GenNext® TECHNOLOGIES

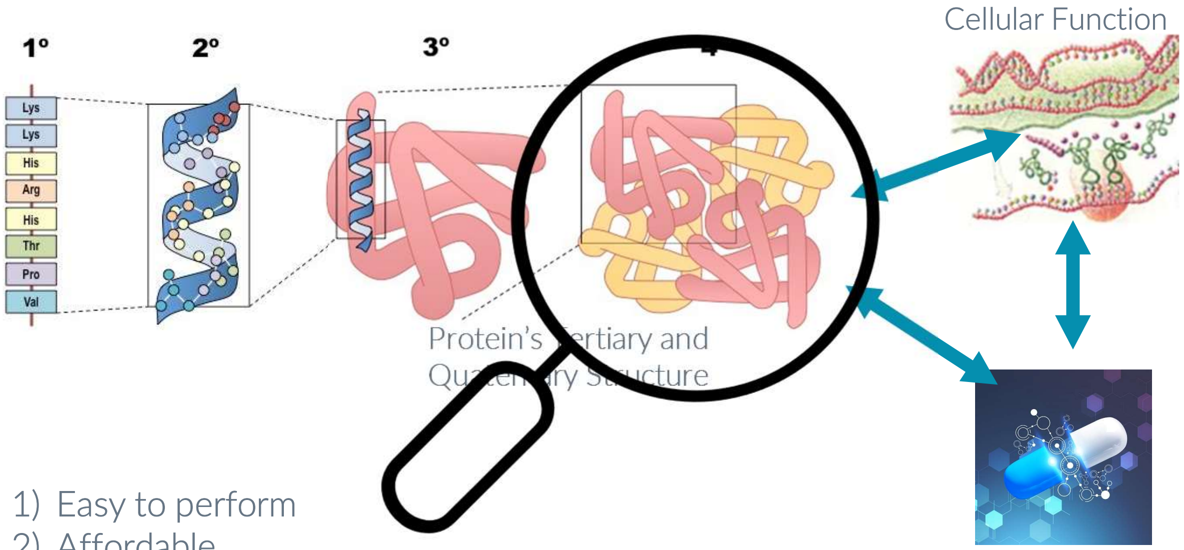
AutoFox® Protein Footprinting System Provides Amino Acid Resolution for Protein HOS Studies

CASSS MS 2024

Emily Chea, Sarah Stow, and Ruwan Kurulugama

Overview of Structural Biology

Higher Order Structure Analysis



Protein's Tertiary and Quaternary Structure

Cellular Function

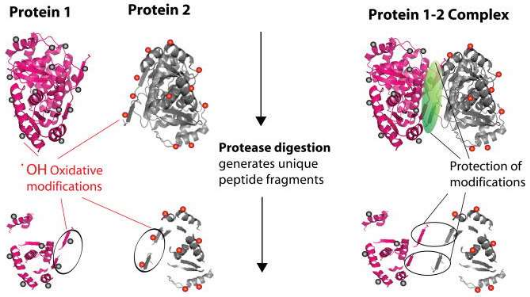
Drug Discovery and Development

1) Easy to perform
2) Affordable
3) Data quality with high resolution
4) Native conditions
5) No protein size or complex limitations
6) Compatible with heterogenous samples

Fox® Technology Applications

Structural Information from HRPF

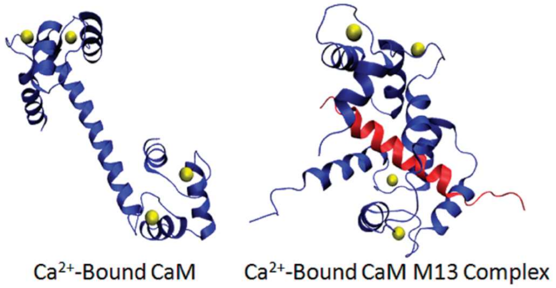
Protein-Protein Interaction Sites



Kiselar JG, Chance MR. Journal of mass spectrometry : JMS. 2010;45(12):1373-82. Epub 2010/09/03.

- Biomolecular Interactions
- Epitope Mapping
- Paratope Mapping
- Identify Aggregation Interfaces

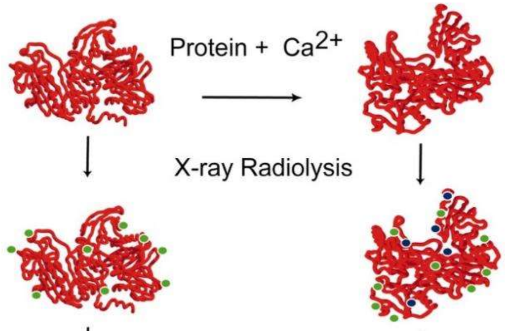
Protein-Ligand Interaction Sites



Zhang H, Gau BC, Jones LM, Vidavsky I, Gross ML. Analytical chemistry. 2010;83(1):311-8.

- Biomolecular Interactions
- Druggable Target Discovery
  - Drug Binding Site

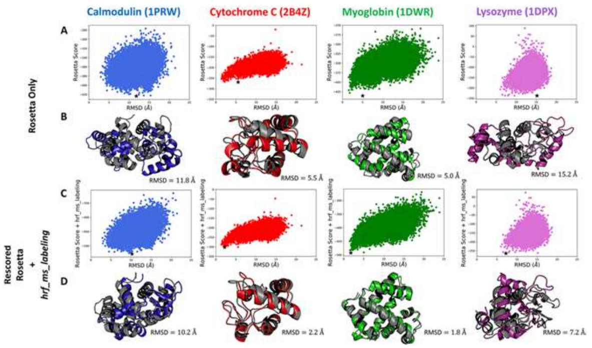
Conformational Changes



Kiselar JG, Janmey PA, Almo SC, Chance MR. Molecular & Cellular Proteomics. 2003;2(10):1120-32.

- Formulation Studies
- Allostery
- Stability Studies
- Biosimilar Development
- Excipient Effects

Protein Structure Predictions



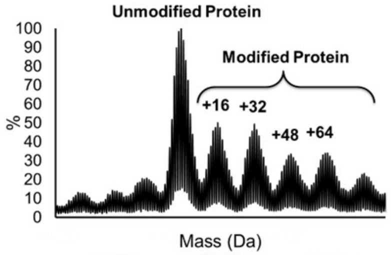
Melanie L. Aprahamian; Emily E. Chea; Lisa M. Jones; Steffen Lindert; Anal. Chem. 90, 7721-7729.

- Improve / confirm computational model
- Improve docking studies

Hydroxyl Radical Protein Footprinting

Enabling HOS Analysis

- Proteins exposed to a pulse of diffusing hydroxyl radicals
    - OH generated by flash photolysis of hydrogen peroxide
- Modifies exposed side chains
- Measures protein topography at peptide to amino acid resolution



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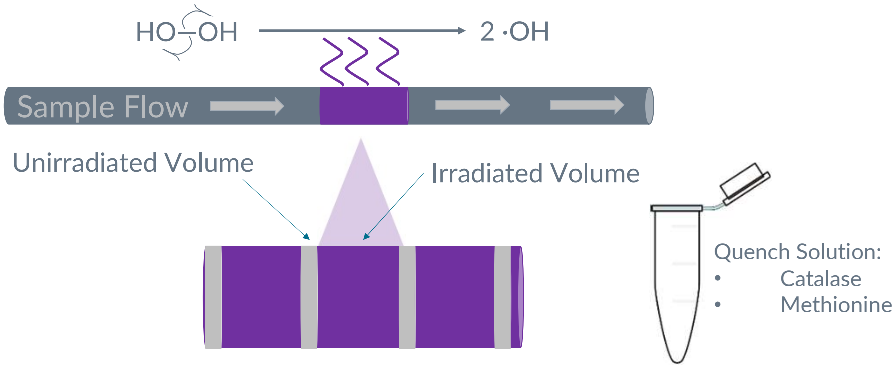
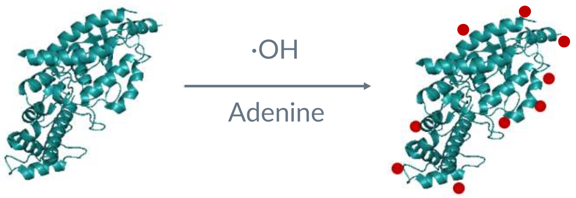


Quench Solution:
- Catalase
- Methionine

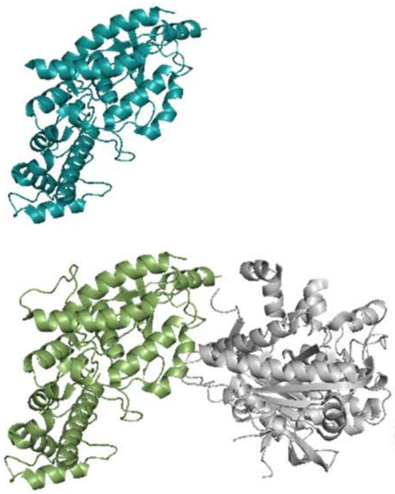


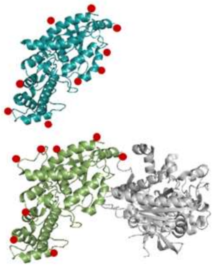
Amino Acid	Rate Constant (L*mol^{-1}*s^{-1})
Cysteine	3.40E+10
Tryptophan	1.30E+10
Tyrosine	1.30E+10
Methionine	8.30E+09
Phenylalanine	6.50E+09
Histidine	5.00E+09
Arginine	3.50E+09
Isoleucine	1.80E+09
Leucine	1.70E+09
Valine	7.60E+08
Glutamine	5.40E+08
Threonine	5.10E+08
Proline	4.80E+08
Lysine	3.50E+08
Serine	3.20E+08
Glutamic Acid	1.60E+08
Alanine	7.70E+07
Aspartic Acid	7.50E+07
Asparagine	4.90E+07
Glycine	1.70E+07

Reference: Buxton et al, J. Phys. Chem. Ref. Data, Vol. 17, No. 2, 1988

Hydroxyl Radical Protein Footprinting (HRPF)

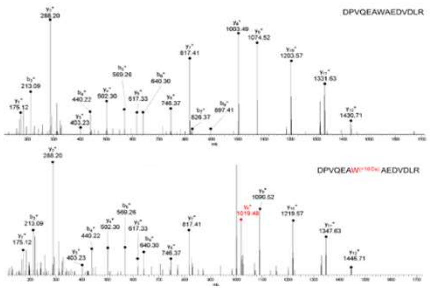
Typical Workflow





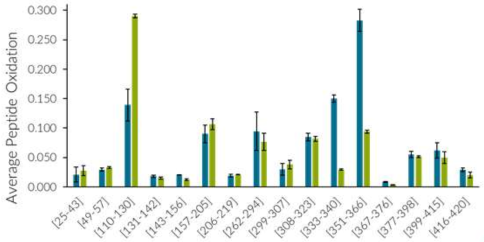
·OH

FPOP



Denature & Digest

Bottom-Up Proteomics



Average Oxidation

Data Process

FoxWare™ Protein Footprinting Software
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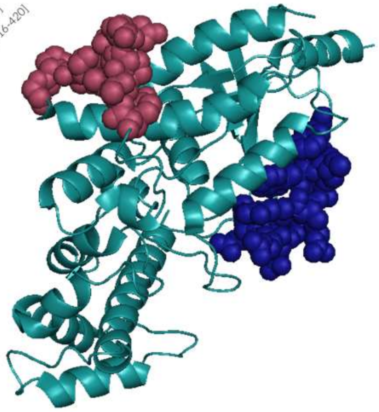

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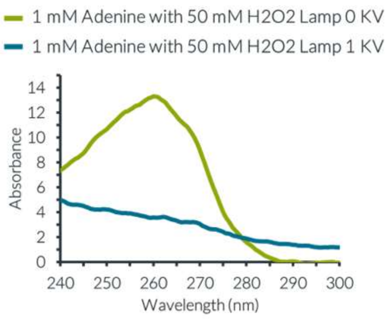
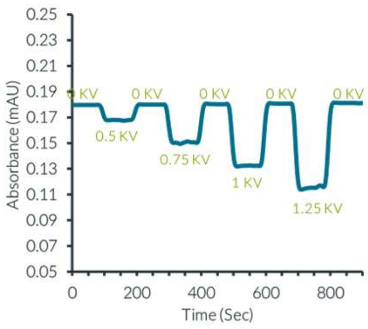
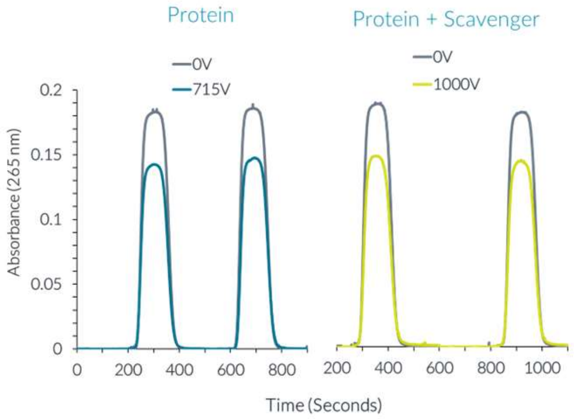
Fox® Protein Footprinting System

Real-time correction for background scavenging







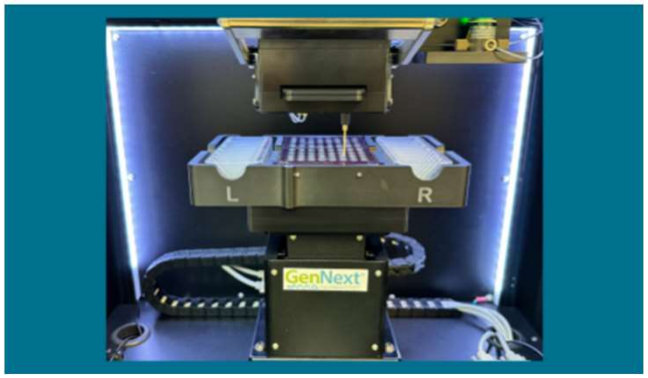
GenNext Fox®, FoxWare®, *in vitro* and *in vivo* FPOP and Radical Dosimetry technologies are protected by US, EU and pending patents: US 10,816,468; US 11,181,529; EP 3630349 and other pending patents.

Introducing the AutoFox® Protein Footprinting System

Fully Automated, High-Resolution Protein Higher Order Structural Analysis

Fully Automated, 96-well microplate

- **AutoFox Robotics** to process up to 48 samples at the push of a button without manual intervention.

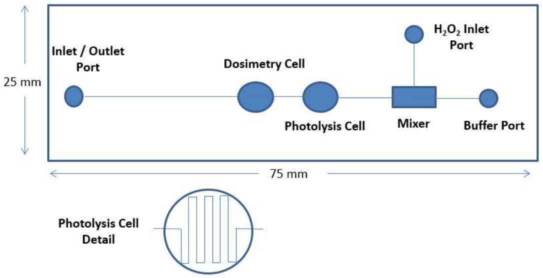


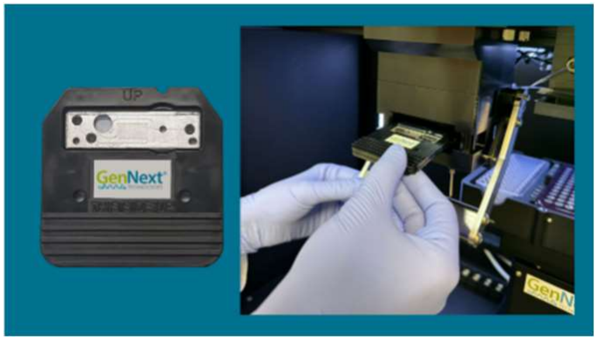
Introducing the AutoFox® Protein Footprinting System

Fully Automated, High-Resolution Protein Higher Order Structural Analysis

Plug-and-Play Opto-Fluidic Chip

- **on-board, high efficiency, microfluidic mixer** simplifies sample preparation, ensuring experimental precision and repeatability.
- **serpentine photolysis cell** that labels up to 30 µL in 10 seconds, and
- **integrated dosimetry cell** ensures robust and reproducible protein structural determination



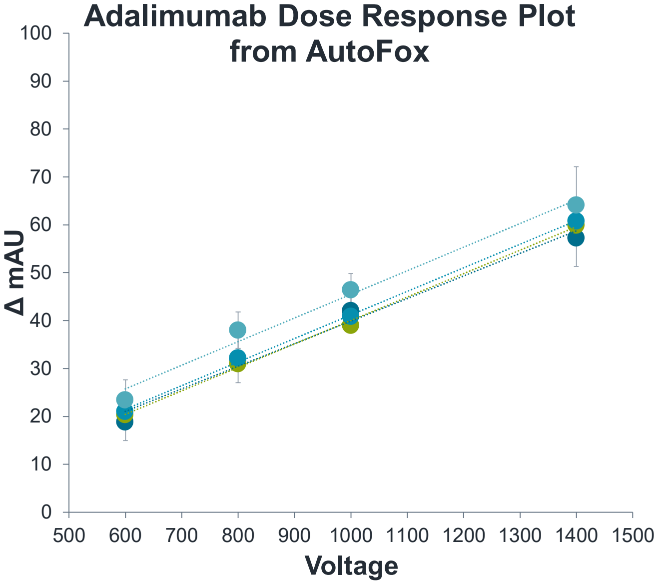


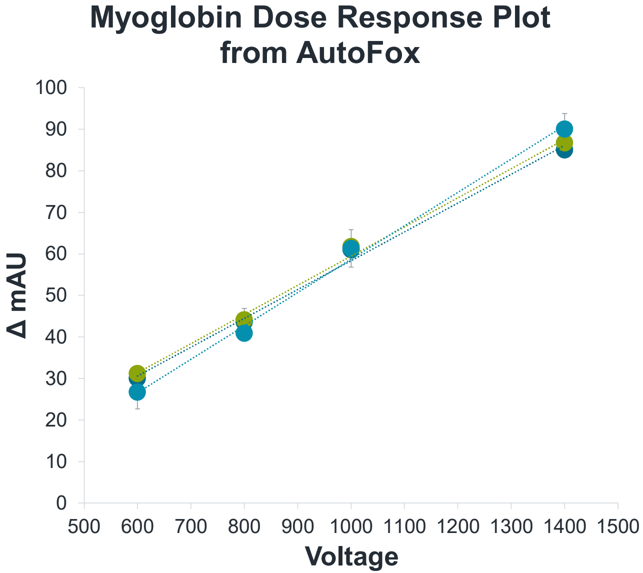
AutoFox® Protein Footprinting System

Proven robust and reproducible labeling

•OH Dose Response during Antibody Labeling

- As the flash voltage increases, more radicals are generated causing an increase in the Δ mAU from adenine.
- The generated radical concentration is very consistent day to day ensuring experimental precision and repeatability





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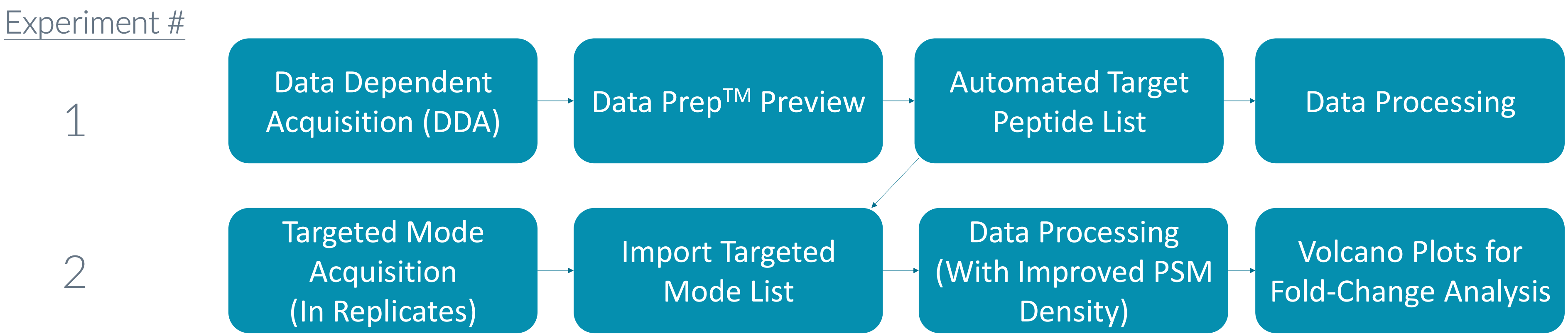
HRPF-LC-MS/MS Workflow

Suggested Experimental LCMS Setup after HRPF Labeling with FOX® System



Experiment #

1

Data Dependent Acquisition (DDA)

Data Prep™ Preview

Automated Target Peptide List

Data Processing

2

Targeted Mode Acquisition (In Replicates)

Import Targeted Mode List

Data Processing (With Improved PSM Density)

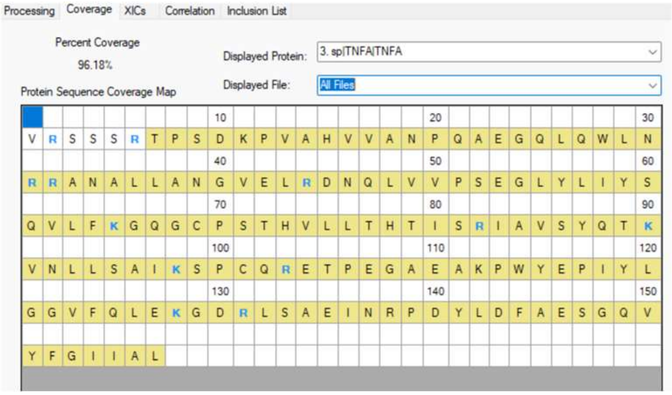
Volcano Plots for Fold-Change Analysis

Data Prep Program Overview

Data Previewing Software for Qualifying Data

Purpose of Data Prep Software:

- Cursory PSM search and review
- Peptide coverage map
- Viewing and reviewing peptide XICs
- Automated Inclusion List for Targeted Mode Analysis



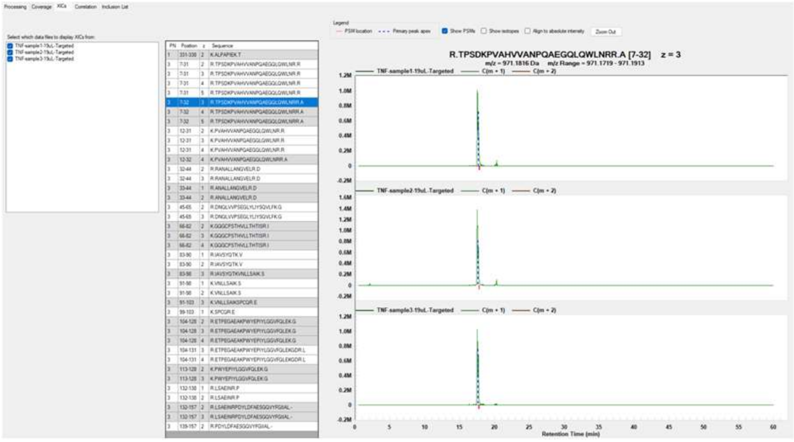
Percent Coverage
96.18%

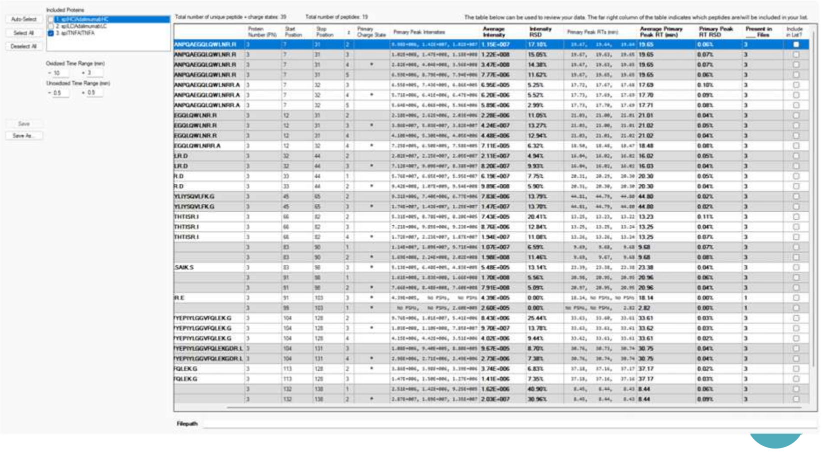
Displayed Protein: 3. sp|TNFA|TNFA

Displayed File: All Files

Protein Sequence Coverage Map

									10										20										30
V	R	S	S	S	R	T	P	S	D	K	P	V	A	H	V	V	A	N	P	Q	A	E	G	Q	L	Q	W	L	N
									40										50										60
R	R	A	N	A	L	L	A	N	G	V	E	L	R	D	N	Q	L	V	V	P	S	E	G	L	Y	L	I	Y	S
									70										80										90
Q	V	L	F	K	G	Q	G	C	P	S	T	H	V	L	L	T	H	T	I	S	R	I	A	V	S	Y	Q	T	K
									100										110										120
V	N	L	L	S	A	I	K	S	P	C	Q	R	E	T	P	E	G	A	E	A	K	P	W	Y	E	P	I	Y	L
									130										140										150
G	G	V	F	Q	L	E	K	G	D	R	L	S	A	E	I	N	R	P	D	Y	L	D	F	A	E	S	G	Q	V
Y	F	G	I	I	A	L																							





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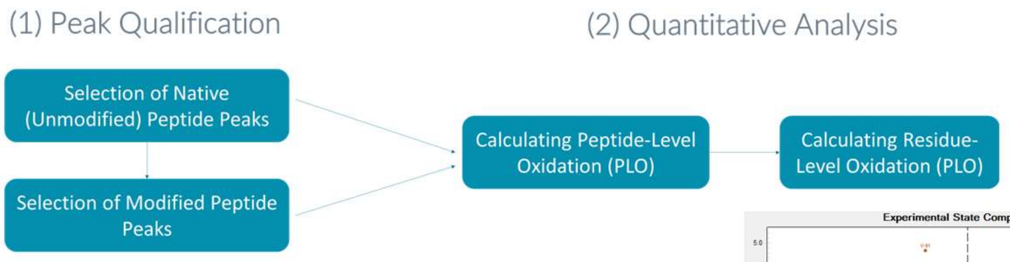
Data Processing Overview

In-Depth Data Analysis and User Review



(1) Peak Qualification

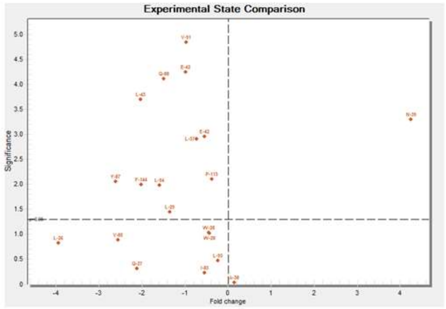
(2) Quantitative Analysis

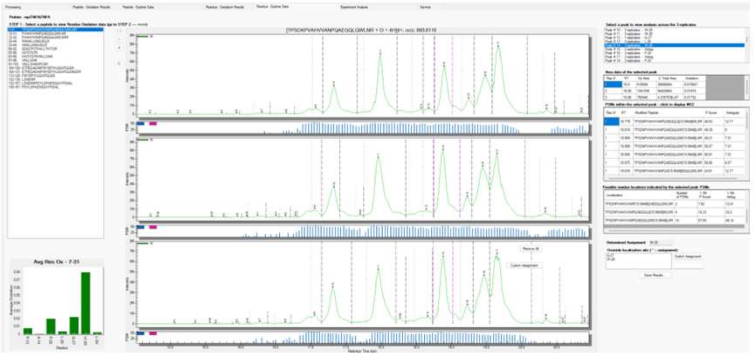
Selection of Native (Unmodified) Peptide Peaks

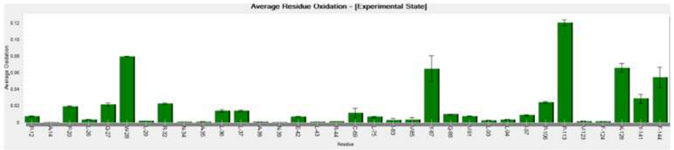
Selection of Modified Peptide Peaks

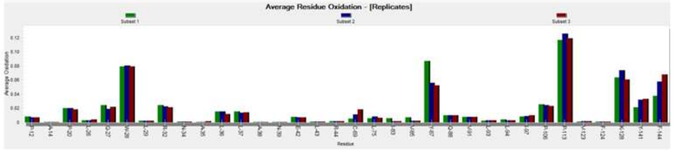
Calculating Peptide-Level Oxidation (PLO)

Calculating Residue-Level Oxidation (PLO)







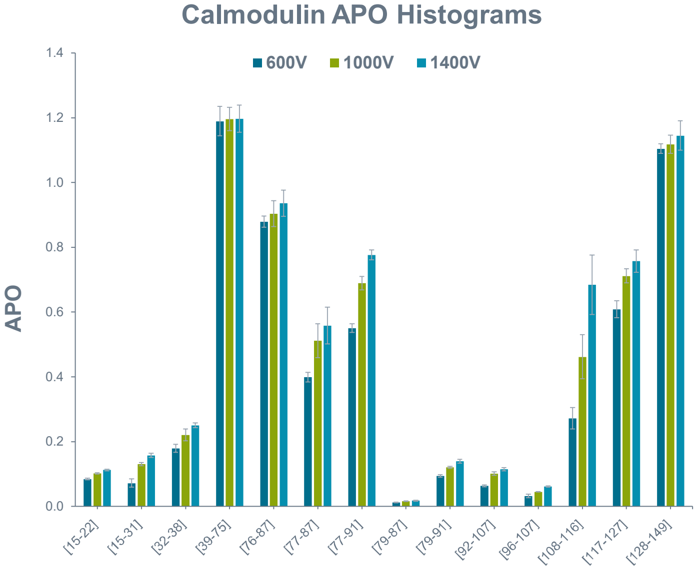


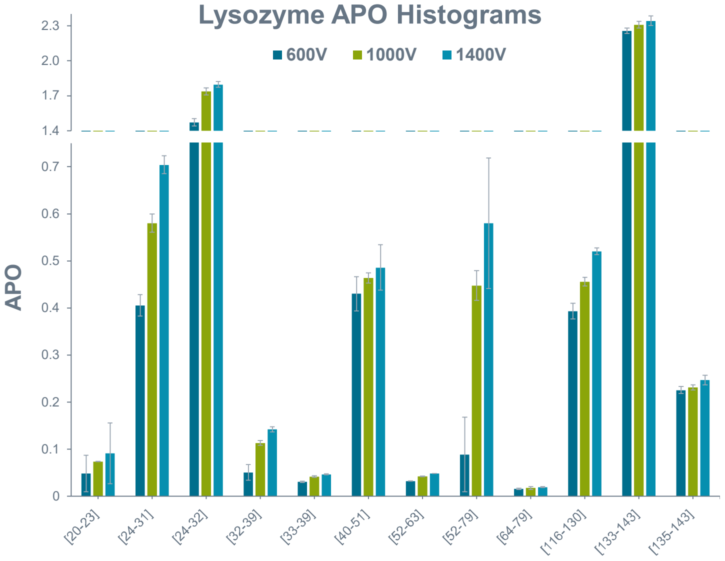
Improved Residue Level Oxidation Calculation:

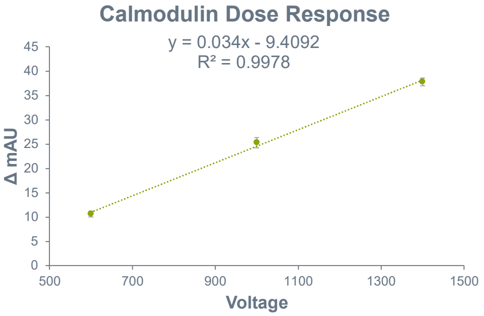
- Provide the user with quantitative capabilities for both peptide and residue-level analysis
- Advanced parameters allows customized controls for automated peak picking, quality filtering and residue-level confidence measurements
- Advanced peak criteria evaluation to automatically determine the validity of XIC peaks and features.
- Automated replicate RT alignment improves data confidence and quality
- Let users review data and make customized selections

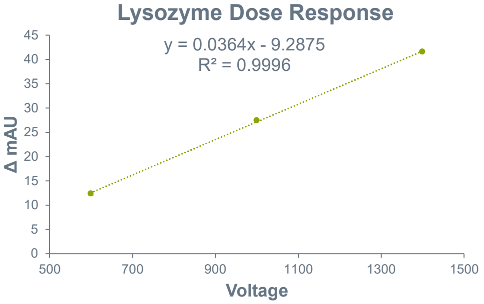
AutoFox® Protein Footprinting System

Lysozyme and Calmodulin Protein Dose Response


Calmodulin APO Histograms


Lysozyme APO Histograms


Calmodulin Dose Response


Lysozyme Dose Response

AutoFox® Protein Footprinting System

Lysozyme and Calmodulin Protein Dose Response

$$Protection\ Factor\ (PF) = \frac{\sum_i R_i}{k_{fp}}$$

Relative intrinsic reactivity (R_i)

Footprinting rate constant (k_{fp})

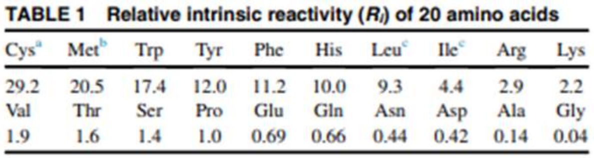
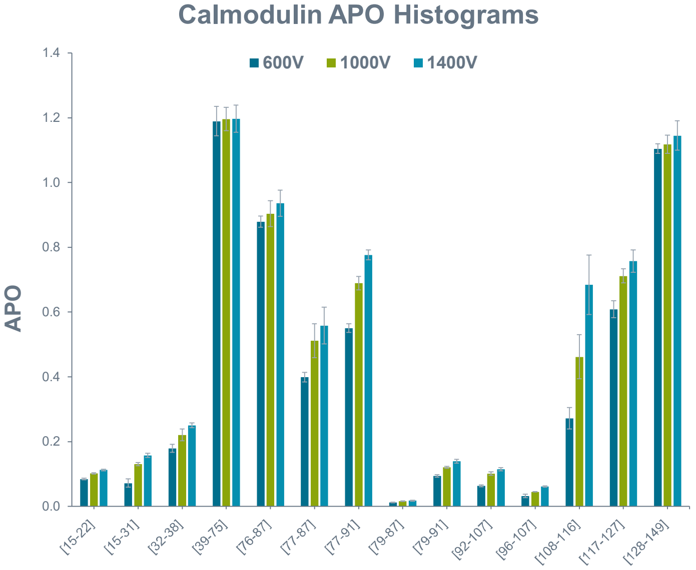
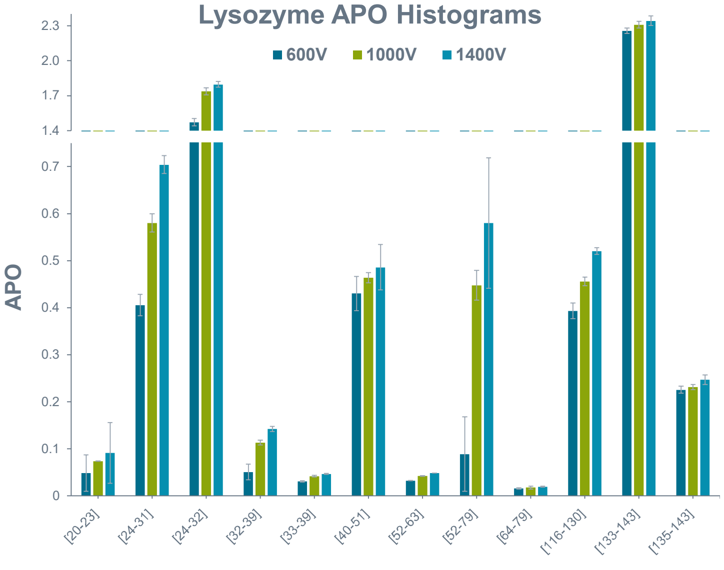


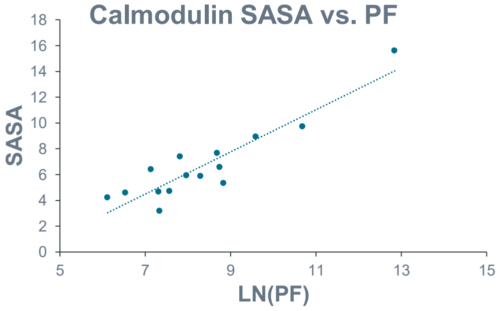
TABLE 1 Relative intrinsic reactivity (R_i) of 20 amino acids

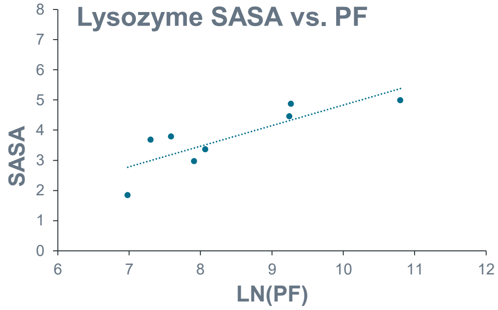
Cys[a]	Met[b]	Trp	Tyr	Phe	His	Leu[c]	Ile[c]	Arg	Lys
29.2	20.5	17.4	12.0	11.2	10.0	9.3	4.4	2.9	2.2
Val	Thr	Ser	Pro	Glu	Gln	Asn	Asp	Ala	Gly
1.9	1.6	1.4	1.0	0.69	0.66	0.44	0.42	0.14	0.04

Quantitative Mapping of Protein Structure by Hydroxyl Radical Footprinting-Mediated Structural Mass Spectrometry: A Protection Factor Analysis. Huang, Wei et al. Biophysical Journal, Volume 108, Issue 1, 107 - 115



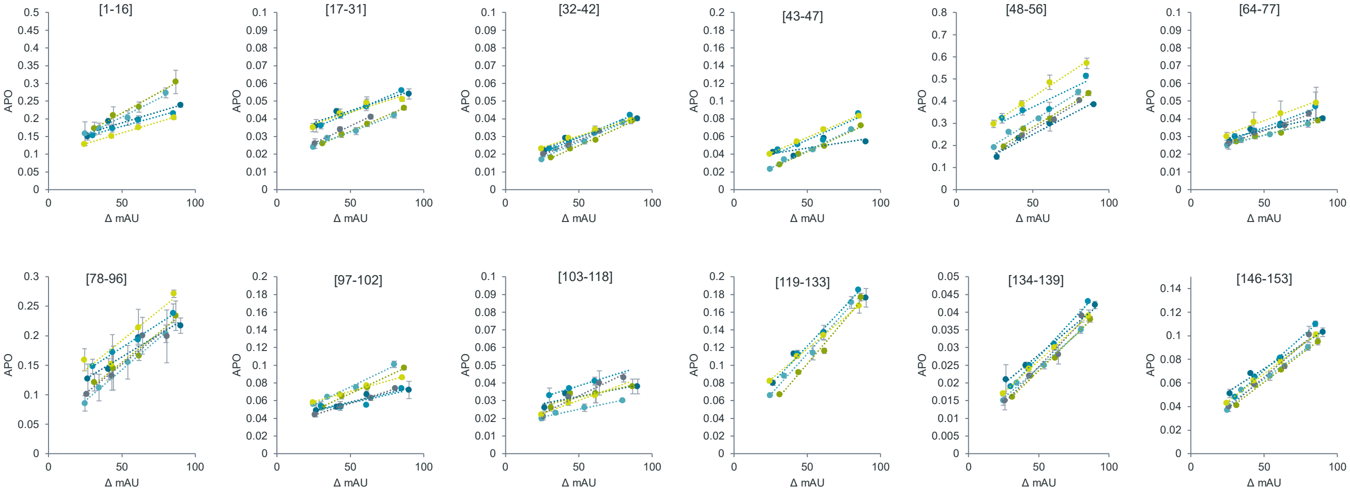






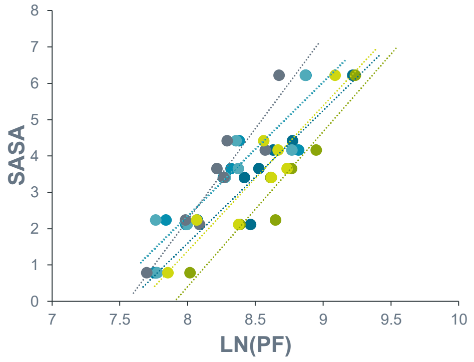
AutoFox® Protein Footprinting System

Myoglobin Protein Dose Response



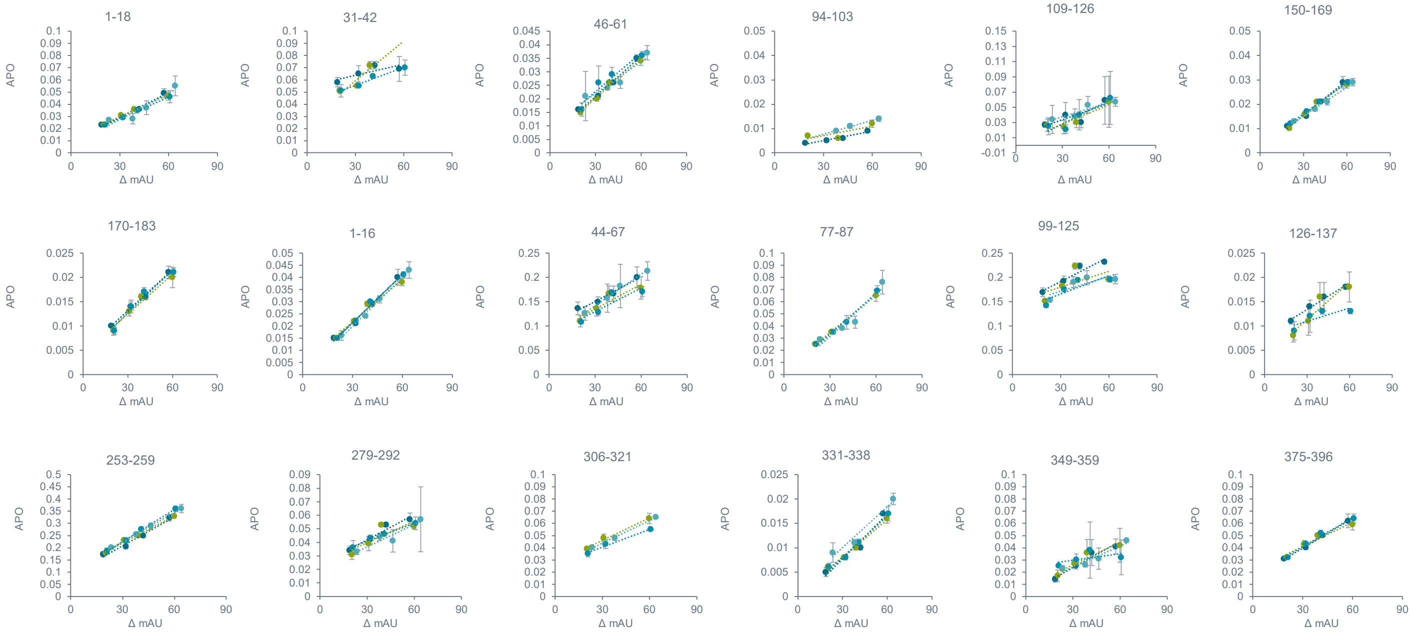
Myoglobin

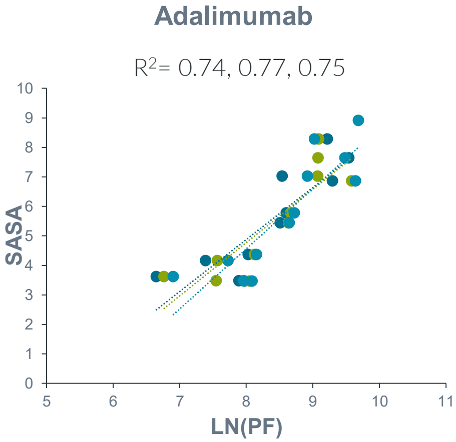
R^2= 0.91, 0.92, 0.84, 0.84, 0.88, 0.87



AutoFox® Protein Footprinting System

Adalimumab Protein Dose Response



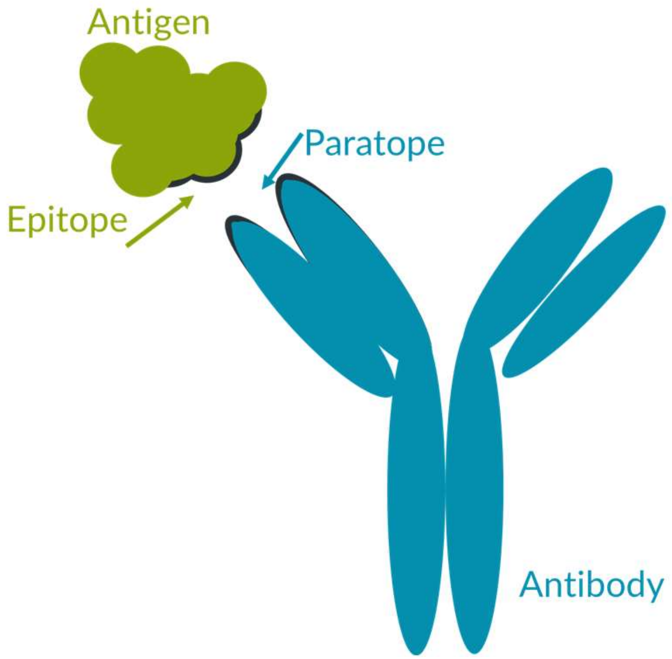


Monoclonal Antibody Therapy

In-depth analytical studies to understand physicochemical and functional characteristics: Epitope and Paratope Characterization

Epitope and Paratope Characterization

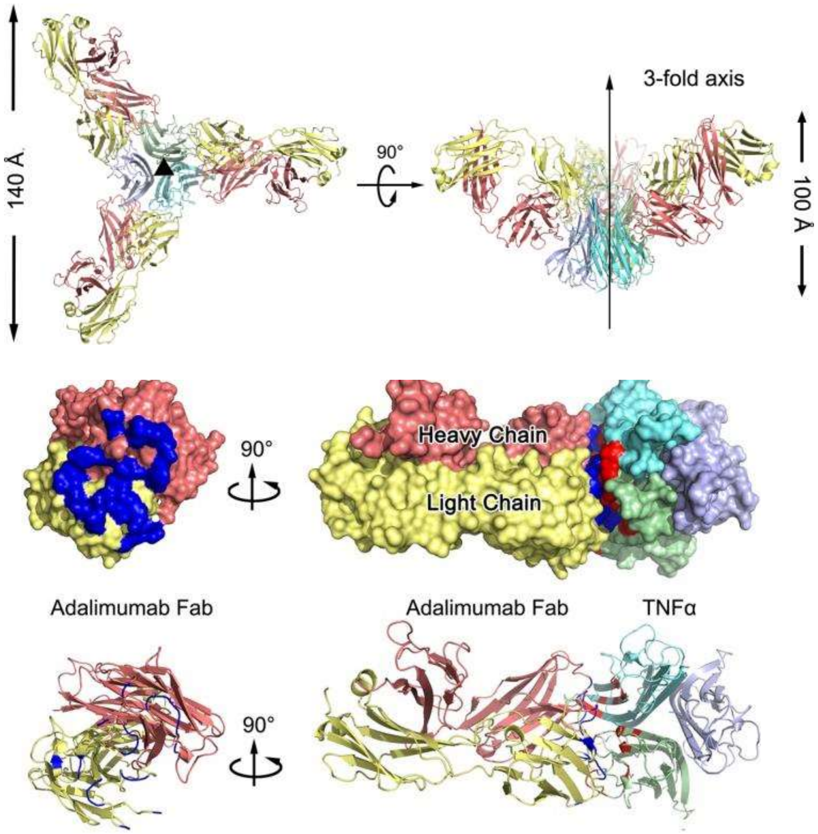
- Identify the specific amino acid sequences or structural features that are critical for antibody-antigen recognition
- Advance the understanding of the precise mechanism of action to make clear and relevant conclusions
- Helps identify vaccine targets
- Helps identify ways to engineer antibodies for improved affinity
- Allows the developer to protect their intellectual property



Epitope Mapping

Fox® System Case Study: TNFα:Adalimumab

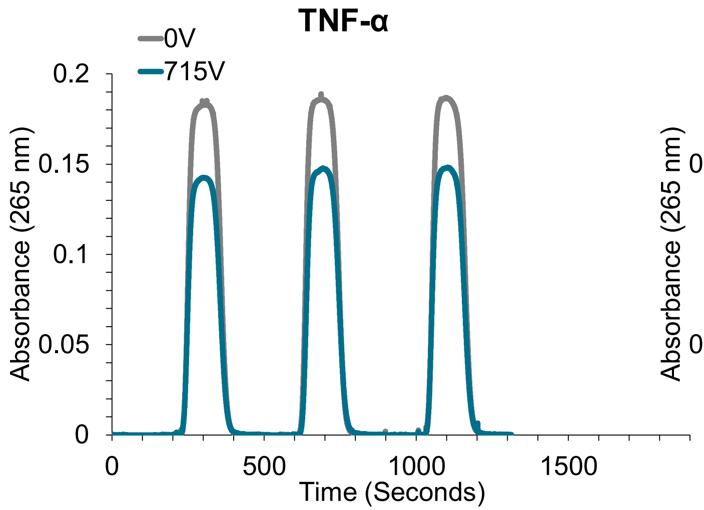
- Tumor necrosis factor α (TNFα) is a pro-inflammatory cytokine
- Adalimumab (Humira) is a monoclonal antibody prescribed to treat inflammatory diseases
- TNFα:Adalimumab epitope and paratope has been well characterized with an available crystal structure
- Can Fox™ Protein Footprinting accurately detect regions involved in the interaction interfaces?

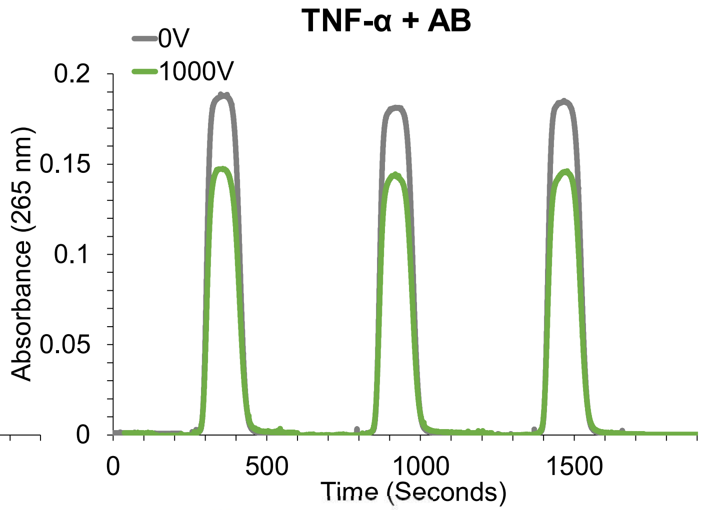


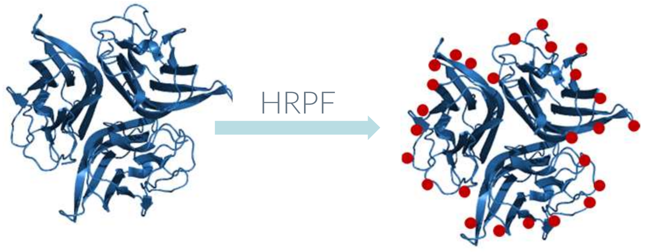
J Biol Chem. 2013 Sep 20; 288(38): 27059–27067.
PDB ID 3WD5

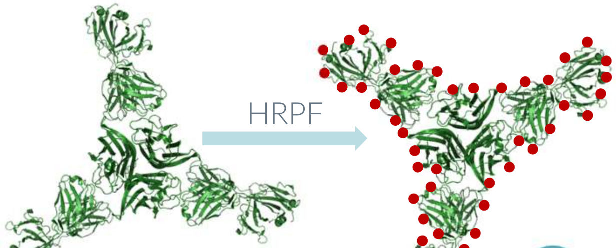
TNFα – Adalimumab HRPF Characterized Epitope

Fox® System Case Study:
TNFα:Adalimumab •OH Dose Response









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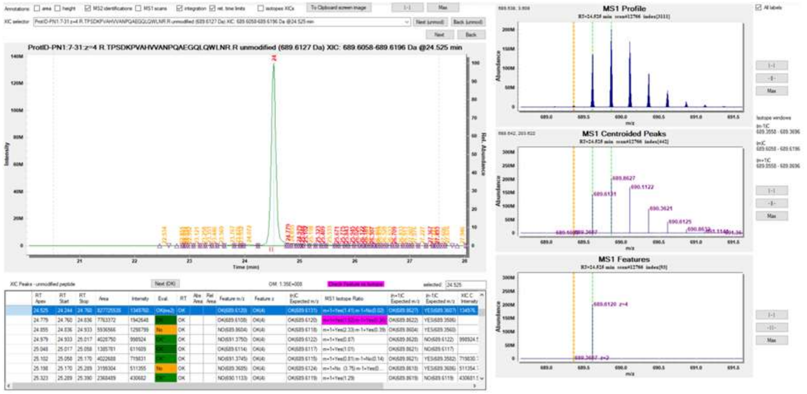
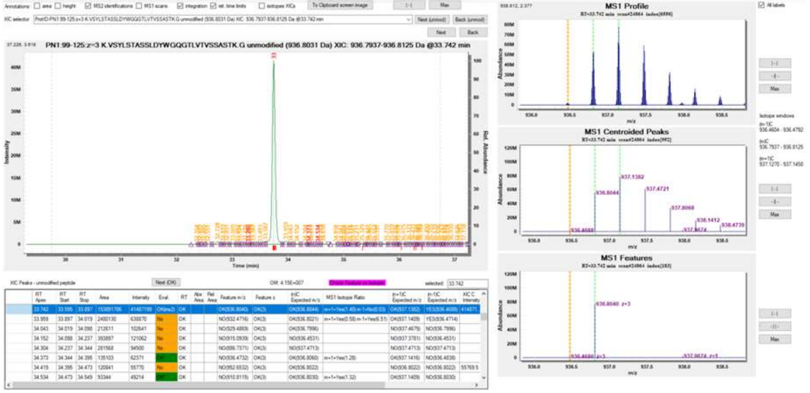
TNFα – Adalimumab HRPF Characterized Epitope

Fox® Case Study: XIC Examples

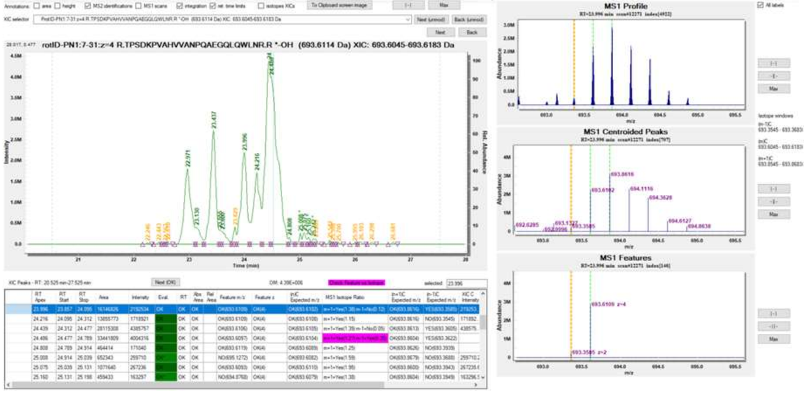
Unmodified Peptide 1



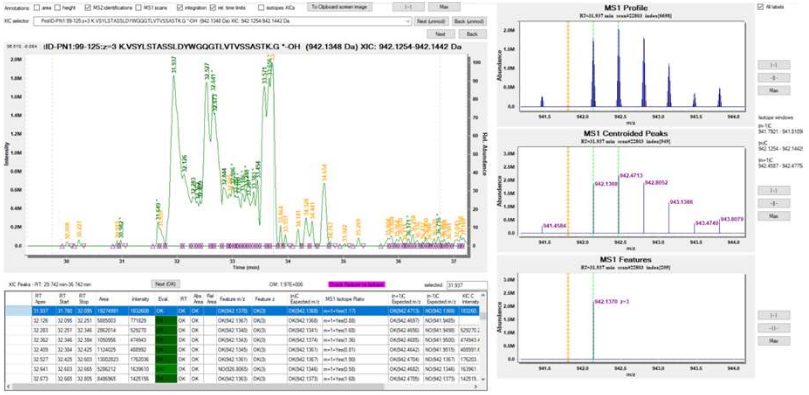
Unmodified Peptide 2



Modified (+16 Da) Peptide 1

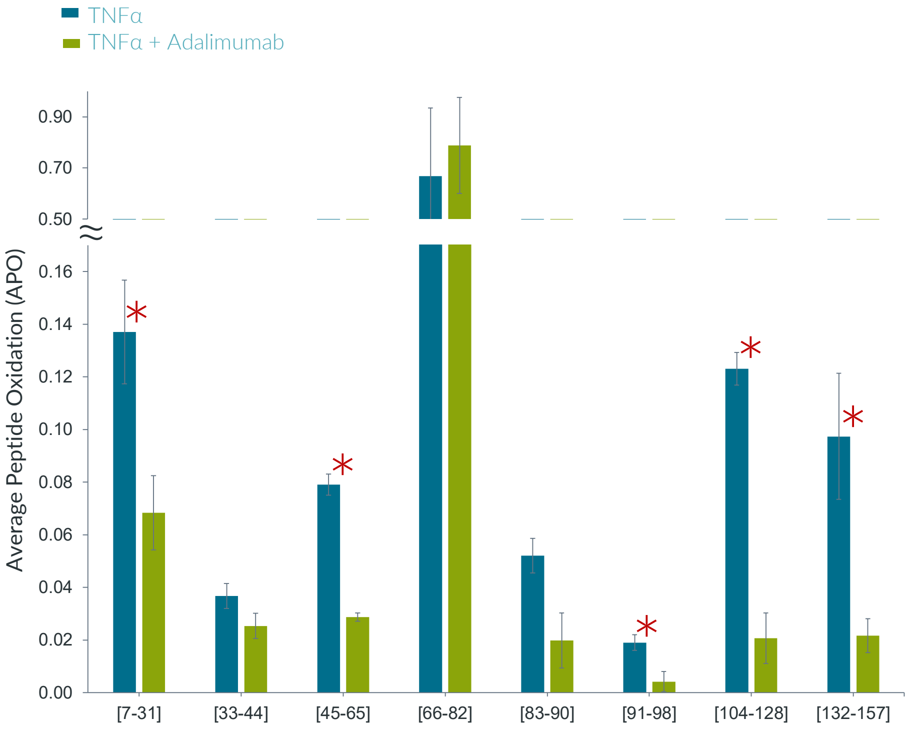


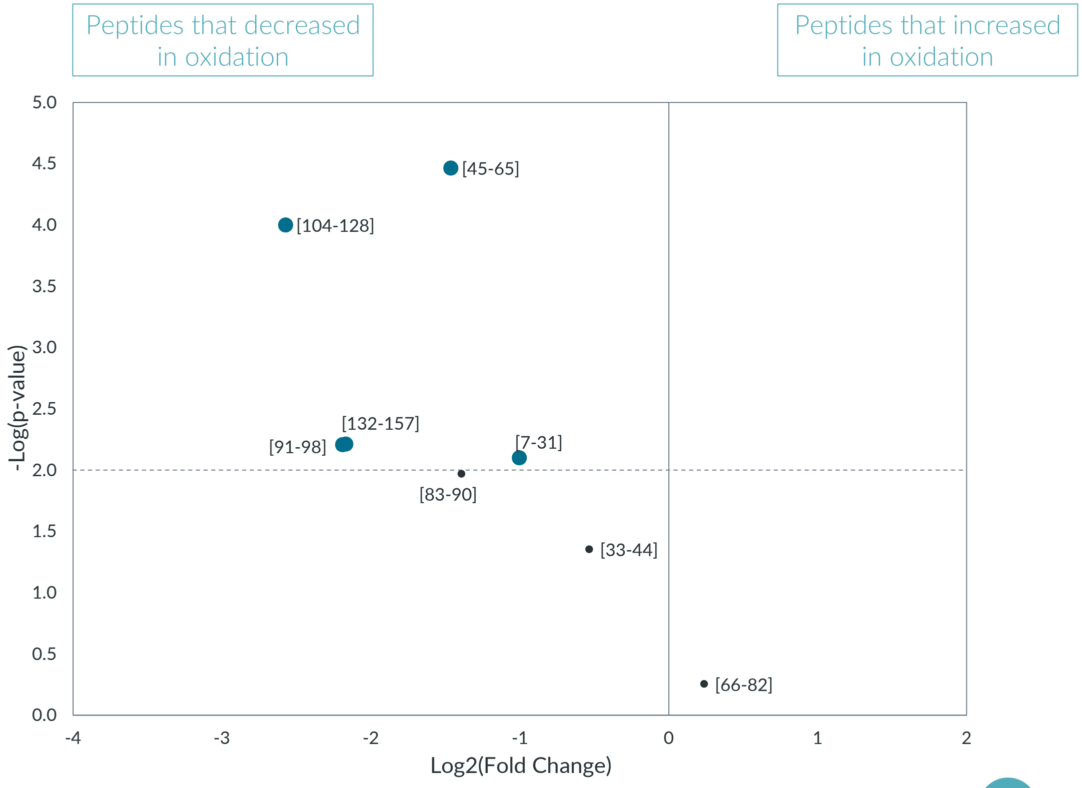
Modified (+16 Da) Peptide 2



TNFα – Adalimumab HRPF Characterized Epitope

Fox® System Case Study: Peptide Histogram and Volcano Plot



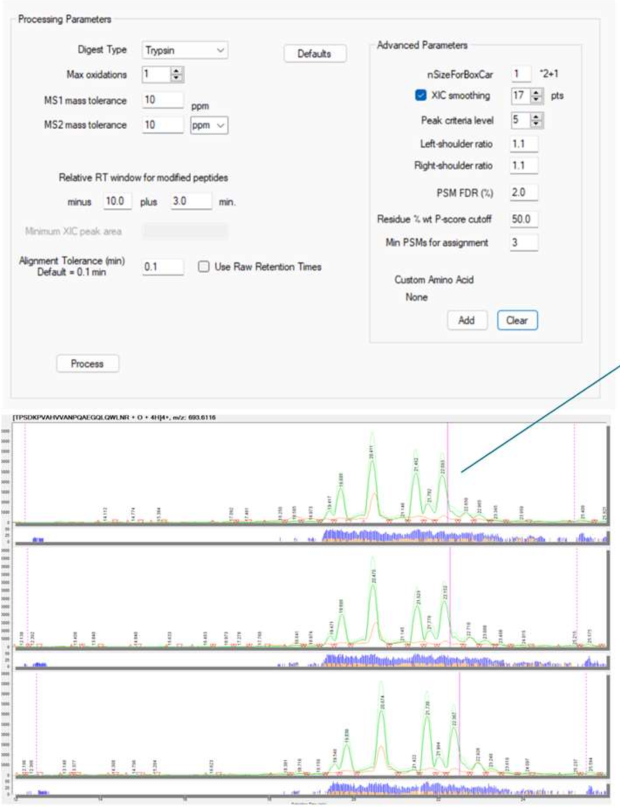


Peptides that decreased in oxidation

Peptides that increased in oxidation

TNFα – Adalimumab HRPF Characterized Epitope

Automated Data Analysis: Localizing Modifications with MS/MS



Processing Parameters

Digest Type: Trypsin

Max oxidations: 1

MS1 mass tolerance: 10 ppm

MS2 mass tolerance: 10 ppm

Relative RT window for modified peptides
minus 10.0 plus 3.0 min.

Minimum XIC peak area

Alignment Tolerance (min) Default = 0.1 min: 0.1

☐ Use Raw Retention Times

Advanced Parameters

nSizeForBoxCar: 1 *2+1

☑ XIC smoothing: 17 pts

Peak criteria level: 5

Left-shoulder ratio: 1.1

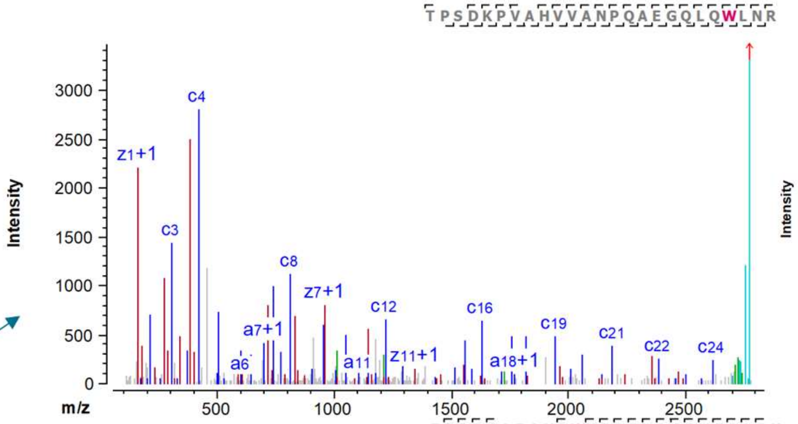
Right-shoulder ratio: 1.1

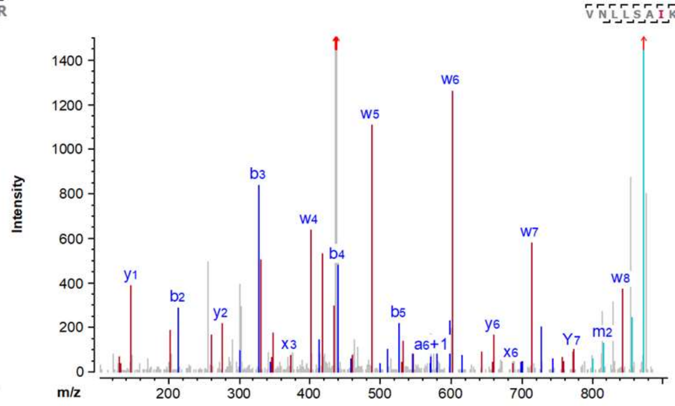
PSM FDR (%): 2.0

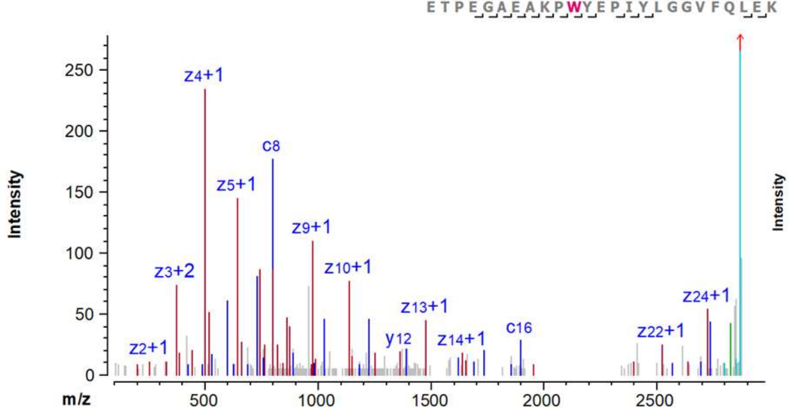
Residue % wt P-score cutoff: 50.0

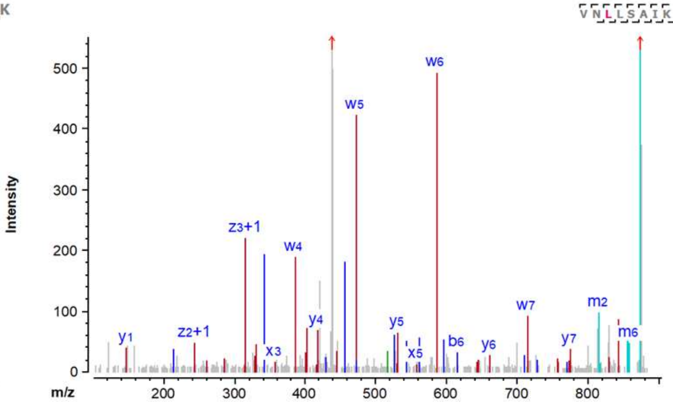
Min PSMs for assignment: 3

Custom Amino Acid
None



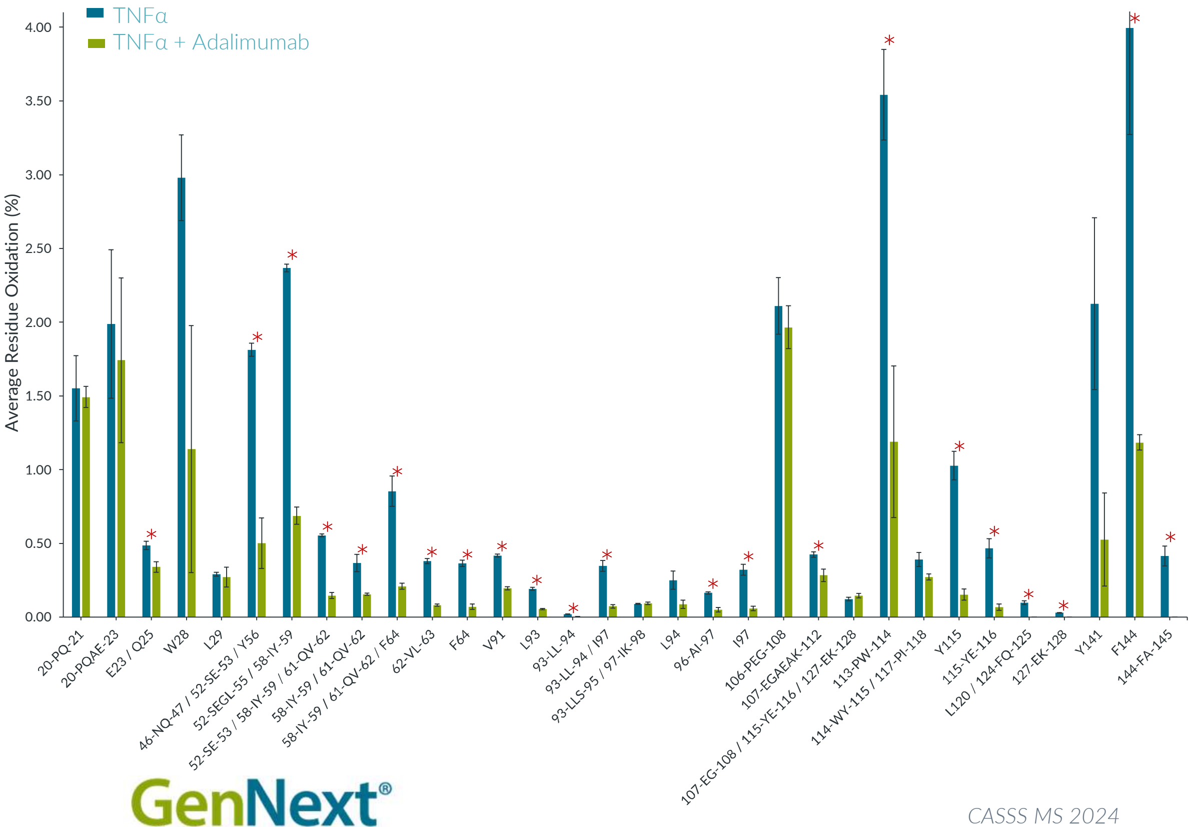






TNFα – Adalimumab HRPF Characterized Epitope

Fox® System Case Study: Residue Histogram and Volcano Plot



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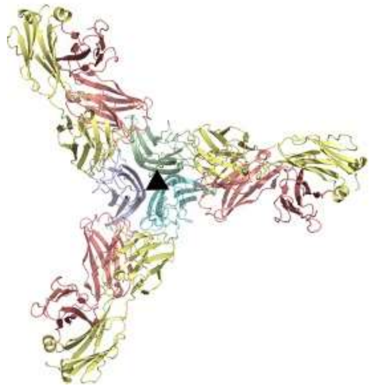
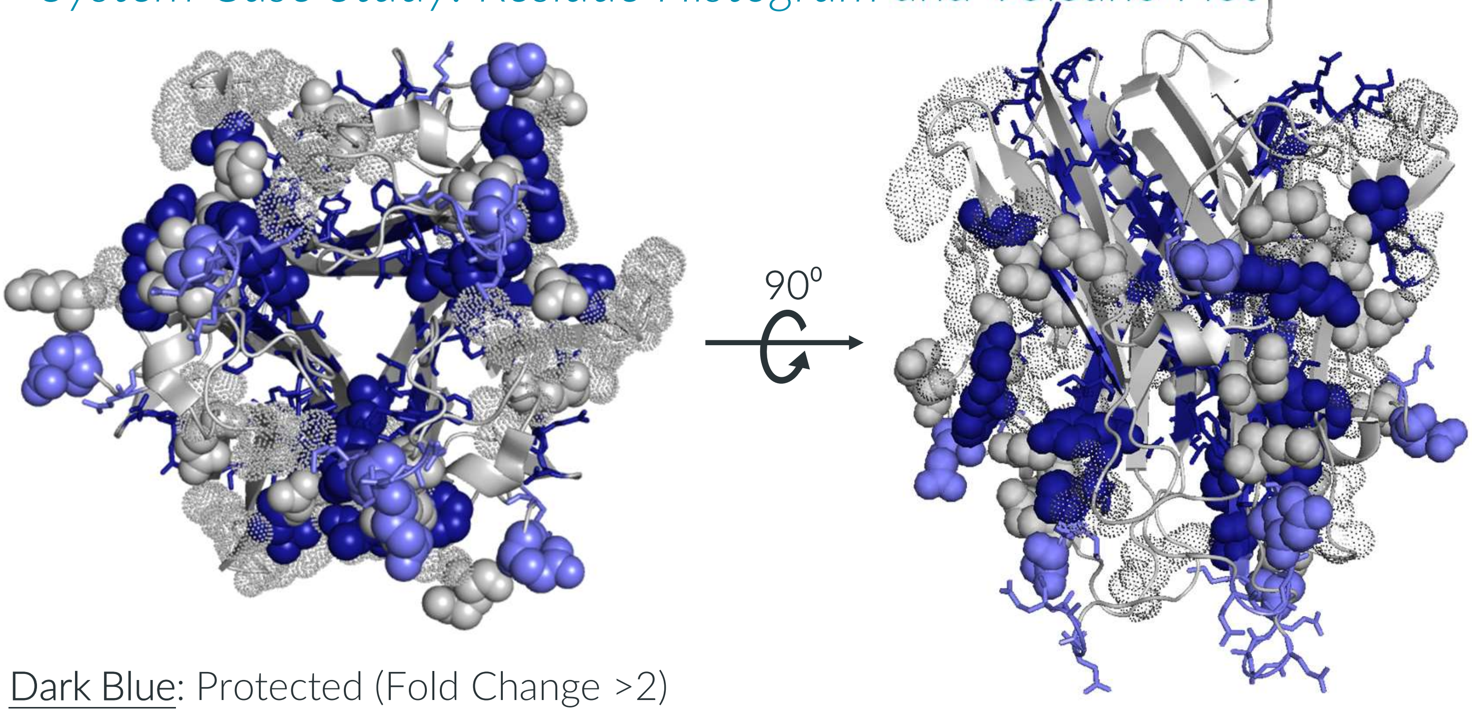
TNFα – Adalimumab HRPF Characterized Epitope

Fox® System Case Study: Residue Histogram and Volcano Plot



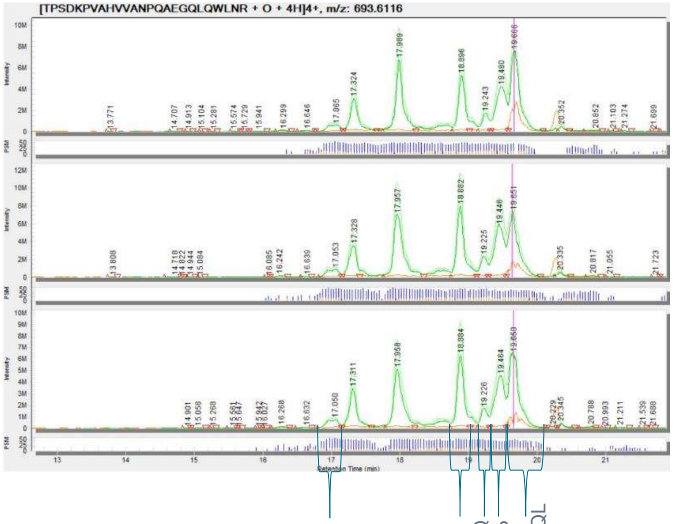
J Biol Chem. 2013 Sep 20; 288(38): 27059–27067. PDB ID 3WD5



Dark Blue: Protected (Fold Change >2)
Light Blue: Protected (Fold Change <2)
Spheres: Substituted residues that resulted in >10-fold decrease in Adalimumab binding
Dots: Other residues IDed but did not decrease Adalimumab binding

TNFα – Adalimumab HRPF Characterized Epitope

Fox® System Case Study: Can Ion Mobility Improve Residue Localization??

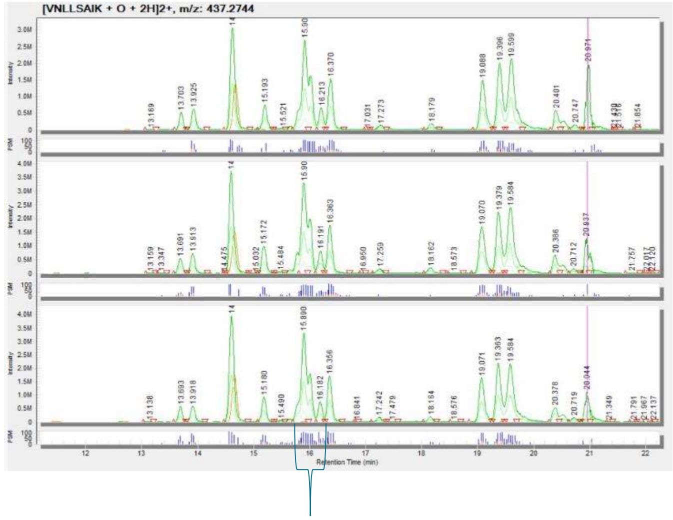


L-26 /
L-29

QW
QAEGQ
KPVANHVVANP
PQAEGQL

O
|
TPSDKPVAHVVANPQAEGQLQWLNR

O
|
TPSDKPVAHVVANPQAEGQLQWLNR



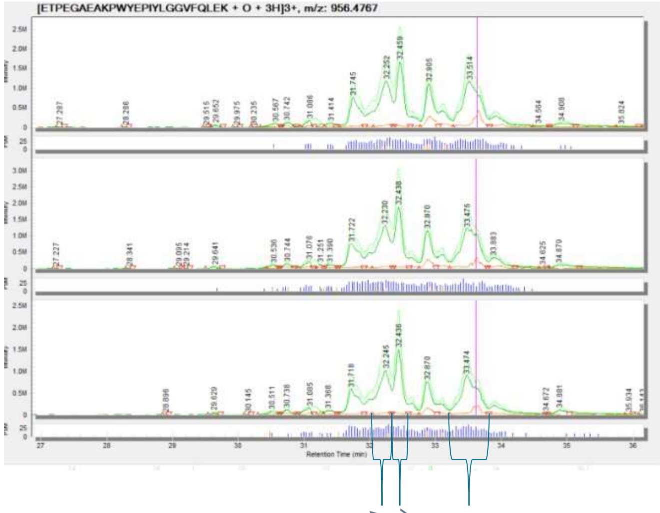
LSAI

O
|
VNLLSAIK

O
|
VNLLSAIK

O
|
VNLLSAIK

O
|
VNLLSAIK



PW
KPWY
PEAGEAK

O
|
ETPEGAEAKPWYEPIYLGGVFQLEK

O
|
ETPEGAEAKPWYEPIYLGGVFQLEK

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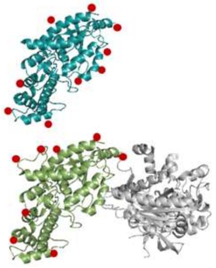
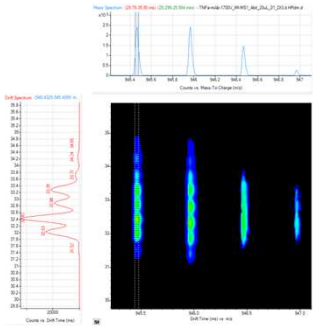
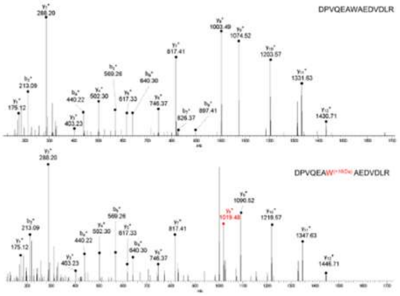
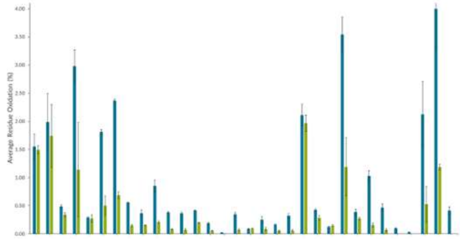
TNFα – Adalimumab HRPF Characterized Epitope

Fox® System Case Study: Can Ion Mobility Improve Residue Localization??









·OH

FPOP

Denature & Digest

Ion Mobility Separation

MS/MS

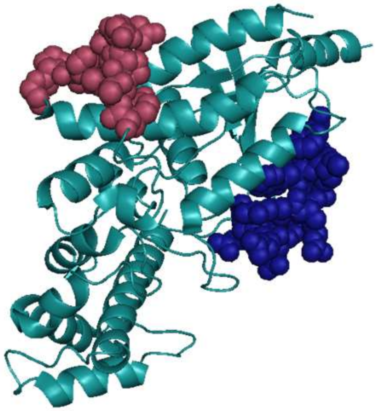
Data Process

Average Oxidation

- Samples were run with 1290 HPLC and 6560 IM-QTOF
  - MS1 with multiplexing
  - All Ions alternating frames with multiplexing

Mass Hunter IM-MS Browser and BioConfirm

- Peptide IDs
- Peak Areas
- Localize Mod Location



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Multiplexing and HRdm

6560 IM-QTOF

- When data is acquired in multiplex mode, 8 packets of ions are sent into drift cell through a series of on and off pulses
- When demultiplexed a boost in sensitivity is observed
- Taking it one step further and applying high resolution demultiplexing (HRdm) the algorithm fits a theoretical peak shape to the data which results in improved resolution
- The example to the right is a set of HRPF labeled peptides which appear as single group of data in the demultiplexed data, when HRdm is applied 6 peptides are separated out

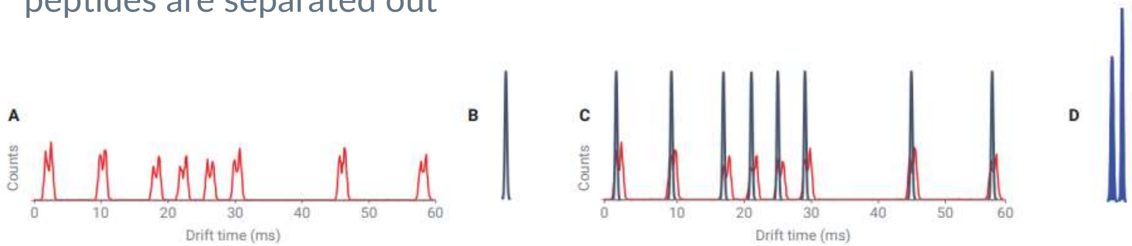
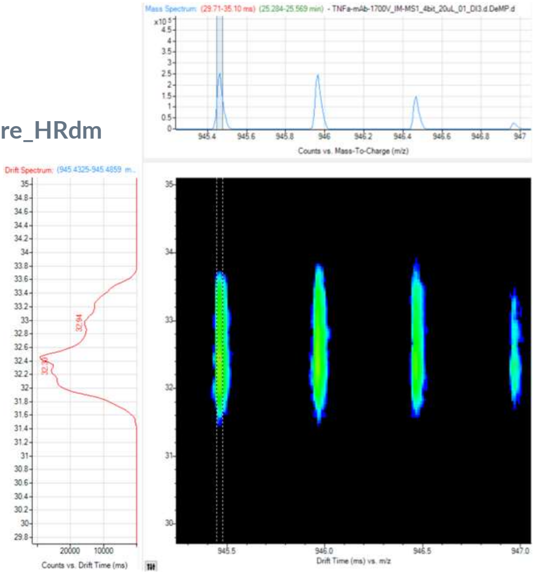


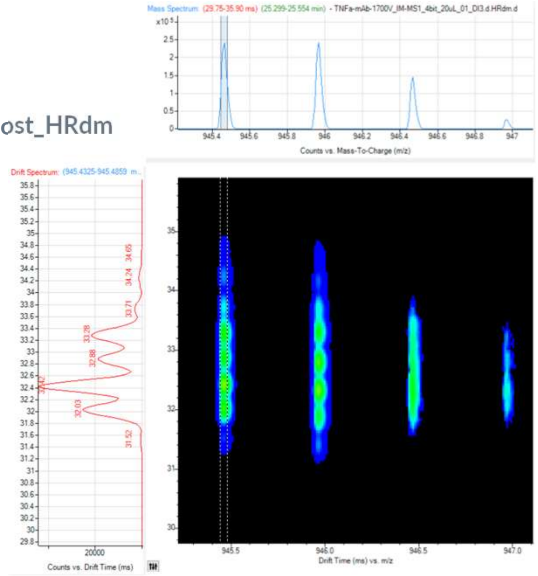
Figure 3. Illustration of demutiplexing and deconvolution process. (A) is the raw data prior to processing. The expected peak shape based on extensive system modeling is shown in (B). In (C) the theoretical peak is overlaid on the experimental data with the resulting deconvoluted and demultiplexed data shown in (D).

Wight, Julia W., et al. *HRdm 2.0: Maximize Your IM Resolution Without Sacrificing Drift Range, Mass Range, or Data Acquisition Time*. Agilent Technologies, Inc., 2022.

Pre_HRdm



Post_HRdm



Ion Mobility Separates Isobaric Peptides

Fox® System Case Study: Can Ion Mobility Improve Residue Localization??

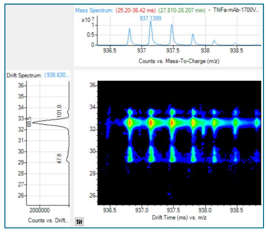
UnModified Peptide

Modified Peptide RT Peak #1

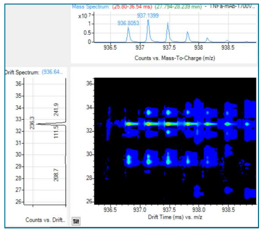
Modified Peptide RT Peak #2

99-125, z:3, VSYLSTASSLDY-WGQGTLVTVSS-ASTK

Pre_HRdm



Post_HRdm



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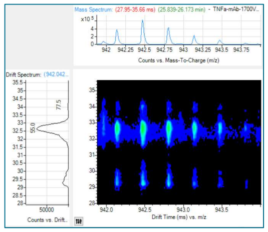

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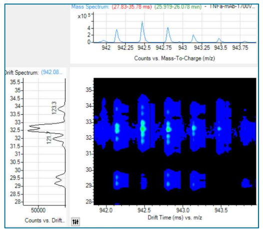

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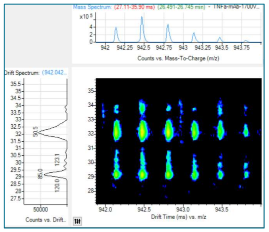

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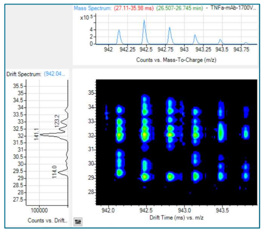
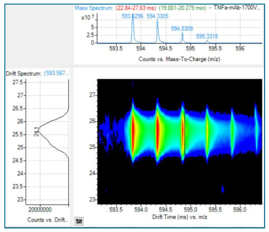
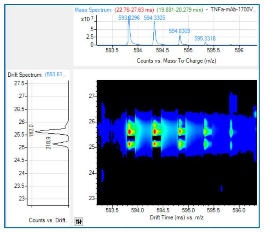


126-137, z: 2, GPSVFPLAPSSK

Pre_HRdm



Post_HRdm



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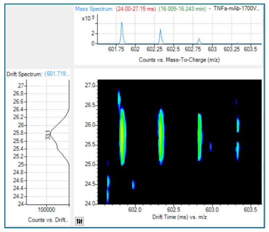

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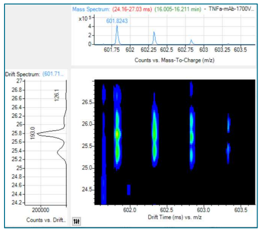

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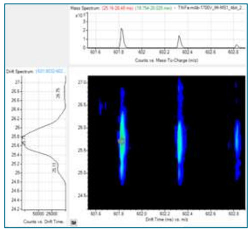

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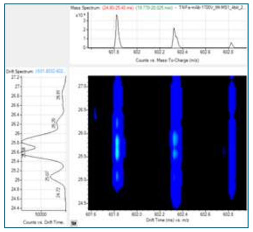

Thank You for Your Attention!

To learn more about HRPF, see us at booth #6 and Poster 123 and 149!

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