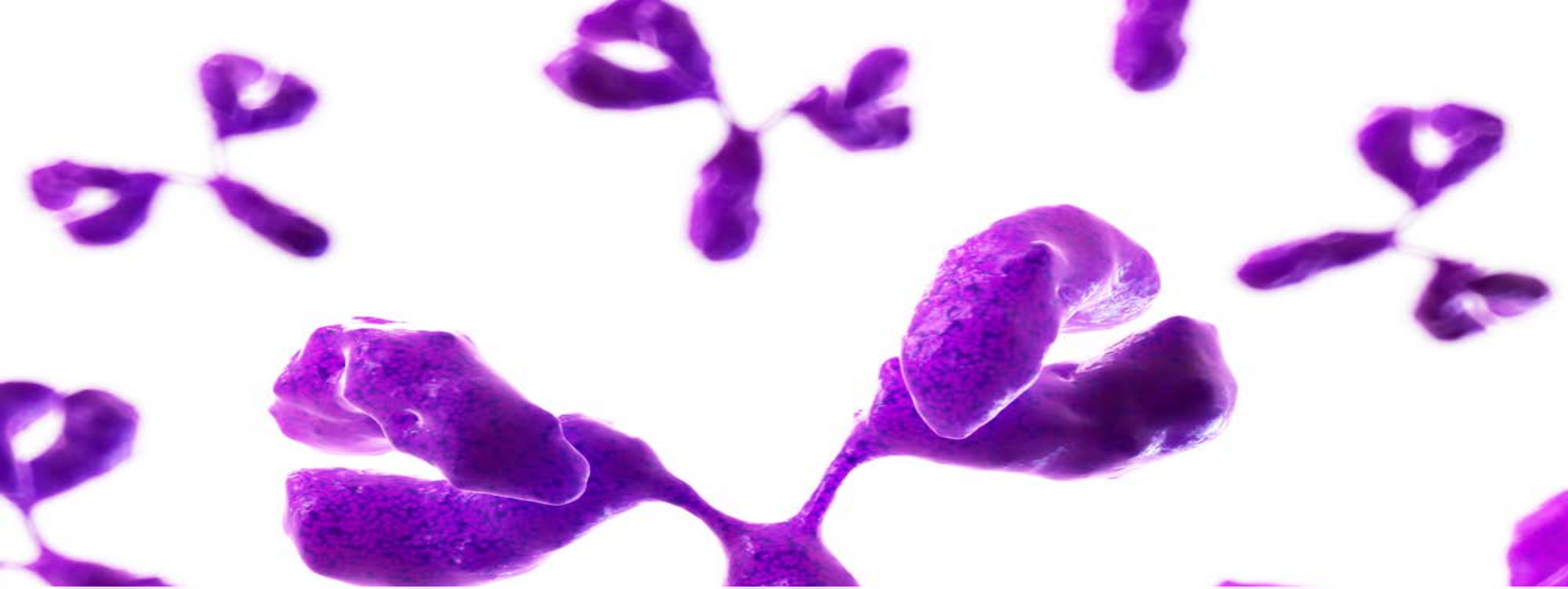


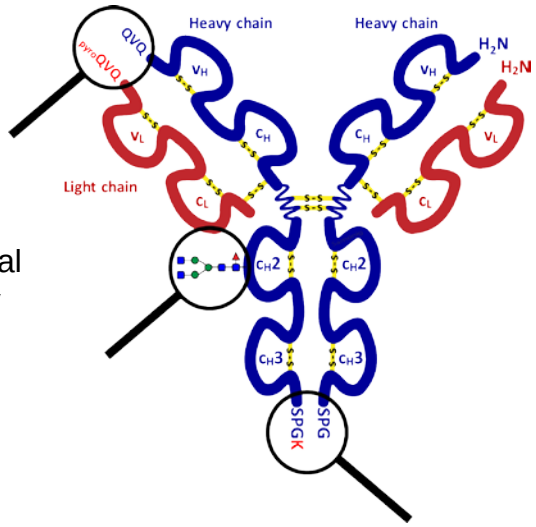
ThermoFisher SCIENTIFIC

Characterization of mAbs Using RP Separations and High Resolution Accurate Mass (HRAM) Mass Spectrometry

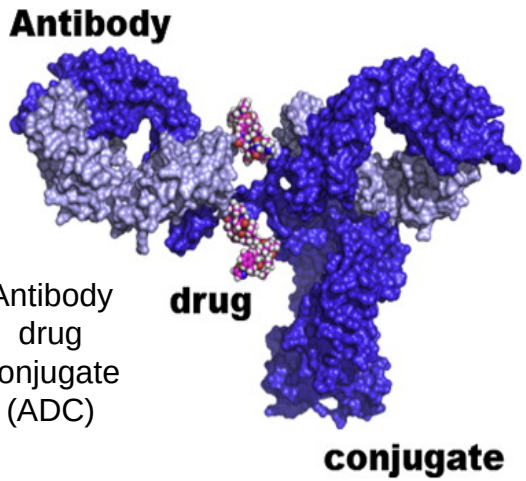
2016 Pharma Tour

Antibody Therapeutics – An Analytical Challenge

Monoclonal antibody (mAb)



Antibody drug conjugate (ADC)



Characterization

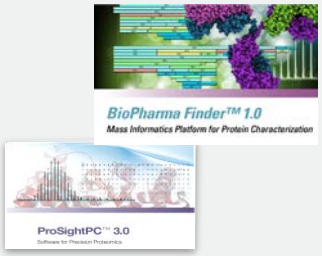
- to ensure proper biomanufacturing of the therapeutics
- to ensure critical quality attributes (CQA's) of a product are identified
  Modifications, impurities, immunogenicity, efficacy, DMPK
- to ensure these CQA's can be routinely measured
  Mass spectrometers, separations, software, reagents

- Deamidation (+1 Da)
- Oxidation (+16 Da)
- Disulfide bond scrambling
- Sequence truncation
- Change in glycosylation
- Low abundance host cell proteins
- Low abundance sequence variants
- Aggregation, protein complex
- Drug Antibody Ratio's
- Linker measurements
- Bound vs unbound drug
- etc

MS Tools for Major Biopharma Characterization Workflows

Thermo Scientific™ Q Exactive™ MS Family

For **all the routine characterization workflows**: intact/top-down, bottom-up, glycan, etc. qualitive and quantitative.



Thermo Scientific™ Exactive™ Plus EMR MS

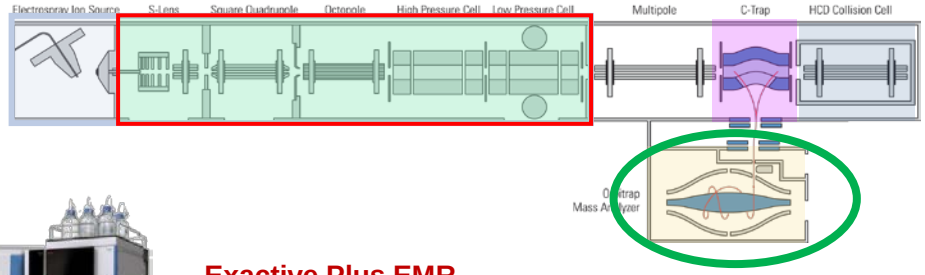
Native MS to analyze large protein complex while preserving non-covalent interactions: antibody-antigen complex, ADCs, antibody mixtures, etc.

Thermo Scientific™ Orbitrap Fusion™ Tribrid™ MS Family

From routine to the most challenging tasks. The most advanced technology that provides the most comprehensive solutions.

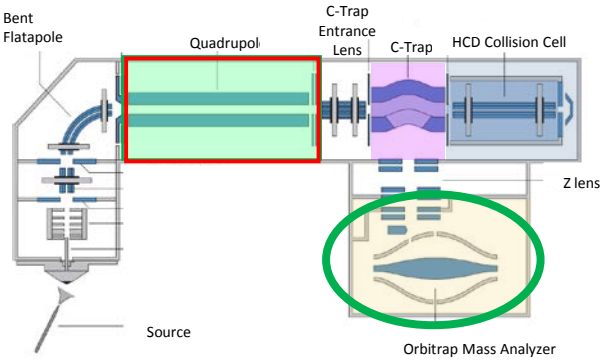
ThermoFisher SCIENTIFIC

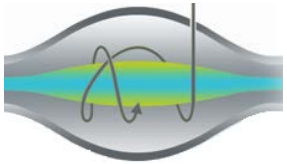
The Orbitrap™ in Hybrid and Non-Hybrid Systems

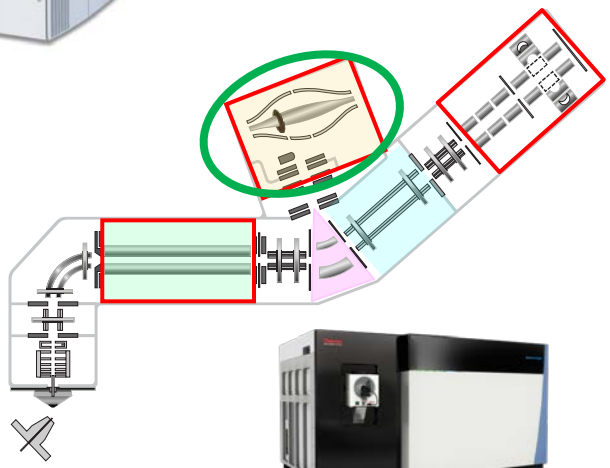


LTQ Orbitrap XL™
Orbitrap Elite™

Exactive Plus EMR
Q Exactive
Q Exactive Plus
Q Exactive HF



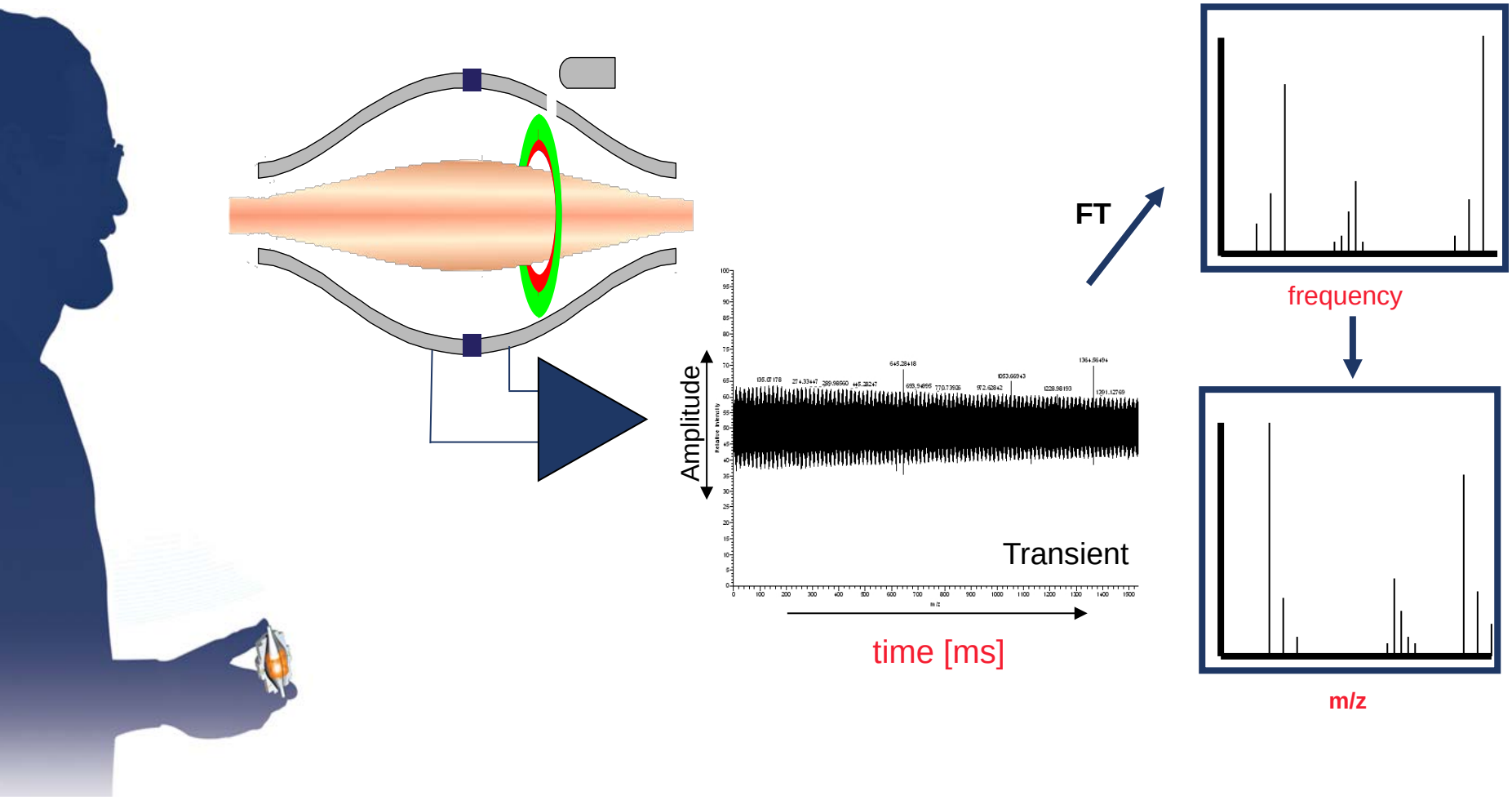




Orbitrap Fusion Tribrid
Orbitrap Fusion Lumos™ Tribrid

ThermoFisher SCIENTIFIC

The Orbitrap Principle



ThermoFisher SCIENTIFIC

The Thermo Scientific™ Vanquish™ UHPLC System… A Perfect Front-end!

Vanquish Platform

- Improved retention time precision
- Biocompatible by default
- Increased sample capacity
- Intelligent sample Pre-compression
- Improved sample cooling
- Multiple heating modes
- Active and passive pre-heating
- 4 Detection options
- Enhanced LC/MS control with Sii and Chromeleon
- Viper™-based, tool-free fluidic connections

Vanquish

- 1500 bar compatible system
- Binary pump (HPG)
- Ultra low Gradient Delay Volume

Separations based on power

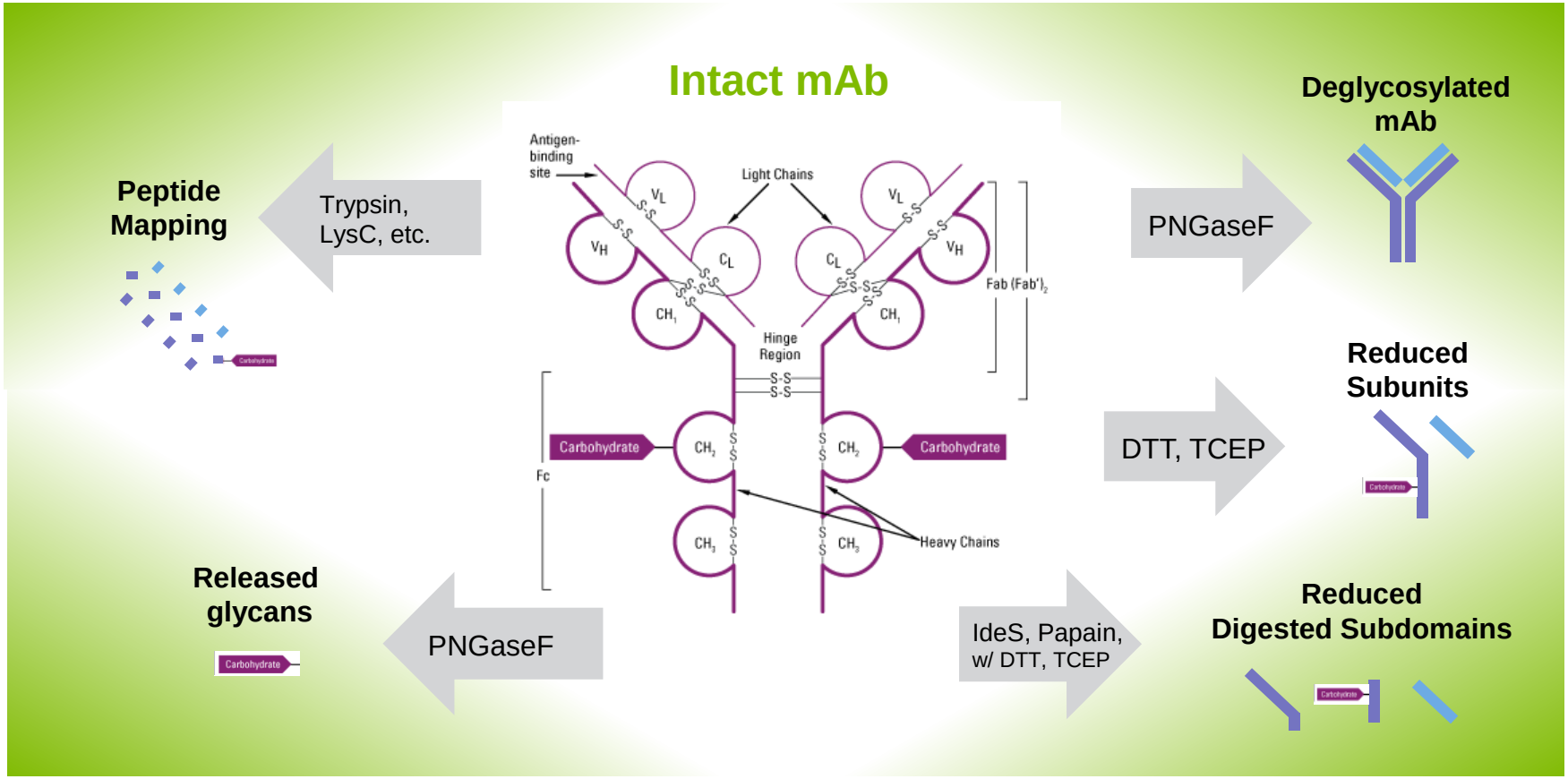
Vanquish Flex

- 1000 bar compatible system
- Quaternary pump (LPG)
- Medium Gradient Delay Volume

Separations based on chemistry

ThermoFisher SCIENTIFIC

Antibody Characterization Roadmap



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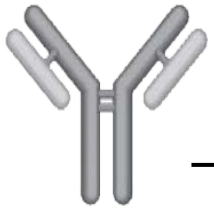
Agenda

Major Characterization Workflows

- Characterization of intact antibody and related products
  - **Intact mAb and ADC mass measurement**
  - Sub-Unit Analysis
  - Top-down and middle-down sequencing

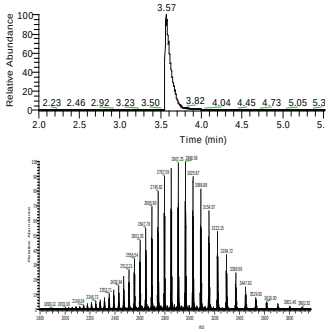
Intact mass Analysis

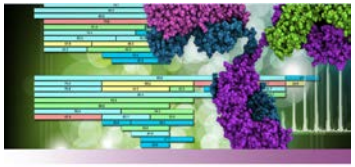


IgG
(150 kDa)

LC-MS

(LC: in native or denaturing conditions)





BioPharma Finder™ 1.0

Mass Informatics Platform for Protein Characterization

Output

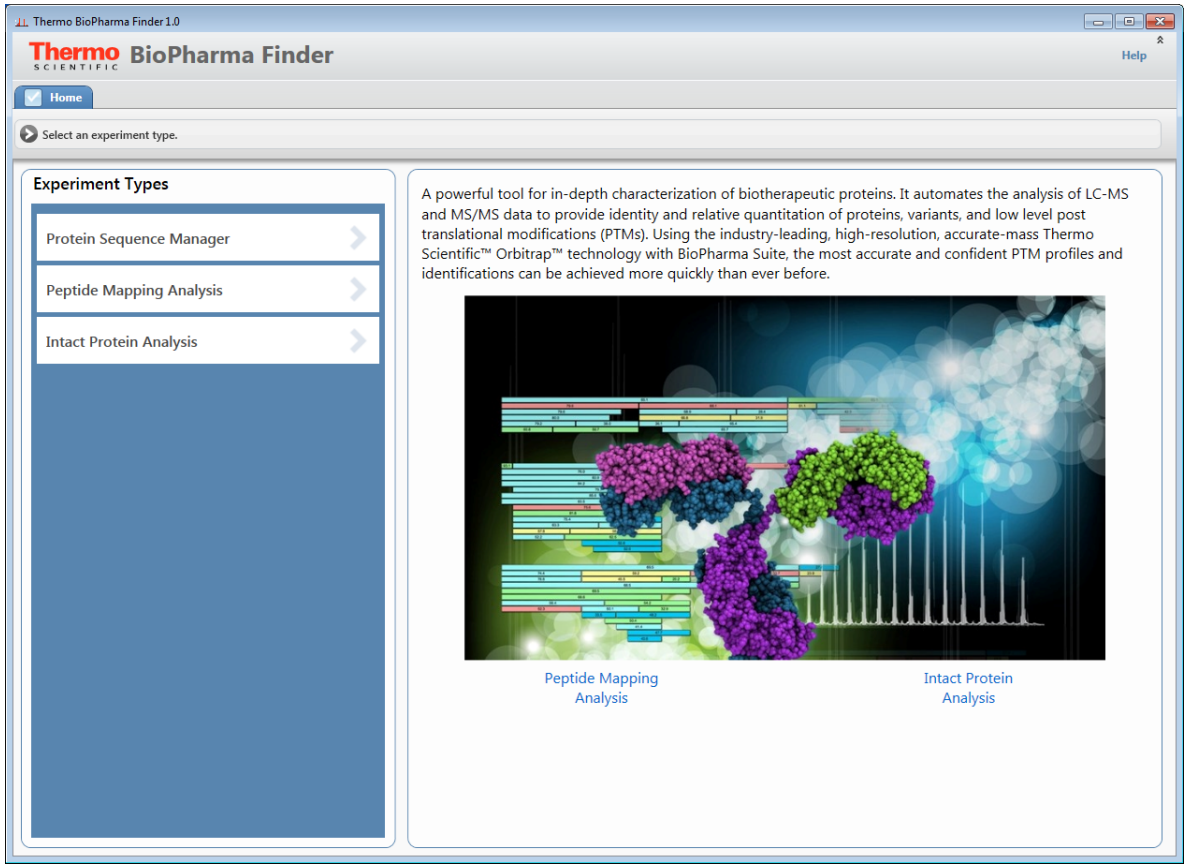
- Confident deconvoluted molecule weight.
- Identify glycoforms using the intact mass.
- Identify Antibody Drug Conjugates (ADC).

ThermoFisher SCIENTIFIC

BioPharma Finder™ 1.0

One software package for

- **Protein Sequence Management**
- **Peptide Mapping**
- **Intact Analysis**



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BPF 1.0 – Protein Sequence Manager

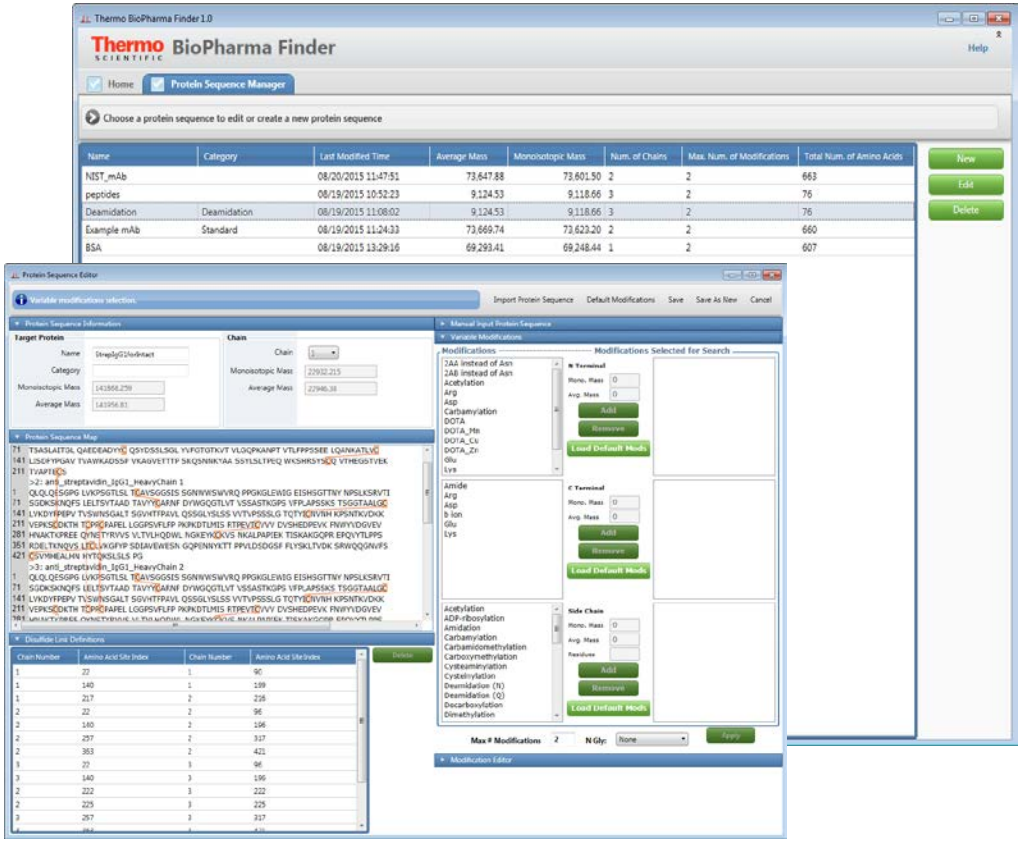
Protein Sequence Manager

Saves time

- Starting point for the software.
- Database for storing protein sequences similar to Proteome Discoverer.
- Both peptide mapping and intact analysis workflow choose protein sequence from this central location.

Easy to use

- Import FASTA file or paste the sequence into a text box.
- Define multiple chains (e.g. two light chains, two heavy chains).
- Define disulfide bonds (information used for intact analysis)
- Choose fixed modifications
- Select variable modifications and list of possible glycosylation structures.
- User definable default modification list.



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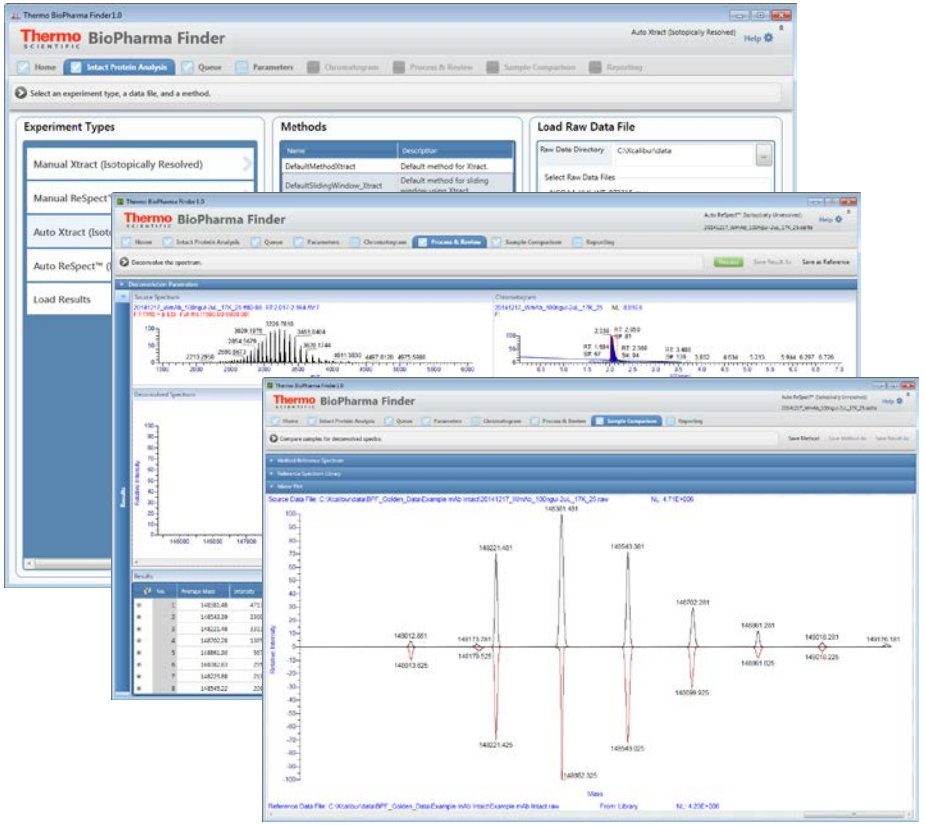
BPF 1.0 – Intact Protein Analysis

Intact Protein Analysis

- Determine profiles of intact masses
- Automated processing, simplified results and improved quantitation
- Sliding window algorithm for improved **ADC** analyses and complex data sets
- Target protein sequence matching – identifies n-linked glycoylations and other common modifications.
- **Native MS**
- Ion trap support (ReSpect™ deconvolution only)

Sliding Window Deconvolution

- Produces a single deconvoluted spectrum of all components in the sample regardless of elution time or profile
- Eliminates the need for the user to define the source spectra for deconvolution.
- Works for both simple and highly complex protein mixtures
- Removes the challenges of chromatographic peak picking
- Works with Xtract™ and ReSpect deconvolution



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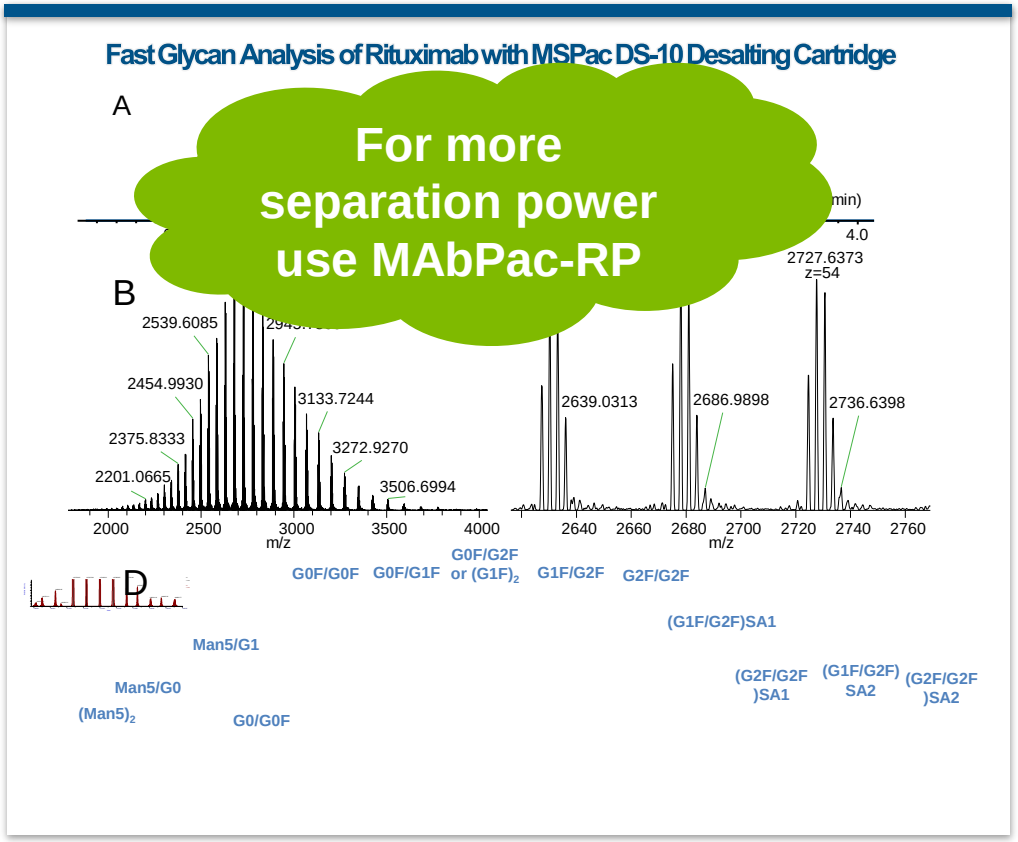
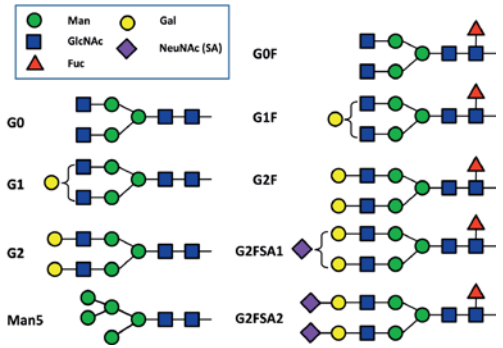
Intact Protein Analysis



Download Application Note 21465: Fast online desalting of mAbs using a reversed phase desalting cartridge for LC-MS analysis

- A fast 4 minutes desalting method for high throughput glycan characterization.
- Intact mass and the relative glycoform abundance of a mAb within 5 minutes.
- In-depth characterization for glycoforms detection below 1% relative intensity.
- Broadly applicable to other mAbs and similar biopharmaceutical compounds.



Carbohydrate structures commonly observed on antibodies.

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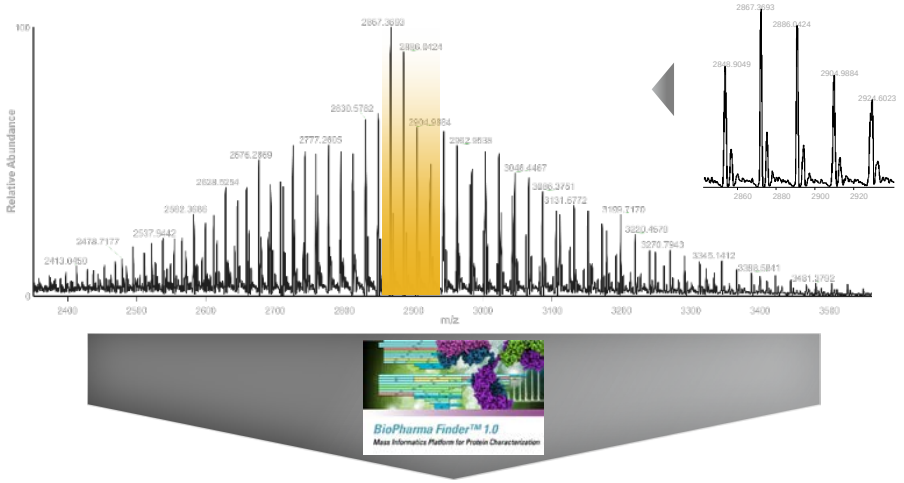
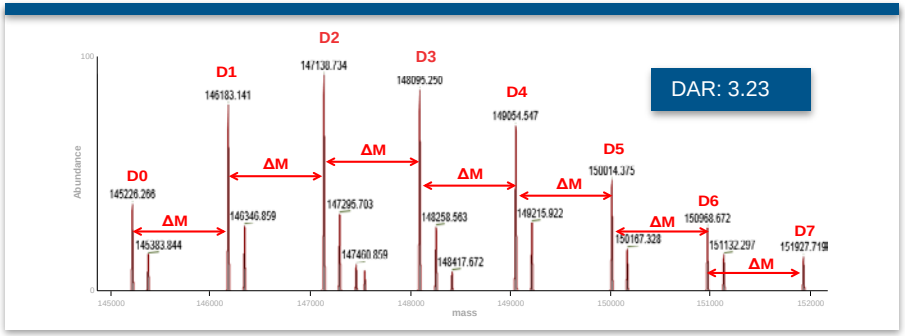
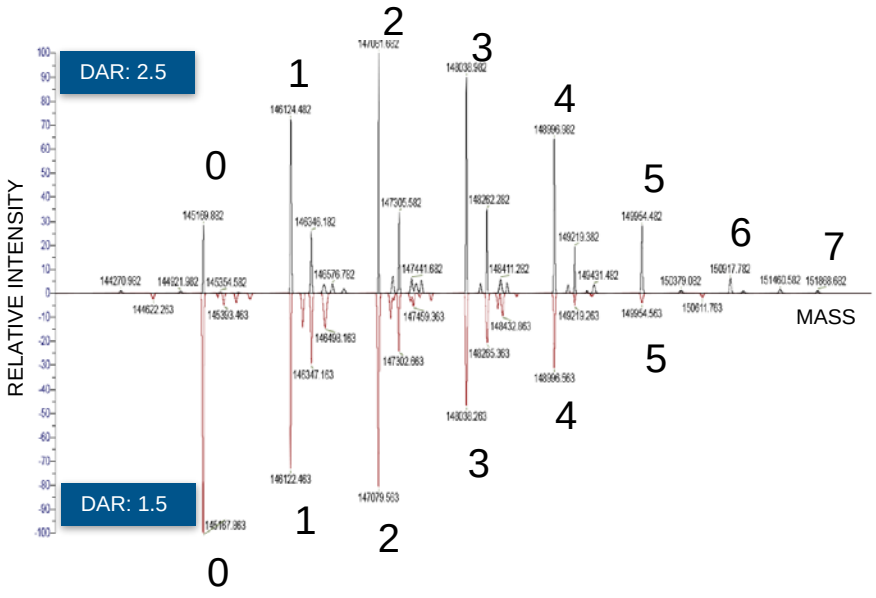
MS of Intact Lysine-linked ADC





Mirror plot of two ADC samples



Q Exactive, Resolution Setting = 17.5 K

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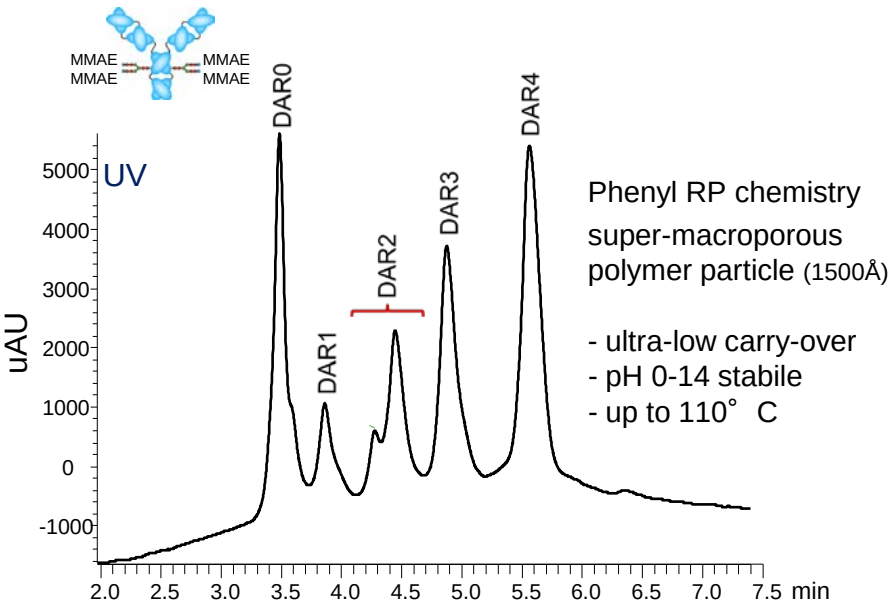
Separation of ADC

by Reversed Phase
for direct coupling with MS

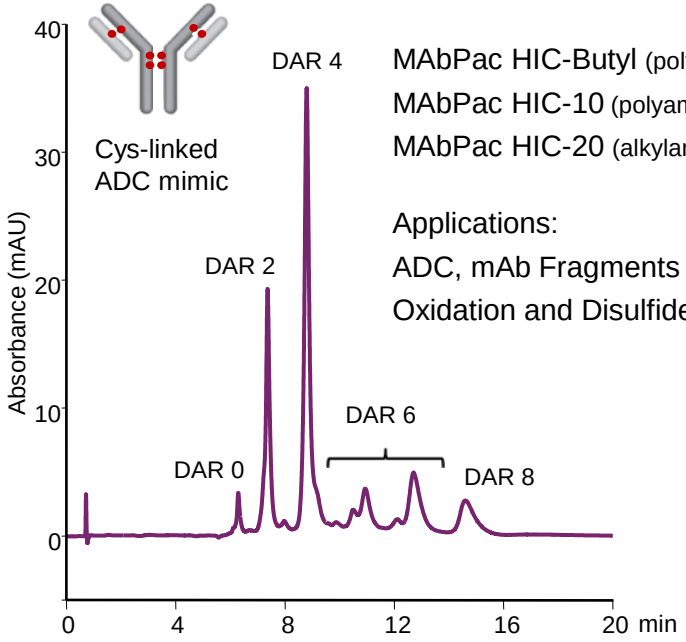


Phenyl RP chemistry
super-macroporous polymer particle (1500Å)

- ultra-low carry-over
- pH 0-14 stabile
- up to 110° C

MAbPac RP (4 µm) 2.1 × 50 mm

by Hydrophobic Interaction (HIC)



MAbPac HIC-Butyl (polymer, non-porous)
MAbPac HIC-10 (polyamide, silica, 1000Å)
MAbPac HIC-20 (alkylamide, silica, 1000Å)

Applications:
ADC, mAb Fragments and Aggregates
Oxidation and Disulfide bridge variants

MAbPacHIC-Butyl (5 µm) 4.6 × 100 mm

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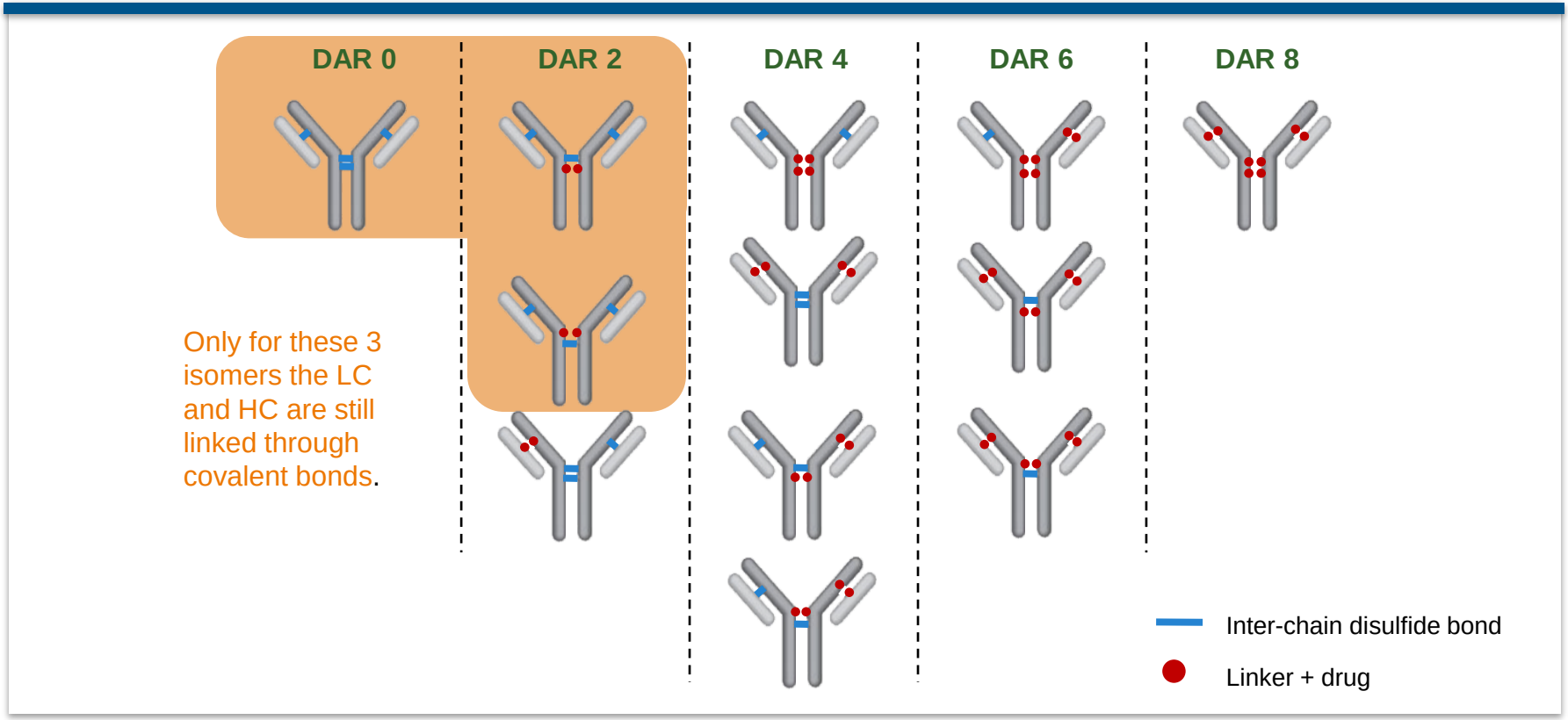
MS of Intact Cysteine-linked ADC



Cysteine linked ADC requires native MS for intact analysis

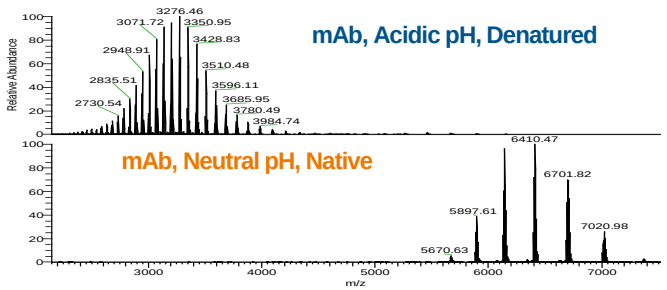
Native MS using Exactive Plus EMR

Performing native MS

- An ESI compatible **volatile solution with near neutral pH** is used to prepare protein samples.
  e.g. 50 mM ammonium acetate pH 6.9
- Under these conditions, the ionized proteins carry fewer charges than those produced by ESI in acidic, denatured condition.
- Therefore, **in native MS, protein ions are detected at a significantly higher m/z range (>5000 -10,000 m/z for antibody complexes)** than conventional MS in acidic condition.

Exactive Plus EMR

- Extended mass range up to m/z 20,000
- Unmatched Desolvation using in-source CID and in HCD cell
- Improved high mass transmission for large protein assemblies



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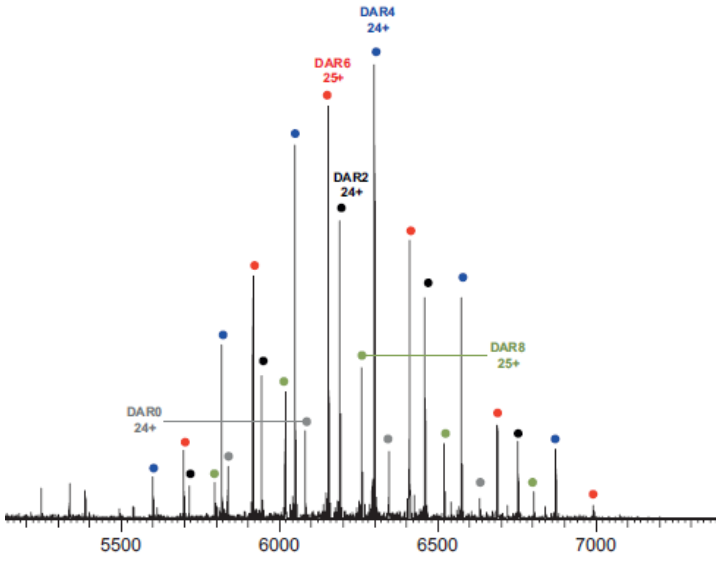
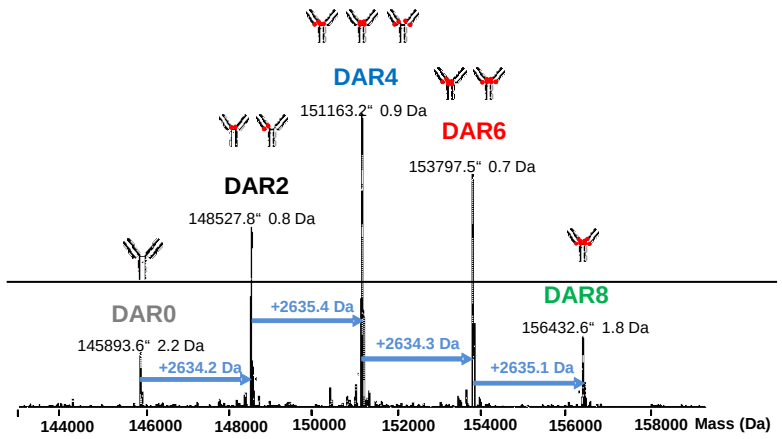
Native MS of an ADC Brentuximab Vedotin

Raw spectrum



Exactive Plus EMR, 35K

Deconvoluted spectrum



$$\overline{DAR} = \frac{\sum_{0}^{8} nA_{DAR_n}}{\sum_{0}^{8} A_{DAR_n}} = 4.2$$

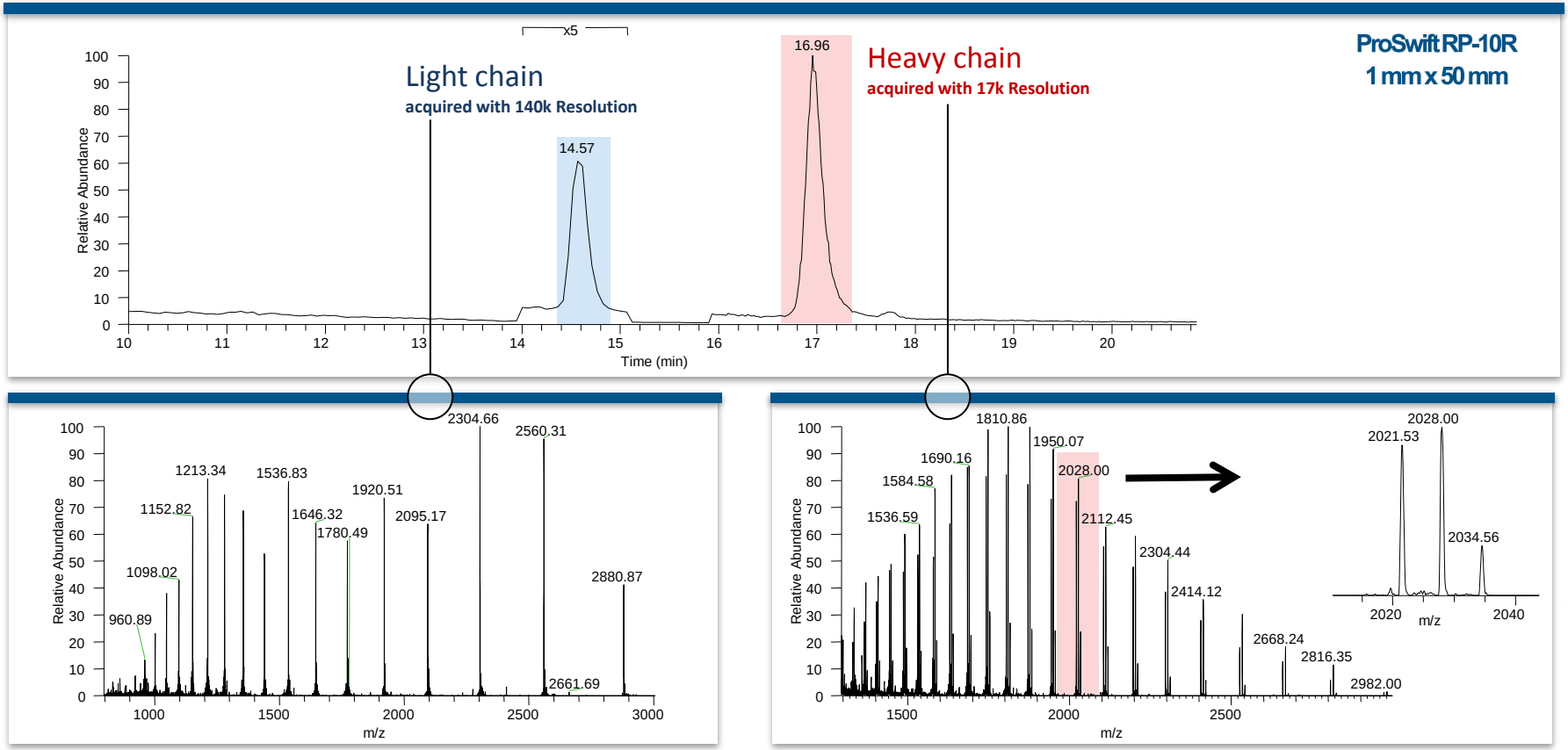
ThermoFisher SCIENTIFIC

Agenda

Major Characterization Workflows

- Characterization of intact antibody and related products
  - Intact mAb and ADC mass measurement
  - **Sub-Unit Analysis**
  - Top-down and middle-down sequencing

Reduced Rituximab



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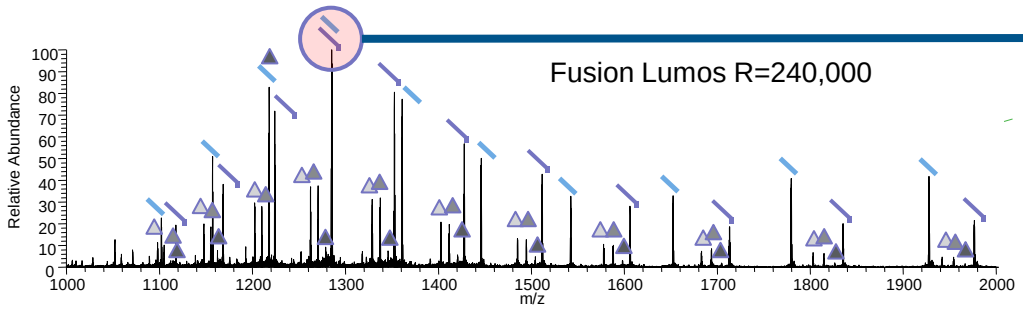
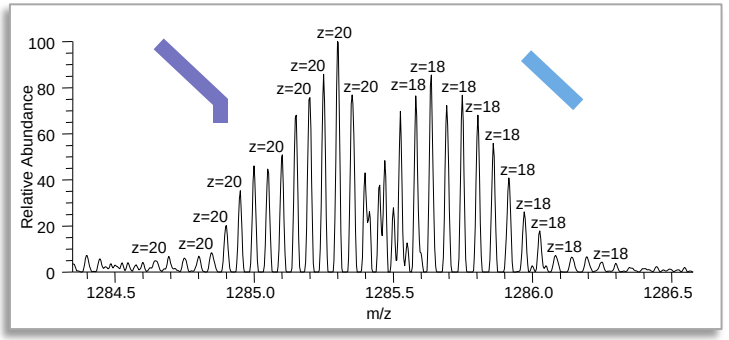
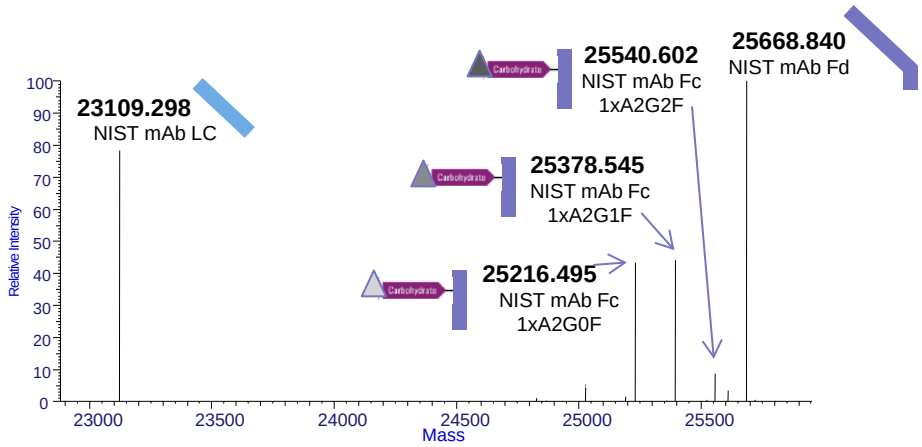
Rapid Subdomain Analysis of NIST mAb







Xtract™ deconvolution

Matched Sequence	Matched Delta Mass (ppm)	Relative Abundance
Fd	**1.05**	100.0000
LC	**2.50**	86.4514
Fc;1xA2G1F	**2.34**	34.9027
Fc;1xA2G0F	**2.47**	34.8168
Fc;1xA2G2F	**2.48**	3.6534

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Agenda

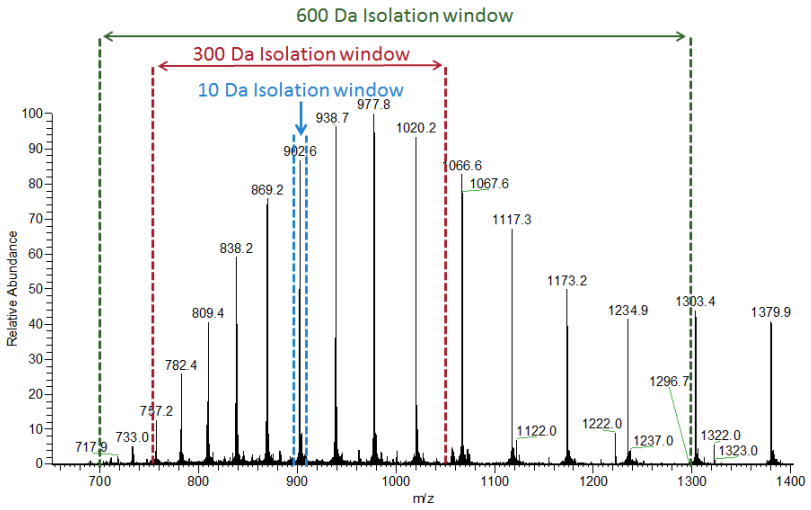
Major Characterization Workflows

- Characterization of intact antibody and related products
  - Intact mAb and ADC mass measurement
  - Sub-Unit Analysis
  - **Top-down and middle-down sequencing**

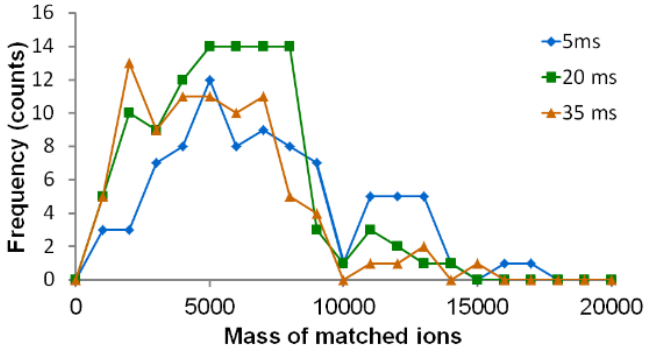
Top-Down Sequencing

Main parameters that can be controlled for ETD fragmentation on an Orbitrap Fusion mass spectrometer

- Isolation window



- AGC target for precursor ions and reagent
- Reaction time
- Supplemental energy



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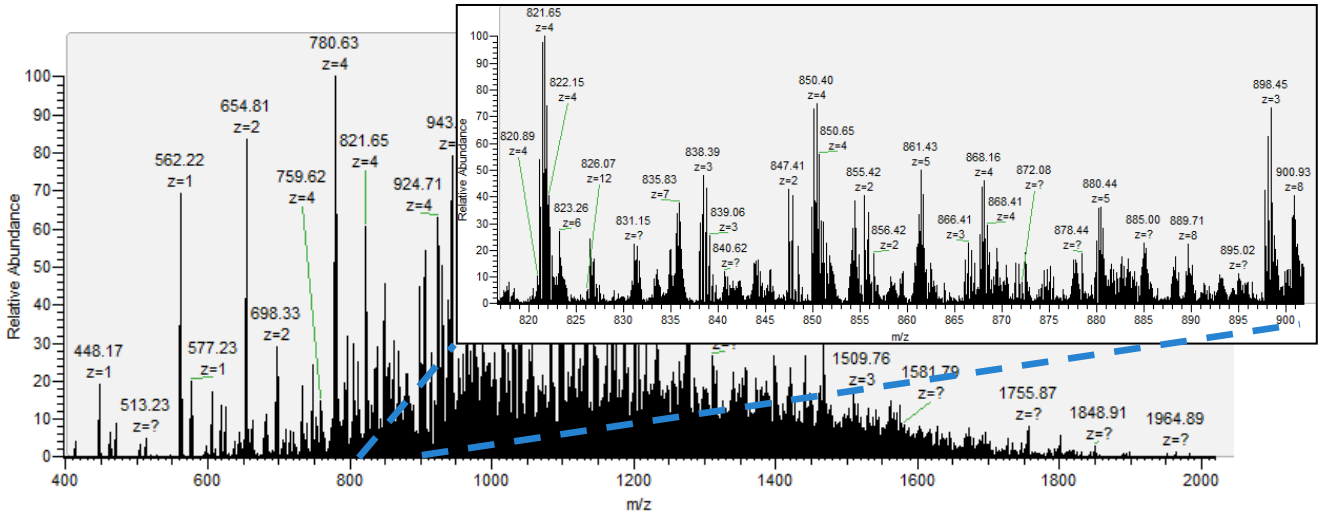
Top-Down sequencing

Light Chain of Trastuzumab

ETD settings: 300 Da isolation window, 3E5 precursor, 7E5 reagent, 10 ms reaction time



Due to the complexity of the spectra in top-down analysis, high resolution is required

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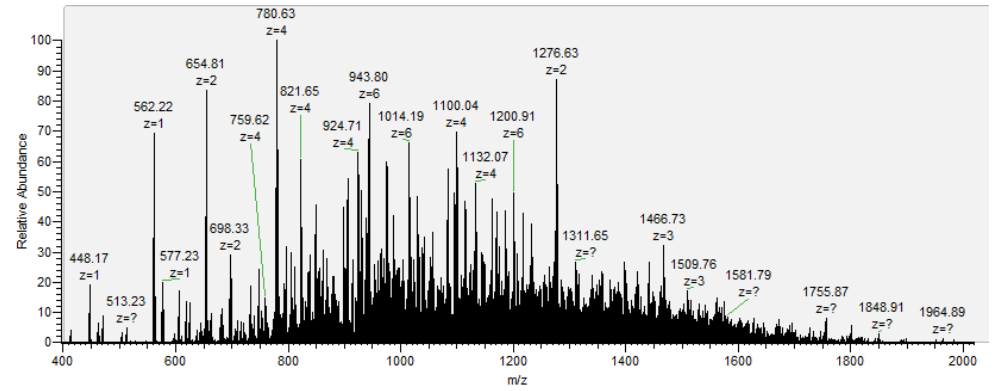
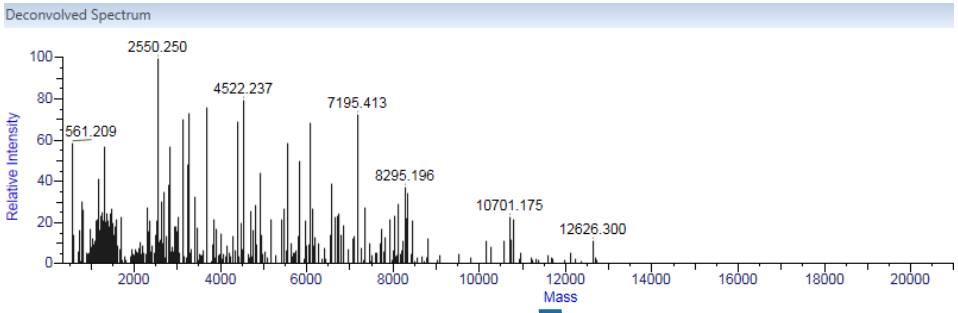
Top-Down Sequencing



Deconvolution



Precursor Mass	
Type:	Monoisotopic
Observed:	23,428.53
Theoretical:	23,428.52
Mass Diff. (Da):	0.001
Mass Diff. (ppm):	0.06

% Fragments Expl...	23 %
% Residue Cleava...	48 %

N [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 25
26 S [Q] D V N T [A] V A W [Y] Q Q K P G [K] A P [K] L [L] I [Y] S 50
51 [A] S [F] L Y [S] G [V] P S [R] F [S] G [S] [R] S G [T] D [F] T [L] T [I] 75
76 [S] [S] L [Q] P E [D] F [A] T Y Y C Q Q H Y T T P P T F [G] [Q] 100
101 [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 125
126 [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 150
151 [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 175
176 [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 200
201 [L] [S] [S] P [V] [T] K [S] F [N] R G E C C

Matching

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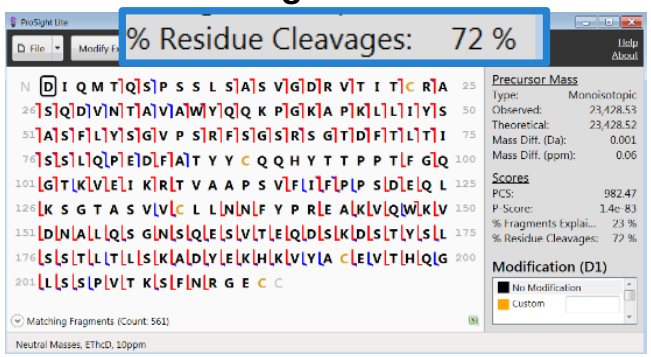
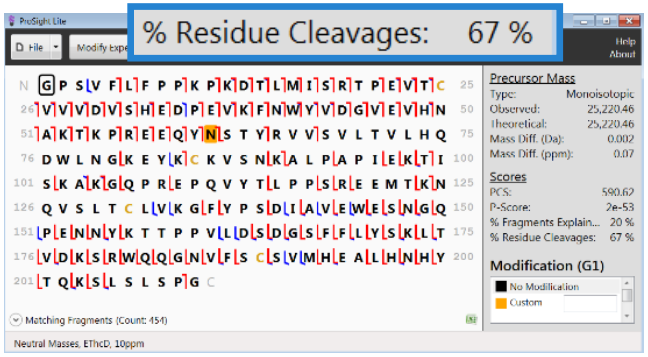
Top-Down sequencing

High sequence coverage for the light chain, Fc and Fd were obtained from the combined ETD and EThcD experiments.

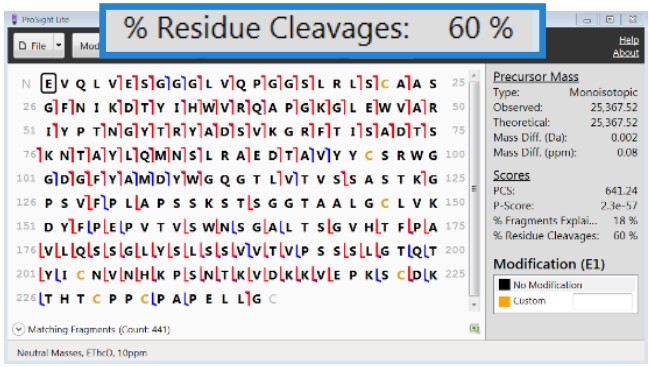
Light Chain



Fc



Fd



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Improved Coverage For Top Down IgG Analysis

Light Chain (LC)

Heavy Chain (HC)

Comprehensive Sequence Map

- Reduced IgG, infusion at 3 ul/min
- Individual charges states of both proteins were isolated and fragmented with CID, ETD and HCD
- 240,000 resolution
- Improved sequence coverage with Advanced Vacuum Technology and ETD HD

	OT FUSION	OTF LUMOS	GAIN
LC	77%	91%	18%
HC	46%	63%	37%

ThermoFisher SCIENTIFIC

Closing

Acknowledgements

North America

- Stephane Houel
- Terry Zhang
- Aaron Bailey
- Michael Blank
- Jonathan Josephs

North America

- Jennifer Sutton
- Seema Sharma
- David Horn
- Shanhua Lin
- Mark Sanders
- Ken Miller

Europe

- Martin Samonig
- Kai Scheffler
- Yue Xuan
- Eugen Damoc
- Remco Swart

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