Demonstration of Adalimumab Structural Stability in Various pHs using High-Throughput Hydroxyl Radical Protein Footprinting with the AutoFox® Protein Footprinting System

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Fully Automated HRPF Labeling using the AutoFox® Protein Footprinting System

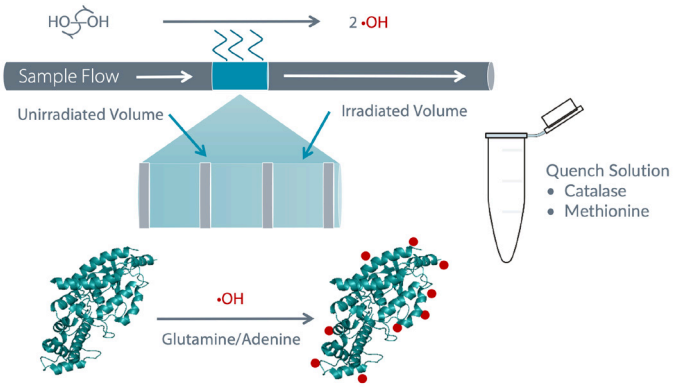
Running buffer is used to push the protein sample passed the photolysis zone which receives light from the flash lamp. The flash lamp has a pulse width at FWHM <10 µs and reliably produces a broad-band spectral output from 200-300 nm wavelength along with some visible light. The broad spectrum of the lamp matches the UV absorbance spectrum of H_2O_2, resulting in effective production of •OH. Immediately downstream of the photolysis zone is the dosimeter module which monitors the effective •OH concentration by detecting the change in a dosimeter molecule's UV absorbance at 265 nm.

HRPF Introduction

The higher order structure (HOS) of a protein plays a critical role in a drug's stability, safety and biological function. Incorrect HOS or protein interactions are linked to adverse drug reactions which can result in further sickness or death.

Advance techniques are required to robustly address the HOS of proteins. One such method is Hydroxyl Radical Protein Footprinting (HRPF).

Figure 1: *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.*



The Fox® Protein Footprinting System is a novel HRPF method that uses a proprietary flash oxidation lamp to generate hydroxyl radicals (•OH) that irreversibly modify solvent exposed amino acid side chains. As solvent accessibility changes, the •OH modification concordantly changes.

Figure 2: *Schematic of an HRPF method, fast Photochemical Oxidation of Proteins (FPOP). With FPOP, protein is mixed with hydrogen peroxide and flowed passed a pulsing light source 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 catalase and methionine.*

Adalimumab pH Stability: Dose Response Plots

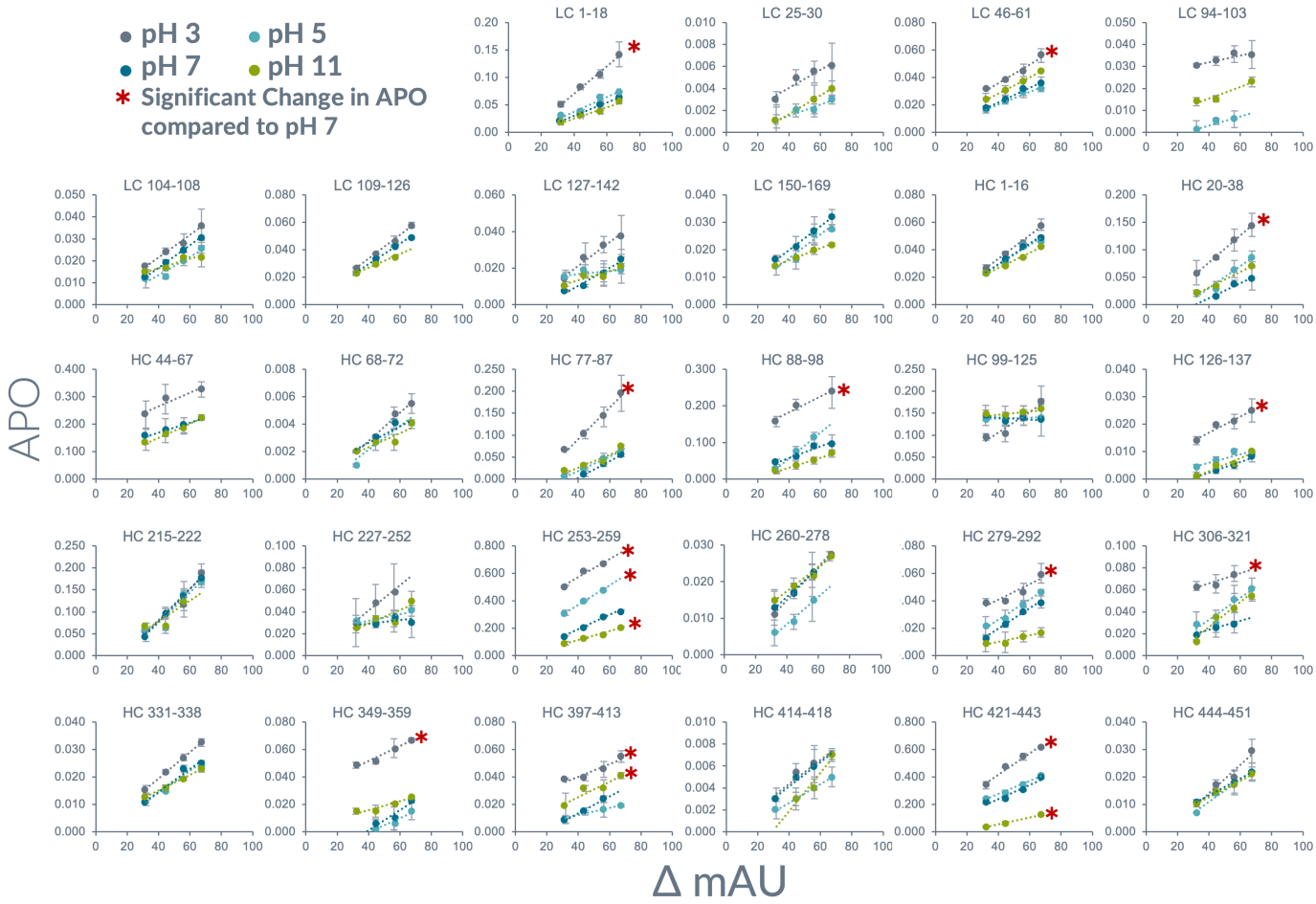


Figure 3: *Adalimumab was incubated in PBS at pH levels of 3, 5, 7, or 11 for three hours. Following incubation, the samples were labeled using the AutoFox System at voltages of 0V, 800V, 1000V, 1200V, and 1400V, which produced dose responses of 32.0, 44.5, 56.2, and 67.4 mAU, respectively. Variations in pH did not affect the radical yield. Across all conditions, as the radical concentration increased, the average peptide oxidation (APO) also increased. The APO at 1000V for Adalimumab at pH 3, 5, and 11 was compared to that at pH 7 using a Student's t-test. A red asterisk indicates conditions with a statistically significant change in oxidation.*

Adalimumab Coverage Map

Adalimumab Light Chain

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q G I R N Y L A W Y Q Q K P G K A P K L L I Y A
A S T L Q S G V P S R F S G S G S G T D F T L T I S S L Q P E D V A T Y Y C Q R Y N R A P Y T F G Q
G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L L N N F Y P R E A K V Q W K V
D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S T L T L S K A D Y E K H K V Y A C E V T H Q G
L S S P V T K S F N R G E C

Adalimumab Heavy Chain

E V Q L V E S G G G L V Q P G R S L R L S C A A S G F T F D D Y A M H W V R Q A P G K G L E W V S A
I T W N S G H I D Y A D S V E G R F T I S R D N A K N S L Y L Q M N S L R A E D T A V Y Y C A K V S
Y L S T A S S L D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S K S T S G G T A A L G C L V
K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G L Y S L S S V V T V P S S S L G T Q
T Y I C N V N H K P S N T K V D K K V E P K S C D K T H T C P P C P A P E L L G G P S V F L F P P K
P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y
N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P
Q V Y T L P P S R D E L T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P
V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G

Figure 4: *Adalimumab coverage map: Peptides highlighted in green were detected under all conditions in at least two replicates. The coverage for the light chain is 51%, while the coverage for the heavy chain is 63%.*

Adalimumab Histograms

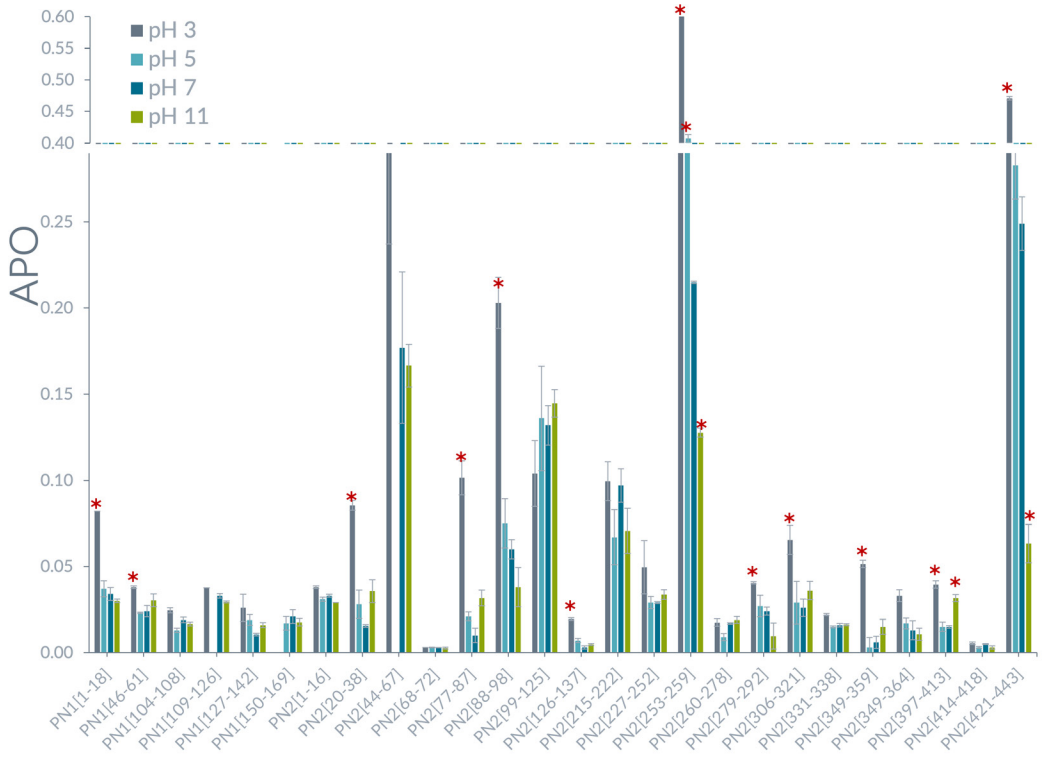


Figure 5: *Histograms showing the Adalimumab Average Peptide Oxidation (APO) for each condition labeled at 1000V. Error bars represent technical triplicates. The APO values for pH 3, 5, and 11 were compared to those at pH 7 using a Student's t-test. Red asterisks indicate a significant change (p-value < 0.01 with a fold change greater than 50%).*

Adalimumab Volcano Plots

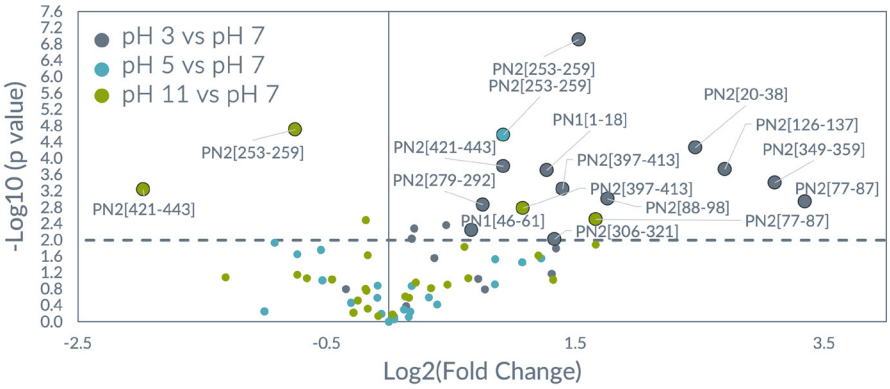


Figure 6: *Adalimumab Volcano Plot comparing the Average Peptide Oxidation (APO) at 1000V for pH 3, 5, or 11 against pH 7. The dotted line represents a p-value threshold of 0.01. Peptides with a p-value < 0.01 and a fold change > 50% are labeled. A positive fold change indicates increased exposure compared to pH 7, while a negative fold change indicates increased protection.*

Adalimumab Crystal Structure

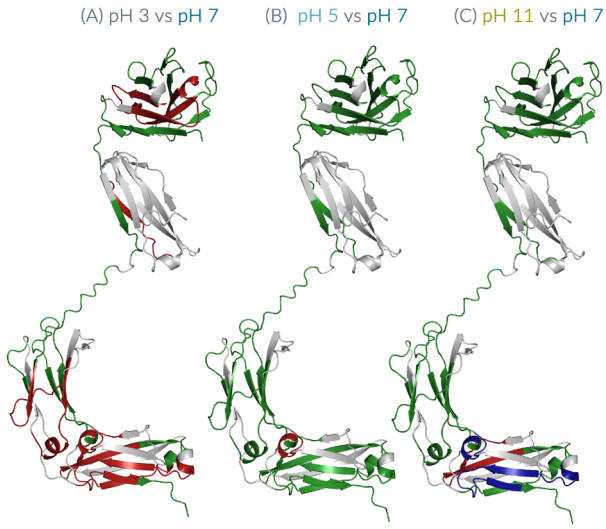


Figure 7: *Structure of Adalimumab Heavy Chain, modeled using AlphaFold, highlighting regions affected by changes in pH. The structures compare the changes in solvent accessibility of the protein at pH 3, pH 5, and pH 11 against a reference condition at pH 7. Regions detected with oxidation under all conditions are colored in green. Regions that become more exposed are highlighted in red, indicating unfolding or increased flexibility, while regions that become more protected are highlightd inblue, suggesting a change in structure or potential interaction site. (A) pH 3 vs. pH 7: Several peptides (12) in both the Fc and Fab regions become more exposed (red), suggesting significant unfolding of the protein structure at the lower pH. (B) pH 5 vs. pH 7: Minimal changes in solvent accessibility are observed, except for a single peptide (260-278) becoming more exposed. This indicates that Adalimumab remains stable under slight pH variations around neutral pH. (C) pH 11 vs. pH 7: One peptide becomes more exposed, and two peptides become more protected, all within the Fc region, indicating initial signs of instability and unfolding starting in this region as pH becomes more basic.*

Conclusions

The AutoFox® System is an automated HRPF platform that uses a microfluidic chip within a 96-well plate format, enabling efficient and reproducible protein labeling for structural analysis.

The AutoFox System effectively facilitates high-throughput HRPF studies to evaluate protein stability and structural integrity under varying environmental conditions. This study specifically examines the stability of Adalimumab across different pH levels.

Results indicate significant unfolding of Adalimumab at pH 3, minimal structural changes between pH 7 and 5, and signs of instability and structural changes in the Fc region at pH 11.

These findings provide valuable insights into the stability and conformational dynamics of Adalimumab under different pH conditions, which are essential for understanding its therapeutic efficacy and safety.