225	0.03039	0.04225
149-157	97.3	97.42	97.27	98.7	98.6	98.77
149-157 | Oxidation [F153]	2.1	2.05	2.104	0.009872	0.02399	0.0009648
149-157 | Oxidation [K156]	0.4121	0.3897	0.4423	0.1263	0.1007	0.1308
149-157 | Oxidation [V149]	0.1899	0.1396	0.1827	1.166	1.273	1.096

Figure 6: Oxidation Changes Across Conditions. *Automated grouping and quantification of oxidized peptide features enable residue-level comparison of oxidation across conditions. Changes in oxidation at individual residues are normalized, statistically evaluated, and visualized directly, facilitating rapid identification of condition-dependent structural differences.*

Application Epitope Mapping of IL-6–Siltuximab Interaction

To demonstrate the utility of the integrated HRPF workflow, the interaction between interleukin-6 IL-6 and a Siltuximab biosimilar was analyzed as a model system for epitope mapping. IL-6 is a clinically relevant cytokine targeted by therapeutic antibodies, providing a well-characterized system for evaluating changes in solvent accessibility upon binding. IL-6 was analyzed in both its free (apo) form and in complex with the antibody, with replicate measurements acquired for each condition. This experimental design enables direct comparison of oxidation patterns to identify regions of protection and exposure associated with complex formation.

IL-6 Epitope Mapping Dose Response Plots

Real-time radical dosimetry enables normalization of hydroxyl radical exposure across samples. By matching effective radical dose between conditions, oxidation differences can be directly compared and attributed to structural changes rather than labeling variability.

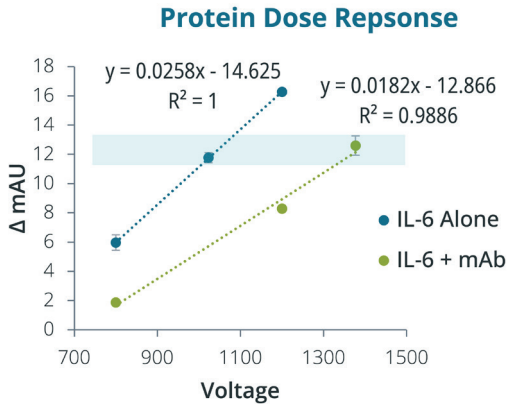


Figure 7: Consistent Radical Dose Across Conditions. *Dose response plots show a linear relationship between lamp voltage and effective radical concentration (ΔmAU; $R^2 > 0.98$). Equivalent radical dosing was achieved at ~1000 V for IL-6 alone and ~1350 V for IL-6 + mAb. These matched conditions were used for all downstream analyses.*

MS/MS Fragmentation to Localize Oxidation

MS/MS fragmentation enables localization of oxidative modifications to specific amino acid residues within each peptide. Fragment ion patterns distinguish between possible oxidation sites, allowing resolution of site-specific modifications even in the presence of multiple oxidation states. This approach increases confidence in residue-level assignment and supports accurate structural interpretation of HRPF data.

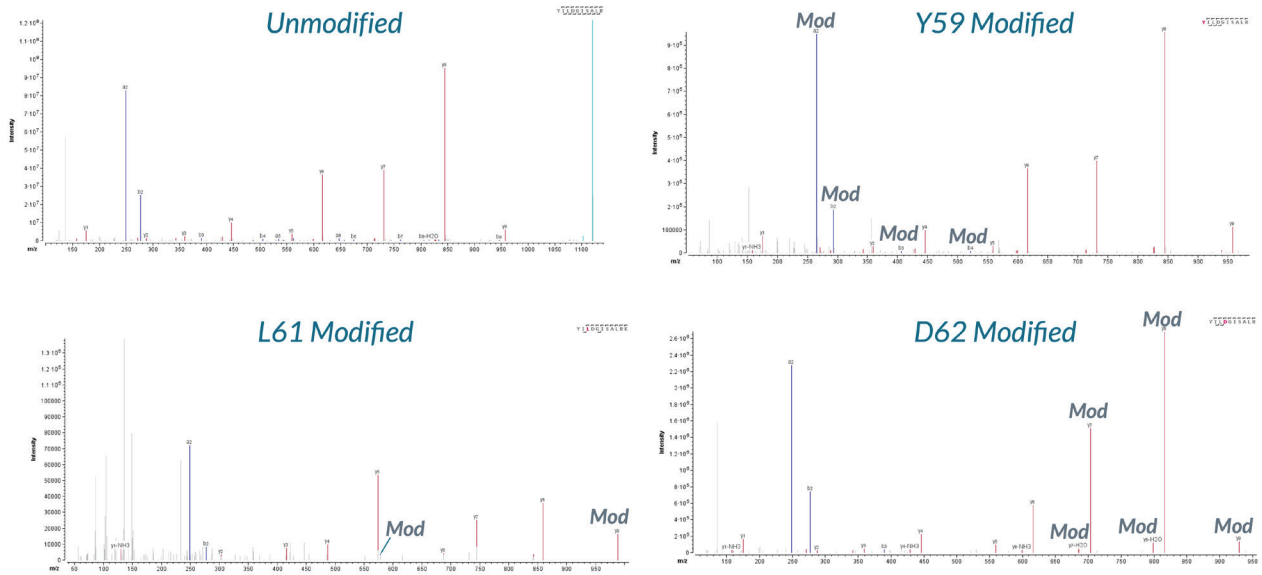
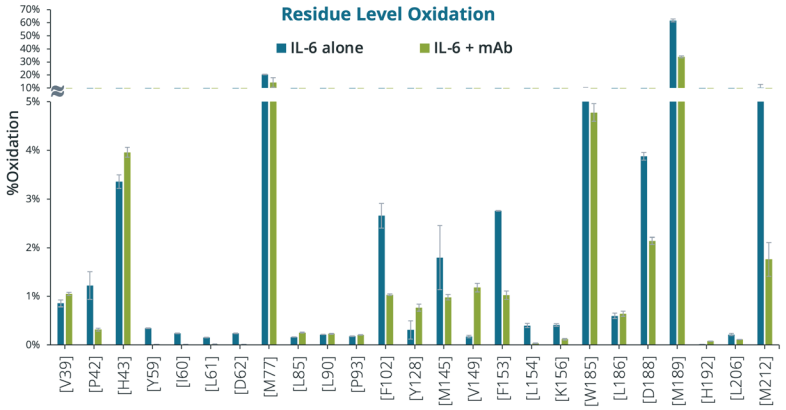


Figure 8: MS/MS Spectra of unmodified and modified peptide 59-68. *Representative MS/MS spectra showing localization of Y59, L61, and D62 modifications. Fragment ion coverage enables discrimination of oxidation sites, demonstrating the ability to assign modifications at the residue level and resolve ambiguity arising from multiple potential oxidation positions.*

Residue-Level Oxidation Changes Across Conditions

Distribution of residue-level oxidation across IL-6 in apo and holo states, highlighting global shifts in solvent accessibility upon antibody binding.

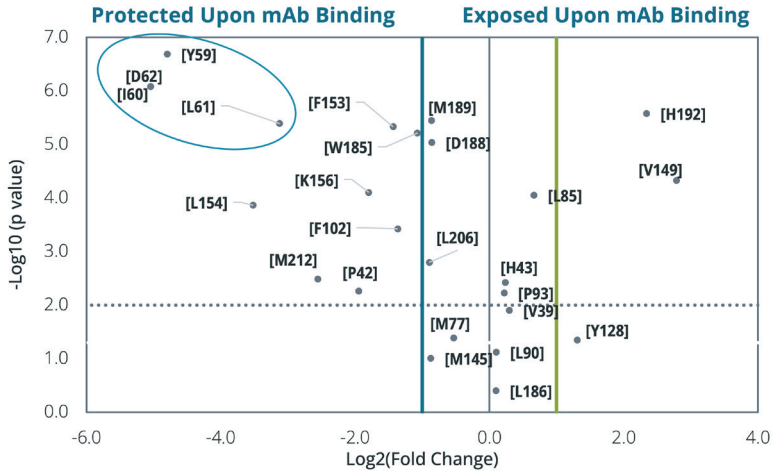
Figure 9: Histogram. *Average residue-level % Oxidation for IL-6 alone (blue) and IL-6 + mAb (green). Error bars represent standard deviation across three replicates. The average and median RSD across all residues are 8.4% and 4.3%, respectively, demonstrating reproducible quantification enabled by automated data processing in Genedata Expressionist.*



Residue-Level Mapping of Oxidation for Structural Insight

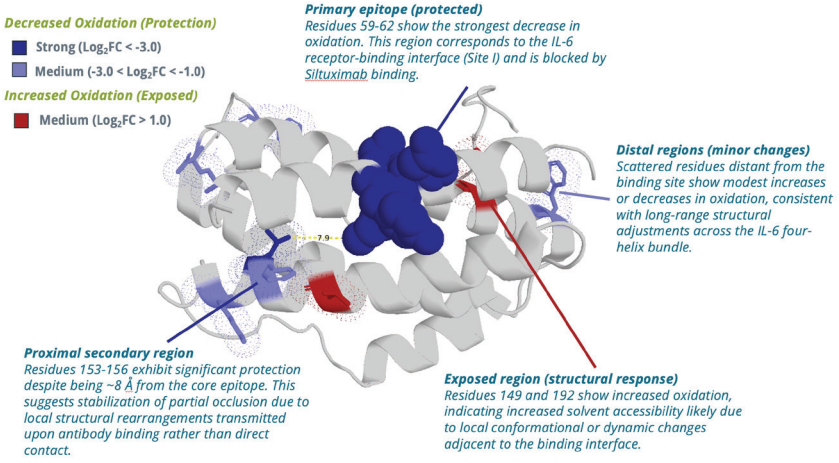
Volcano plot illustrating residue-level changes in oxidation between apo and holo IL-6, enabling identification of statistically significant structural perturbations upon antibody binding.

Figure 10: Volcano Plot. *Log_2(fold change) in oxidation is plotted against $-log_{10}$(p-value) for IL-6 alone versus IL-6 + mAb. Residues above the horizontal threshold ($p < 0.01$) are considered statistically significant. Vertical thresholds indicate ≥2-fold decreases in oxidation (protection, blue) and ≥2-fold increases (exposure, green). The most protected residues (circled) are consistent with antibody binding.*



HRPF Reveals a Conformational Epitope & Allosteric Structural Response Upon Siltuximab Binding to IL-6

Residue-level oxidation changes mapped onto the IL-6 structure PDB 1P9M reveal a conformational epitope centered on residues 59–62, consistent with the receptor-binding interface Site I. A second protected region at residues 153–156, located ~8 A away, suggests local structural stabilization rather than direct antibody contact. In contrast, increased oxidation at residues such as V149 and H192 indicates enhanced solvent accessibility due to binding-induced conformational changes. Minor changes observed in distal regions further support a model of long-range structural rearrangement across the IL-6 four-helix bundle.



Conclusions

- The integration between AutoFox and Genedata Expressionist transforms HRPF from a specialist technique into an automated, scalable, and comparative structural biology workflow.
- Automated alignment, feature grouping, and statistical analysis address key HRPF challenges and enable consistent and unambiguous interpretation of complex datasets.
- The workflow supports robust residue-level comparison across conditions, enabling confident identification of structural changes associated with protein-protein interactions.
- Application to the IL-6–Siltuximab system demonstrated consistent detection of protection and exposure patterns, illustrating the ability to resolve both epitope engagement and broader structural responses.
- Together, this approach enables rapid, high-confidence structural biology studies with reduced manual intervention, supporting scalable structural characterization of complex protein systems.

By combining AutoFox's controlled labeling with Genedata Expressionist's automated, enterprise-scale data processing, HRPF is transformed from a specialist technique requiring extensive manual data interpretation into a scalable, push-button structural biology workflow capable of delivering reproducible, publication-quality epitope mapping and conformational analysis with minimal hands-on intervention.