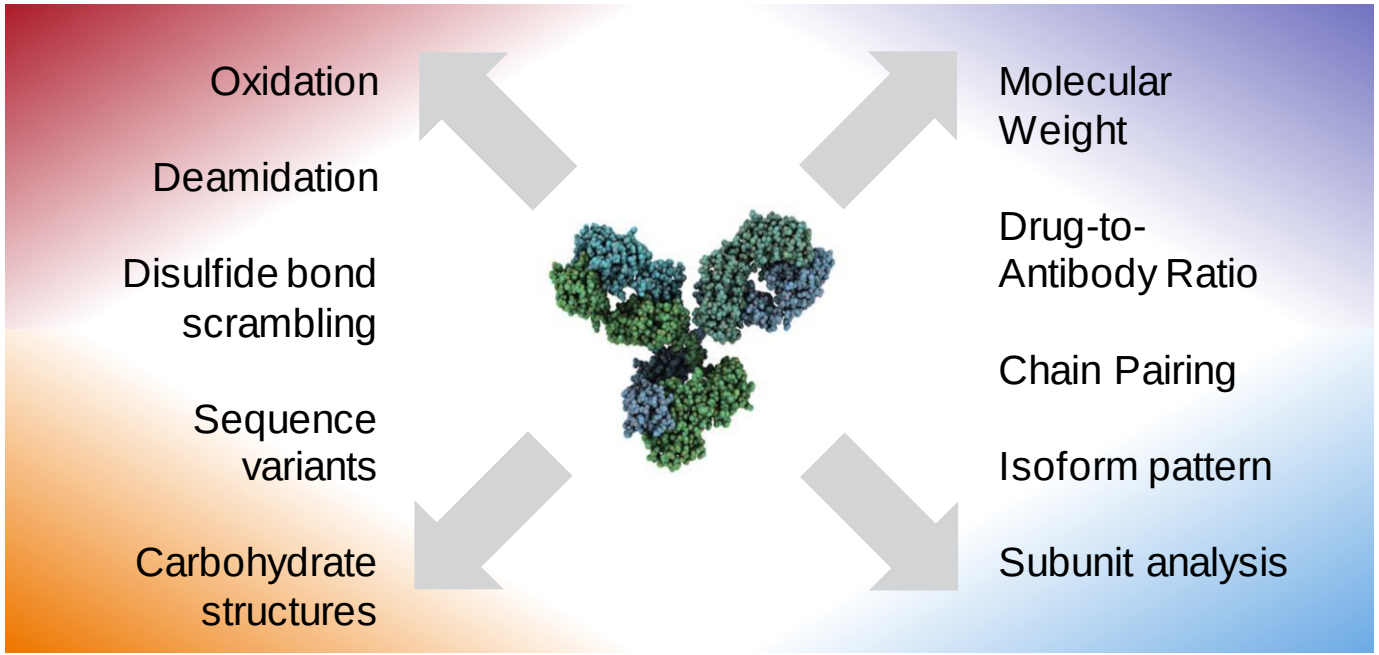
ThermoFisher SCIENTIFIC

ADC characterization on a single platform with Q Exactive BioPharma

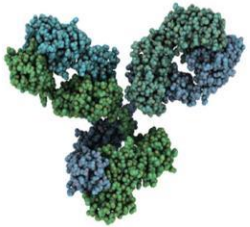
Global BioPharma Summit

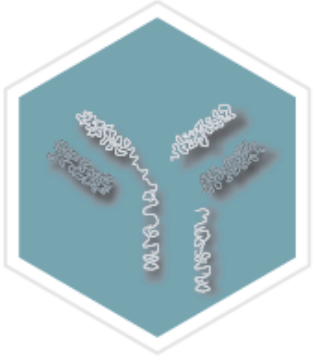
Understanding Microheterogeneity and Critical Quality Attributes (CQAs)

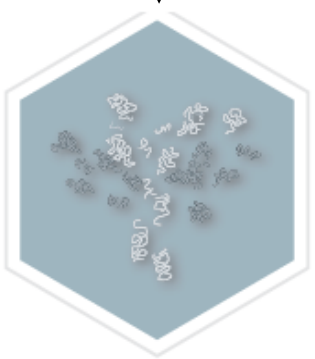


Comprehensive characterization of therapeutic protein CQAs requires analysis from multiple perspectives.

Characterization at the Intact, Subunit and Peptide Level



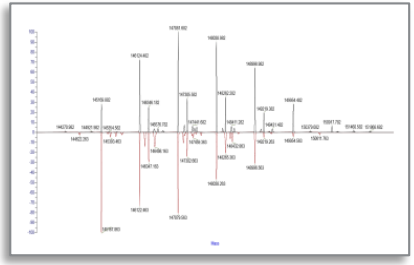


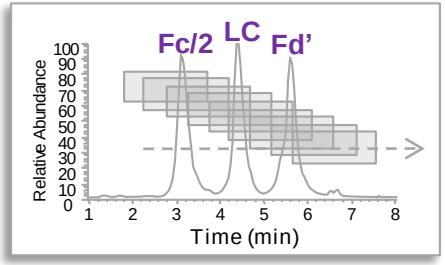


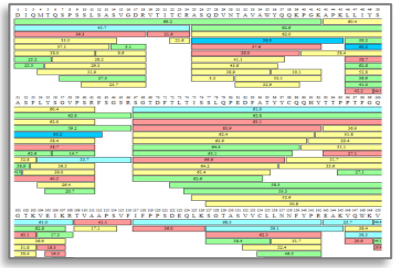
Intact Analysis

Subunit Analysis

Peptide Mapping







ThermoFisher SCIENTIFIC

Q Exactive BioPharma Allows a Complete Solution for BioPharma Characterization

One-Stop Shop

- Denatured Intact Analysis (MS1)
- Peptide Mapping (MS/MS)
- Middle/Top-Down (MS/MS)

Q Exactive™ MS

Dedicated to Deliver

- Denatured Intact Analysis (MS1)
- **Native** Intact Analysis (MS1)
- With ***Tune-able Optics***

Exactive™ Plus EMR MS

Highest performing for intact analysis

Q Exactive BioPharma Allows a Complete Solution for BioPharma Characterization

One-Stop Shop

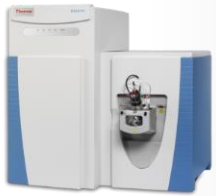
- Denatured Intact Ana
- Peptide Mapping (M
- Middle/Top-Down (M

Q Exactive

Complete Solution

- Denatured Intact Analysis (MS1)
- **Native Intact Analysis (MS1)**
- Peptide Mapping (MS/MS)
- Middle/Top-Down (MS/MS)

Q Exactive™ BioPharma MS



nalysis (MS1)

ysis (MS1)

ptics

Plus EMR MS

g for intact analysis

A Powerful Solution to the Challenge of BioPharma Characterization

Thermo Scientific™ :

Vanquish™ Horizon
UHPLC system

Q Exactive™ Plus/HF BioPharma
Orbitrap™ Mass Spectrometer

BioPharma Finder™
Data Analysis Software Platform

ThermoFisher SCIENTIFIC

Q Exactive BioPharma has Pre-Optimized Workflows for BioPharma Characterization

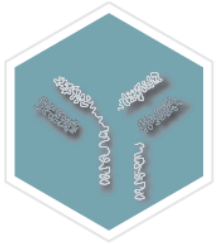
High Mass Range (HMR) Mode

Intact mAb and ADC Analysis

Optimized intact protein analysis under both native and denaturing conditions assures the highest quality and most informative spectra for the widest range of therapeutic proteins.

Protein Mode

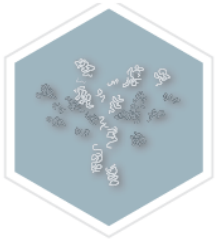
Subunit Analysis Top/Middle-Down



Extreme resolving power of the Orbitrap™ mass analyzer ensures isotopic resolution of subunits and facilitates top/middle-down sequencing.

Standard Mode

Peptide Mapping



Perform peptide mapping with Orbitrap technology for unparalleled acquisition speed, mass accuracy, and spectral quality.

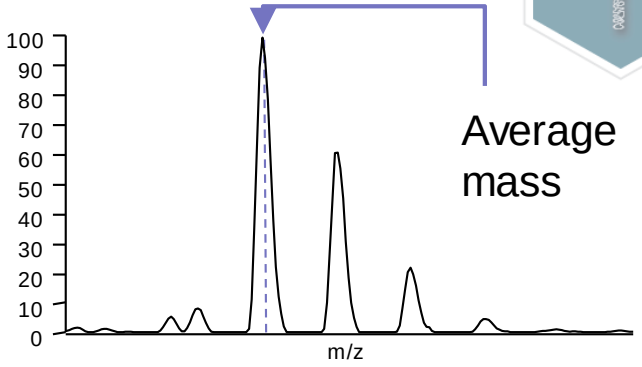
BioPharma Finder™ Mass Spectrometry Protein Characterization Data Software

- Screen, identify and characterize intact proteins with higher productivity and confidence using Intact Protein workflow.
- Xtract and ReSpect™ deconvolution algorithms take full advantage of the high-quality HRAM intact protein data produced by Thermo Scientific™ Orbitrap™ mass spectrometers.
- Confirm amino acid sequence, identify site and type of known/unknown PTMS while providing relative amounts using Peptide Mapping workflow.
- Additional features include disulfide linkage, sequence variant analysis and sequence alterations.

Two Types of Orbitrap Measurements for Large Molecules

Intact mAb

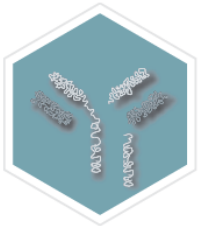
- Average mass measurement
- ***High Mass Range (HMR) mode***
- Works for all protein sizes
- Low resolving power

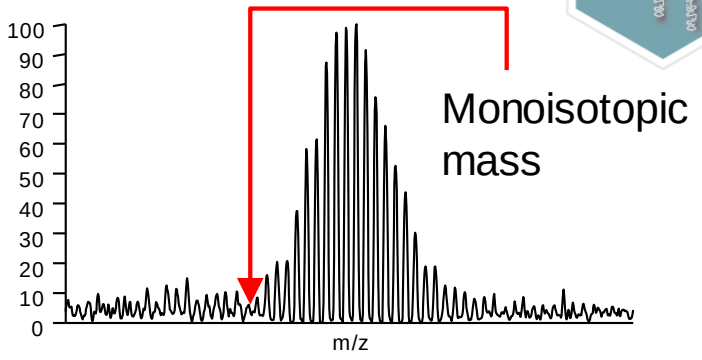


ReSpect deconvolution algorithm

mAb Subunit

- Isotopic resolution
- **Protein mode**
- Small-medium size proteins
- High resolving power



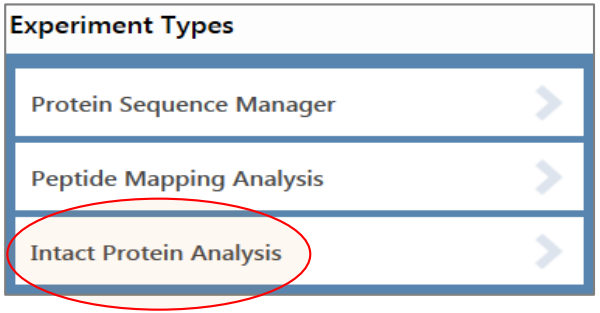


Xtract deconvolution algorithm

ThermoFisher SCIENTIFIC

Intact Protein Analysis in BioPharma Finder

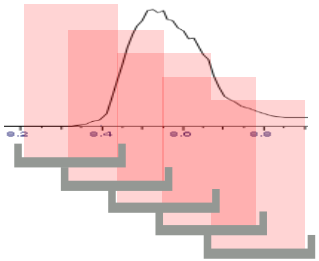
- Workflow software for intact protein mass determination

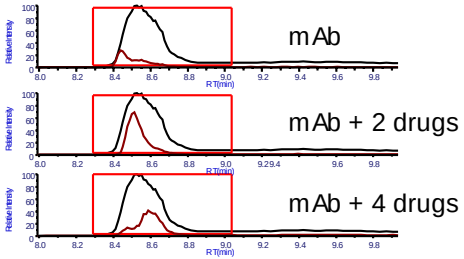


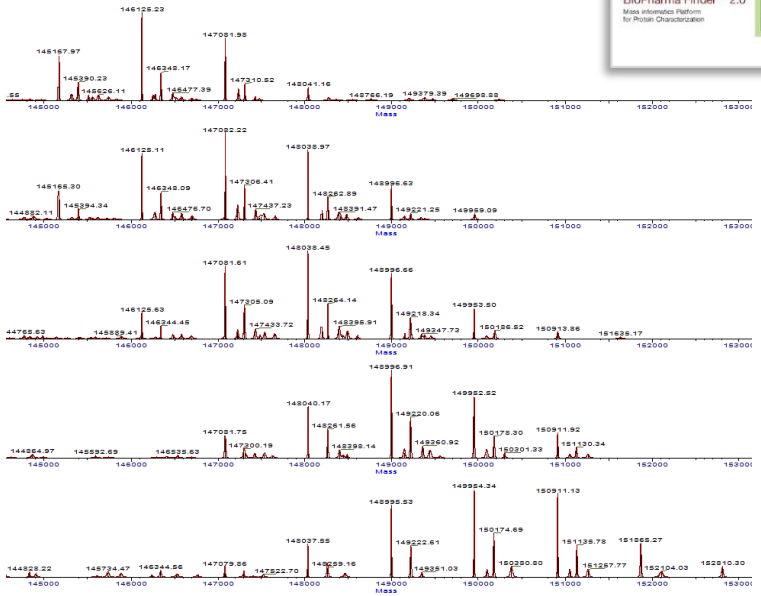
- Supports all **Orbitrap** mass spectrometers
- Includes 2 deconvolution algorithms:
  - **ReSpect** for isotopically-unresolved, average masses for intact mAb analysis
  - **Xtract** for isotopically-resolved, monoisotopic masses for mAb subunit analysis
- Batch processing/automation enhanced with **Sliding Window deconvolution**

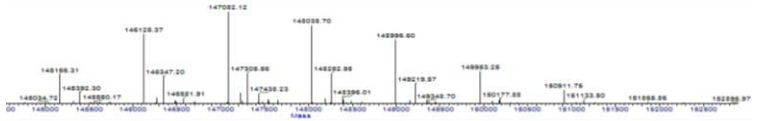
Sliding Window Deconvolution

- **Automatically** performs Xtract or ReSpect deconvolution along separations timescale
- **Removes user bias** from deconvolution analysis
- Improves quality of **batch analyses**
- Sensitive and confident identification + relative quan
- **All biologics benefitted** - simple mAbs, complex ADCs





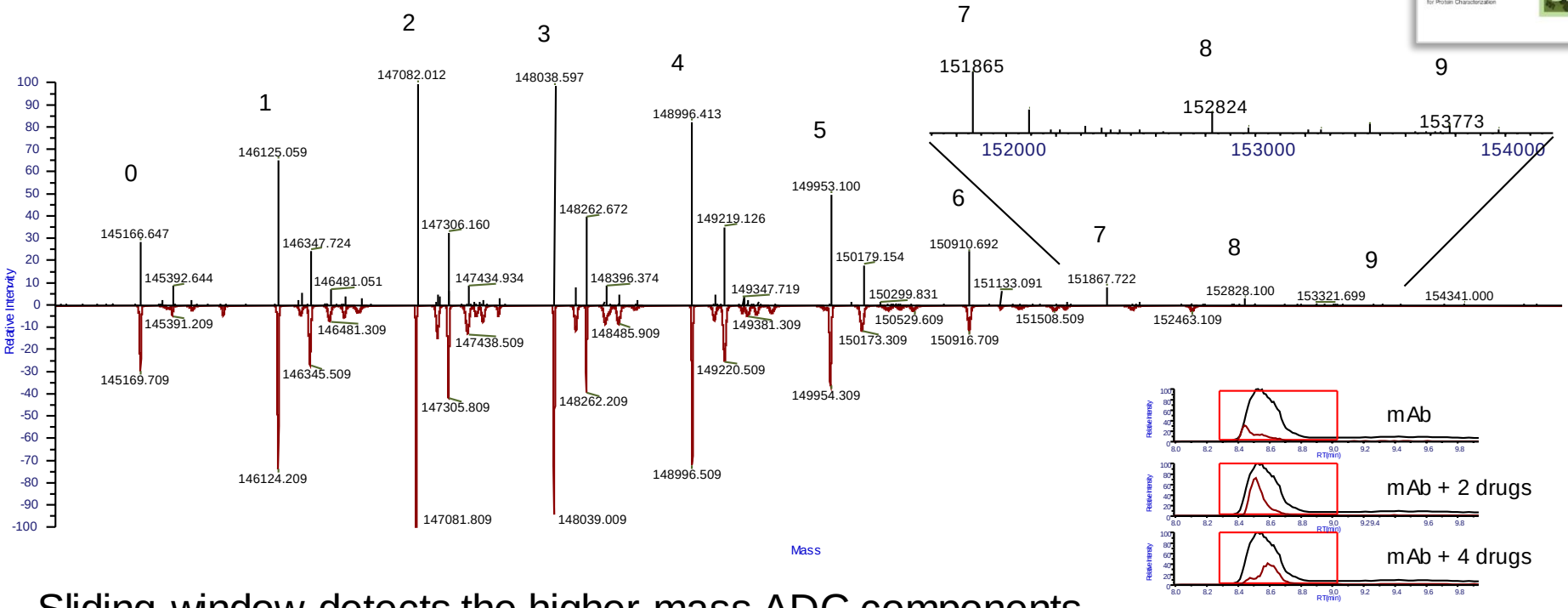




LC peak area automatically integrated

ThermoFisher SCIENTIFIC

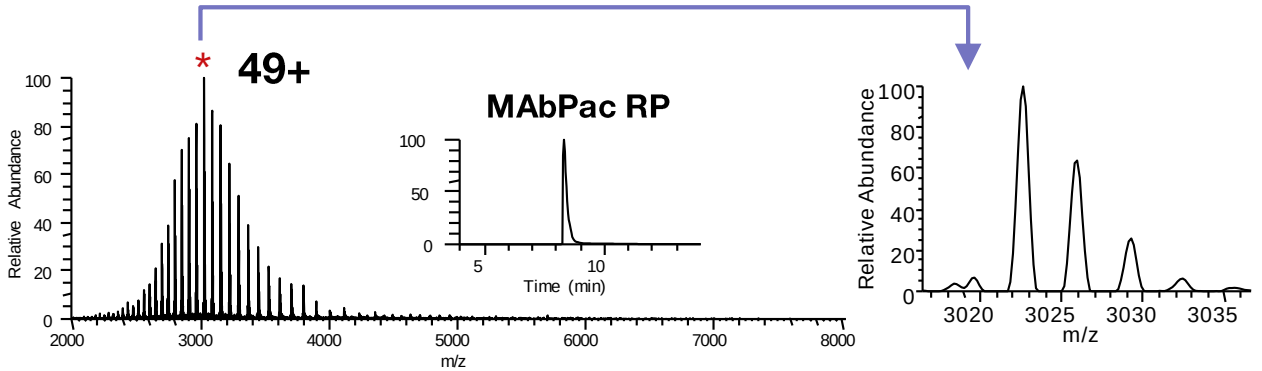
Comparison of Sliding Window and Conventional Deconvolution



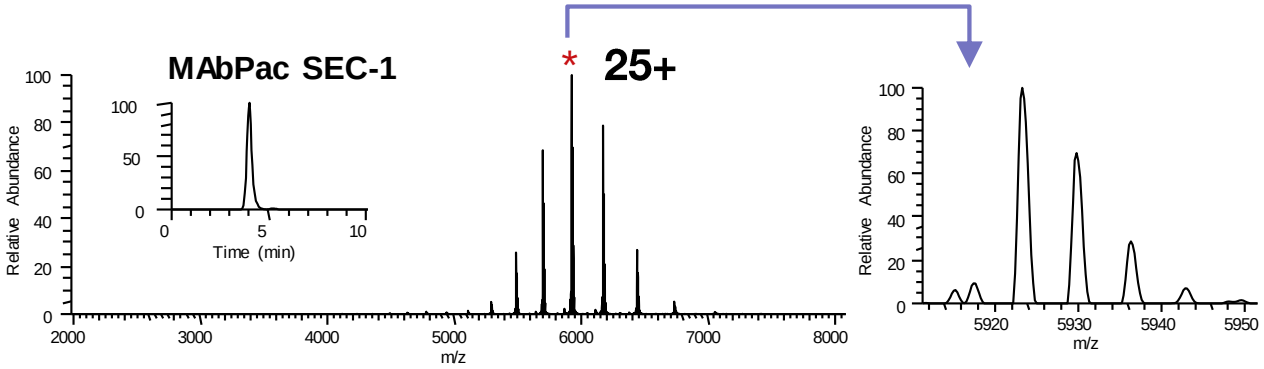
Sliding window detects the higher mass ADC components missed by conventional deconvolution – **works with both Xtract and ReSpect**

ThermoFisher SCIENTIFIC

Navigating Intact Protein Complexity with High Mass Range (HMR) Mode



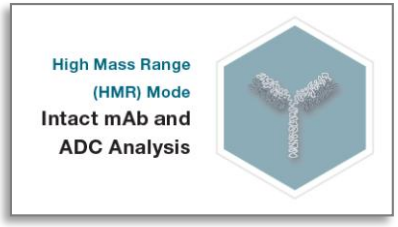
Denaturing MS is compatible with reverse phase HPLC separation.

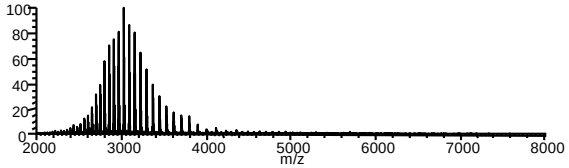


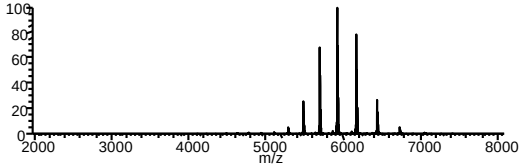
Native MS is best for co-eluting high complexity samples like ADCs

HMR mode intact analysis of Trastuzumab shows same answer in denaturing or native conditions

Choosing the Right Strategy for Intact Protein Analysis



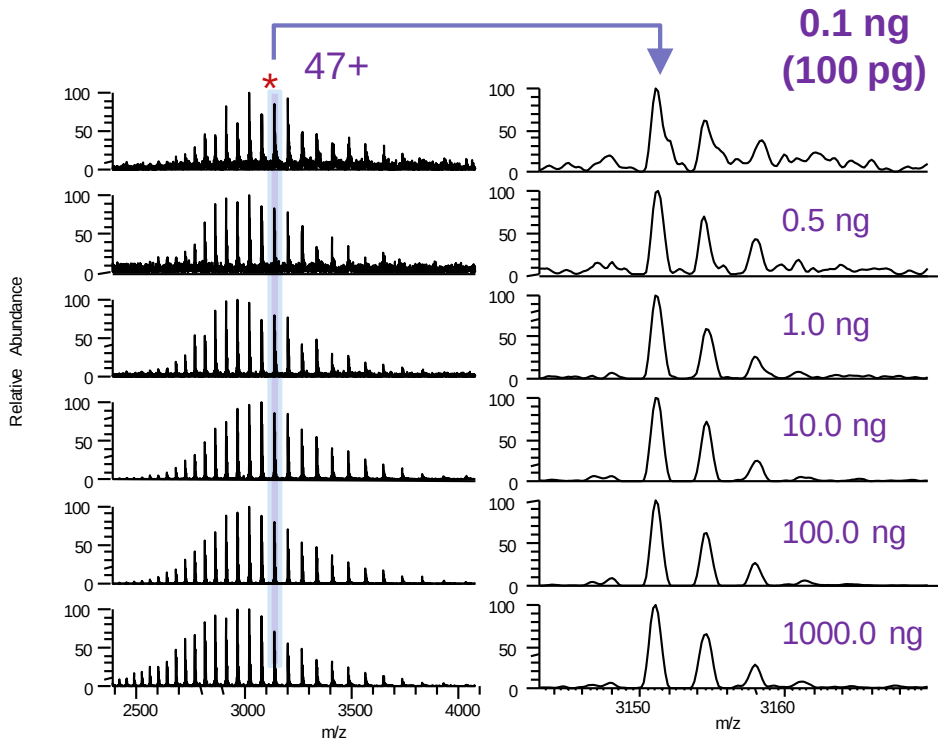




	Denaturing MS	Native MS
***Most sensitive* detection** of simple mAbs and subunits, low abundance protein isoforms	★	
High resolution separations Reverse Phase (RP)	★	
***Most confident* analysis** of heterogeneous mixtures, ADCs, glycoproteins, PEGylation		★
On-line desalting Size Exclusion (SEC)		★

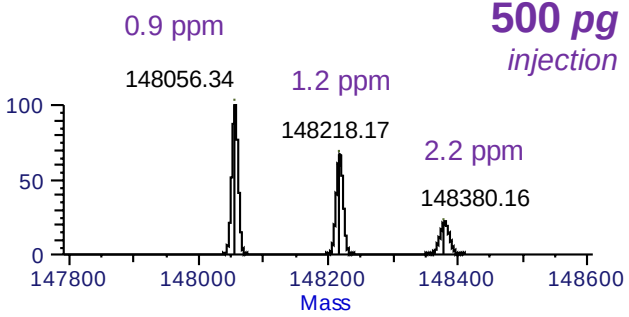
Denaturing LC-MS in HMR Mode
Sensitive Intact Analysis of Trastuzumab Antibody

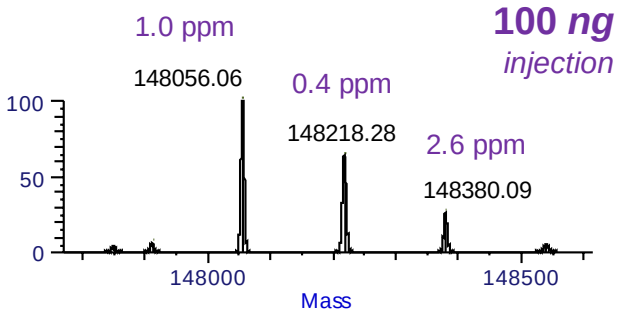
High Mass Range (HMR) Mode
Intact mAb and ADC Analysis



Q Exactive Plus in HMR Mode with MAbPac RP 2.1 x 50mm, 250 µl/min

ReSpect deconvolution





ThermoFisher SCIENTIFIC

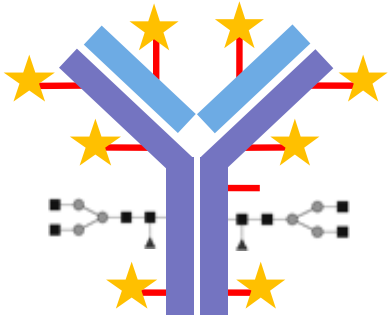
Understanding Antibody Drug Conjugate (ADC) Complexity
Native MS in HMR Mode

*ADC construction can create **layers** of sample **heterogeneity***

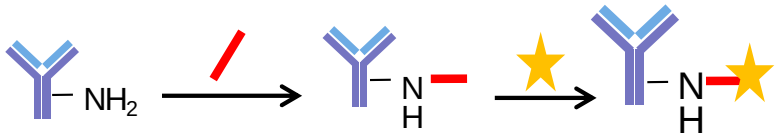
Intact ADC analysis:
Goal is to look at unaltered molecule

<u>Many diverse ADC forms possible</u>

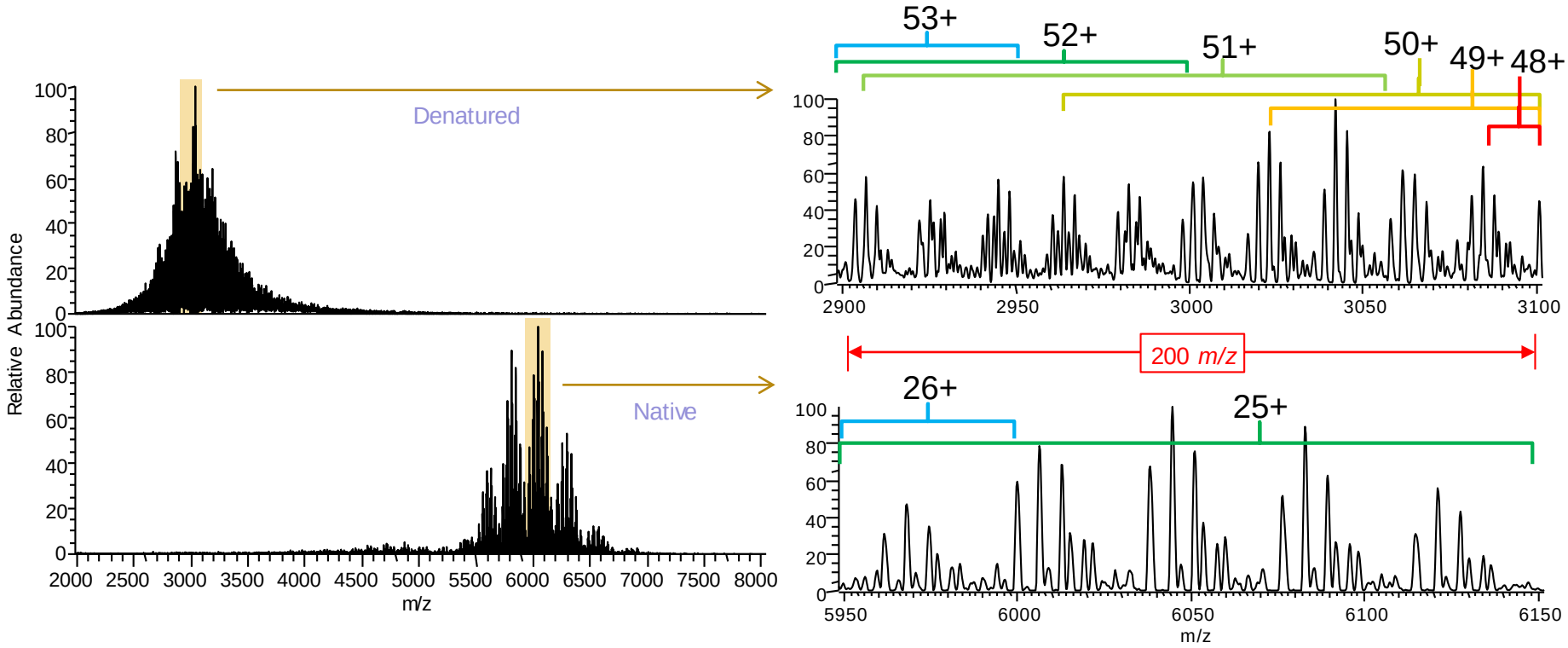
- Antibody
  - Glycan variants
  - Minor PTM variants
  - Sequence/clipping variants
- Conjugation
  - Linker-payload forms
  - Free-linker forms



Trastuzumab Emtansine
Lysine-linked ADC



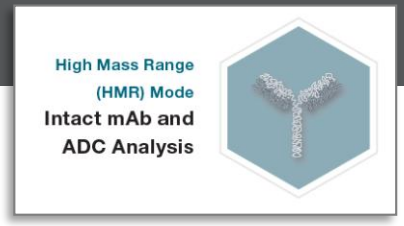
Intact Analysis of *Trastuzumab Emtansine* Lysine-inked ADC Denaturing vs. Native Conditions



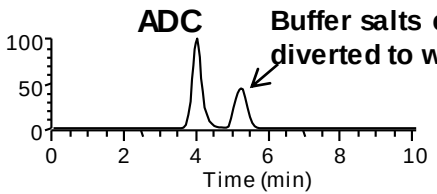
Native MS allows greater m/z separation of co-eluting species' charge states

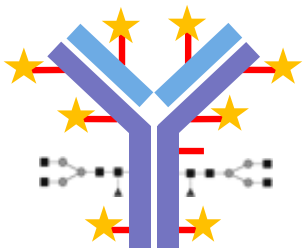
ThermoFisher SCIENTIFIC

Native LC-MS in High Mass Range (HMR) Mode
Intact ADC Analysis of *Trastuzumab Emtansine*

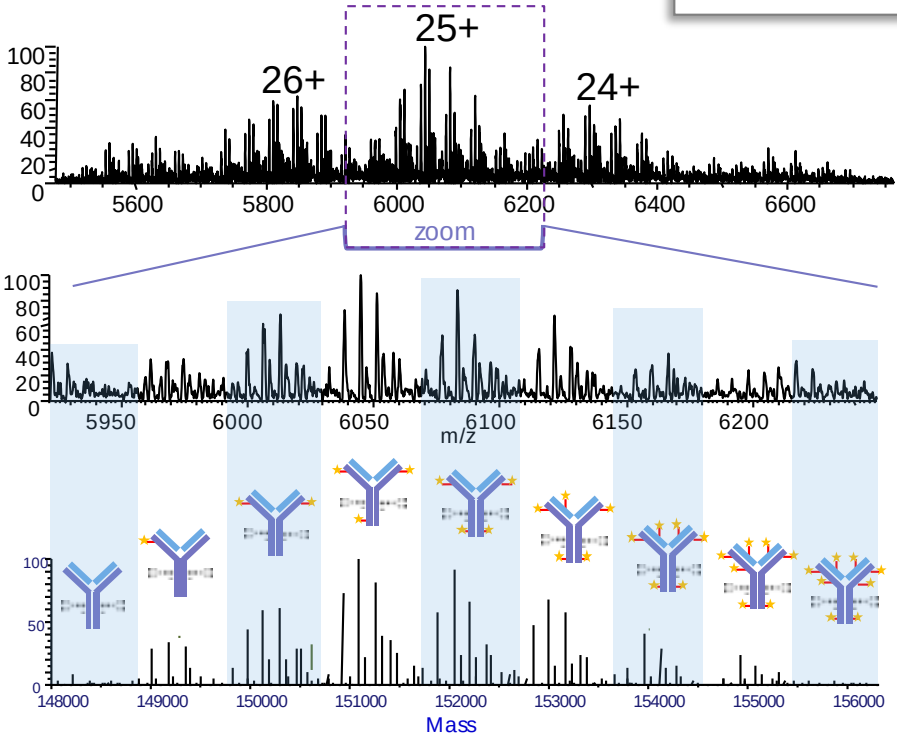


Size Exclusion Chromatography (SEC)-MS



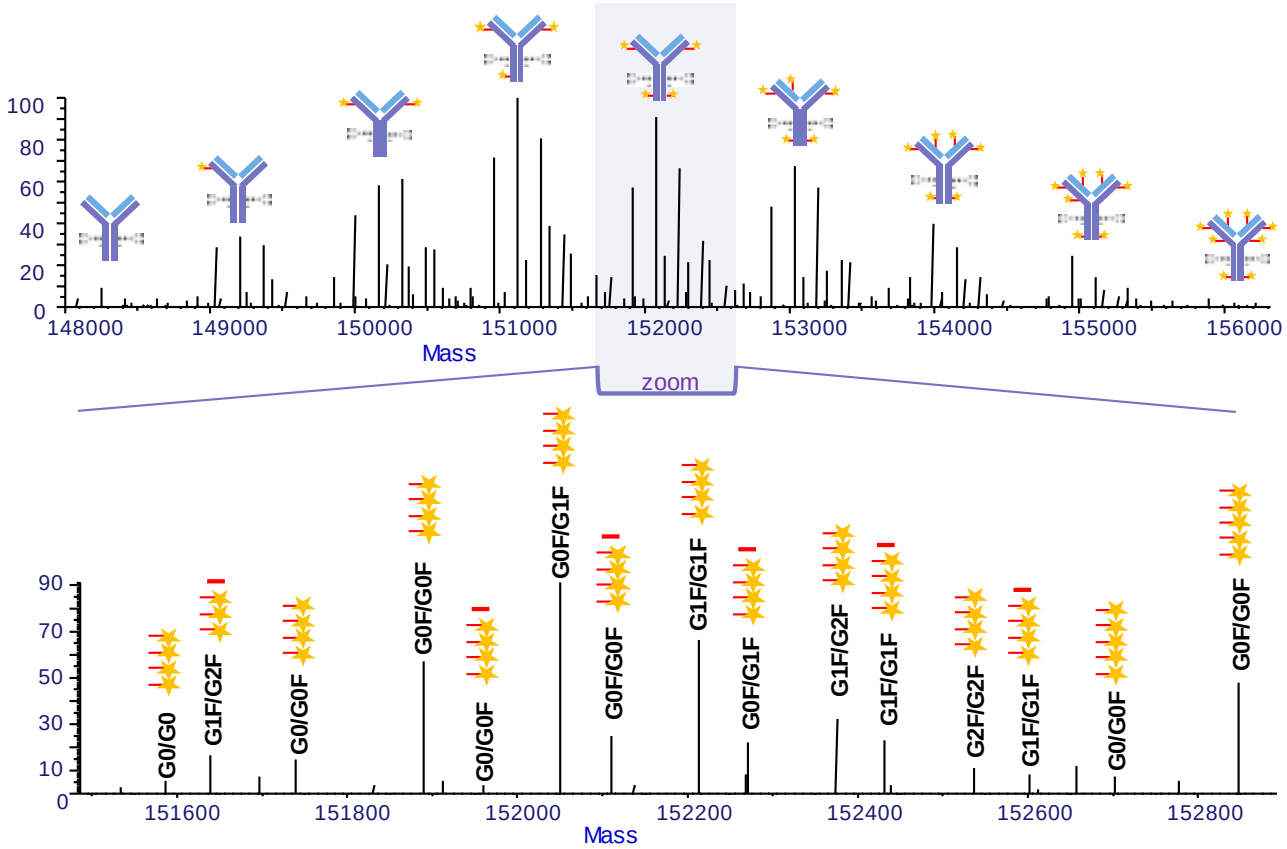


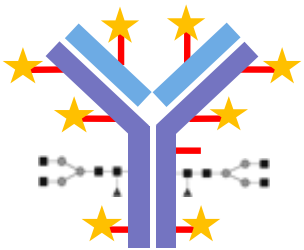
Trastuzumab Emtansine
Lysine-linked ADC



ThermoFisher SCIENTIFIC

Native LC-MS in High Mass Range (HMR) Mode
Intact ADC Analysis of *Trastuzumab Emtansine*

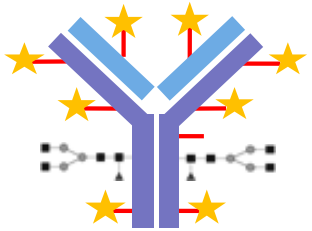




Trastuzumab Emtansine
Lysine-linked ADC

ThermoFisher SCIENTIFIC

Native LC-MS in High Mass Range (HMR) Mode
Intact ADC Analysis of *Trastuzumab Emtansine*

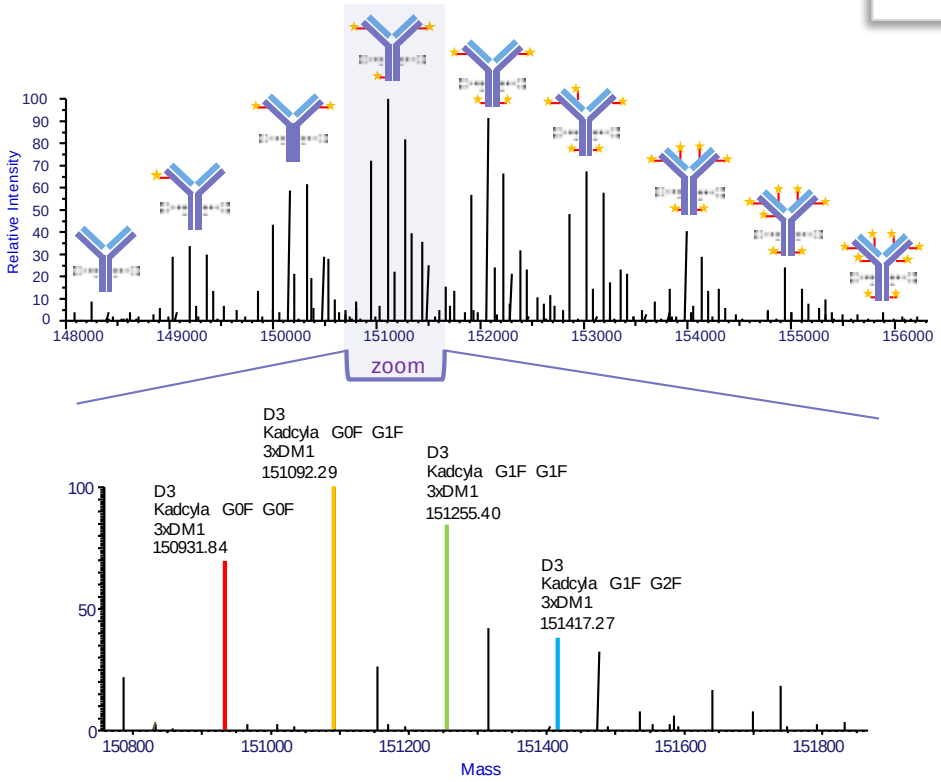


Average Drug-to-Antibody Ratio (DAR)

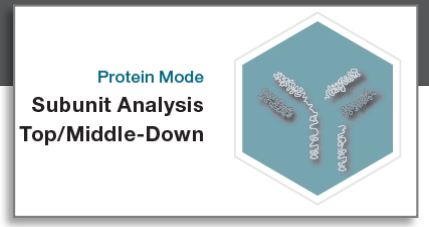
3.65

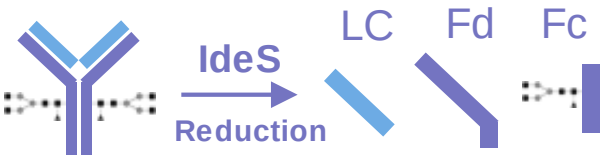
Glycoform	DAR
G0F/G0F	3.58
G0F/G1F	3.71
G1F/G1F	3.64
G1F/G2F	3.65
Top 4 avg	3.65

Automatic calculation

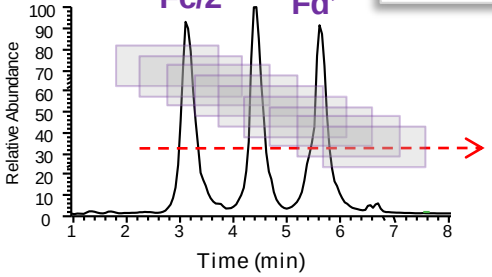


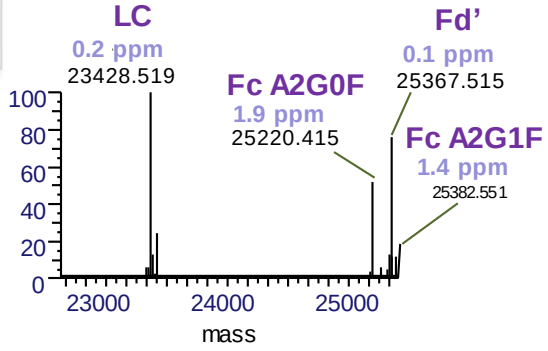
Subunit Analysis in Protein Mode
LC-MS Analysis of IdeS-digested, Reduced *Trastuzumab*



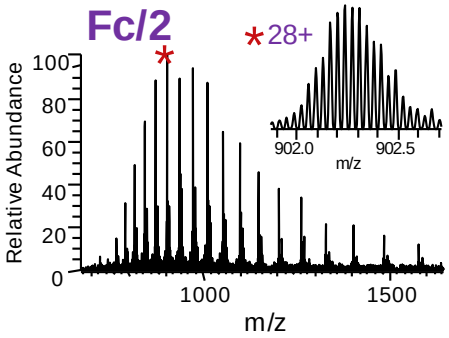


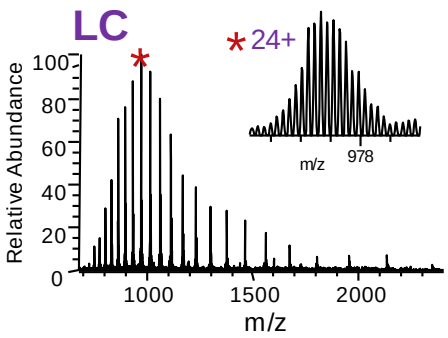
***Sliding Window* Xtract deconvolution**
for monoisotopic masses

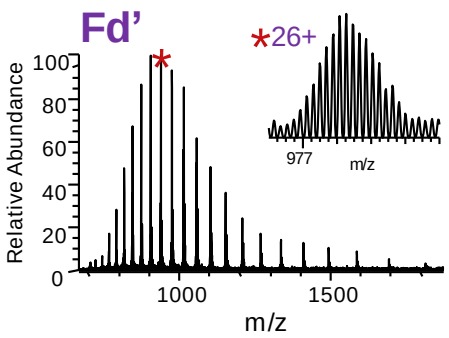




R=140,000

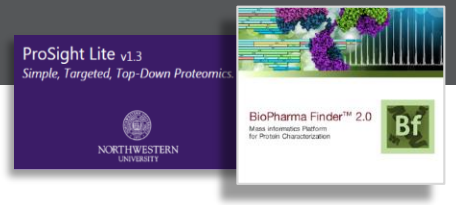


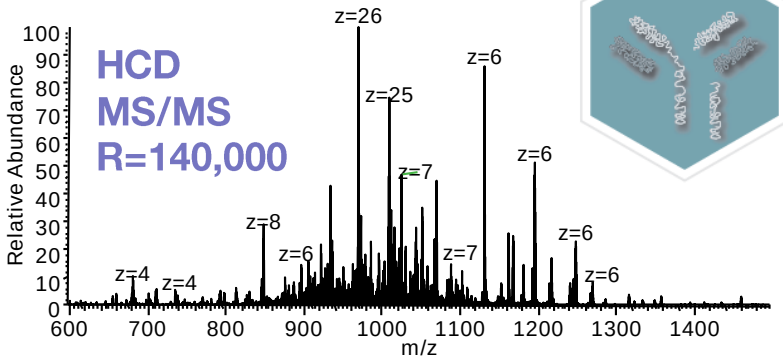




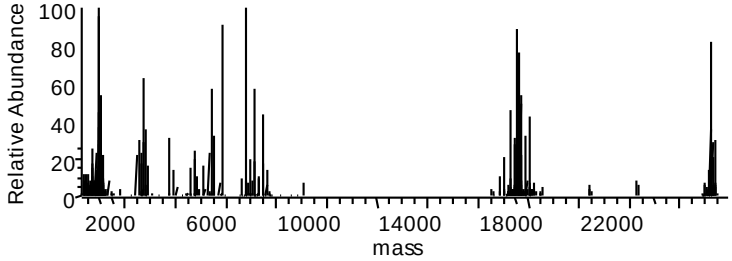
ThermoFisher SCIENTIFIC

Subunit Analysis in Protein Mode
LC-MS Analysis of IdeS-digested, Reduced *Trastuzumab*



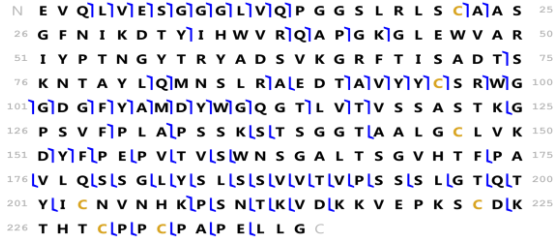


Xtract deconvolution

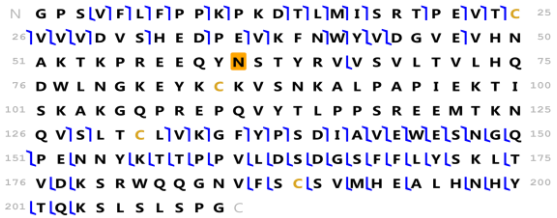


Light Chain
49% residue cleavages

Fd'
38% residue cleavages



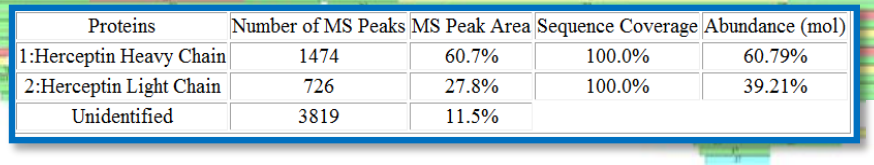
Fc/2
39% residue cleavages



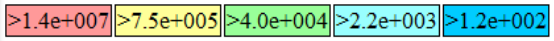
ThermoFisher SCIENTIFIC

Peptide Mapping in Standard Mode
Deep characterization of *Trastuzumab* with HCD Fragmentation

Standard Mode
Peptide Mapping

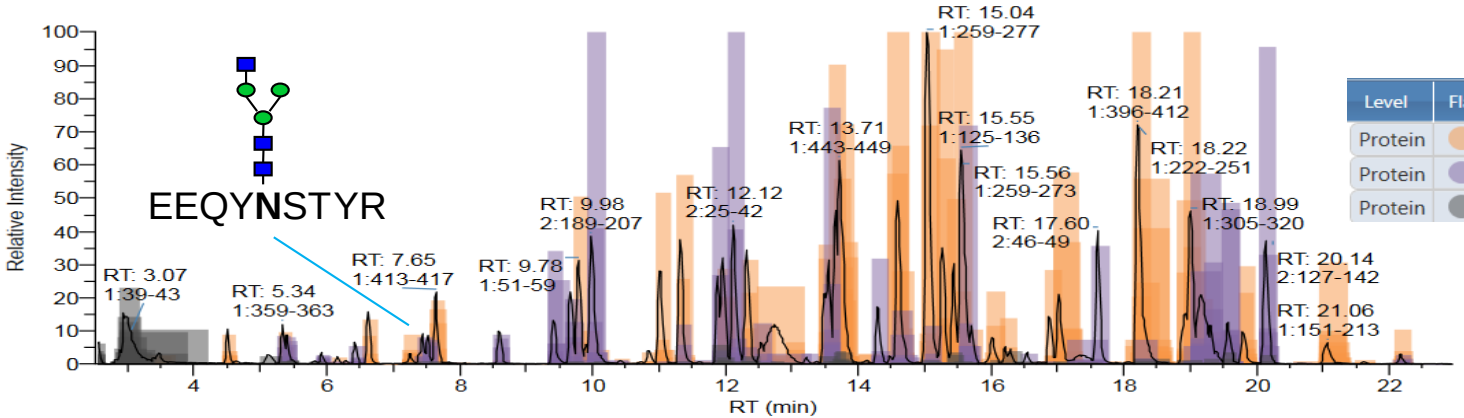


Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:Herceptin Heavy Chain	1474	60.7%	100.0%	60.79%
2:Herceptin Light Chain	726	27.8%	100.0%	39.21%
Unidentified	3819	11.5%		



Sensitivity and rapid high resolution scanning of the Orbitrap allows discrimination between components which are near-isobaric or co-eluting.

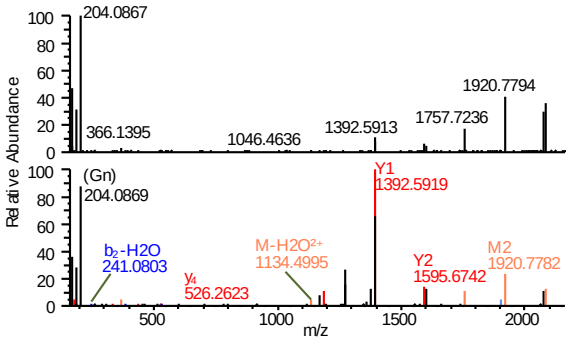
Accurate peptide IDs are based on high fidelity MS/MS spectra visualized in a color coded display by BioPharma Finder software.



Level	Flag	No.	Protein
Protein		1	Herceptin Heavy Chain
Protein		2	Herceptin Light Chain
Protein		3	Unidentified

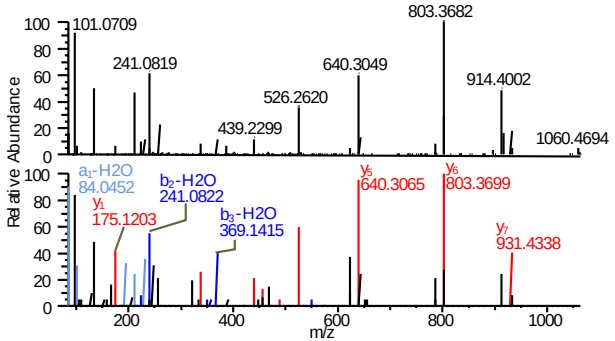
ThermoFisher SCIENTIFIC

Glycopeptide Analysis in Standard Mode
Signature Ion Detection with HCD Fragmentation

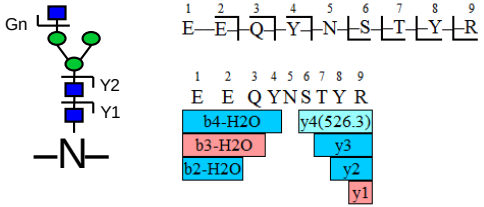


predicted spectra

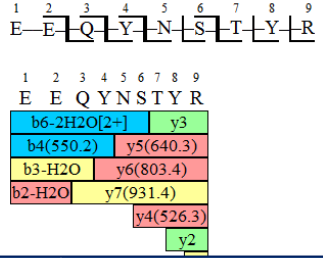
acquired spectra



Glycosylated peptide (A1G0)



Unmodified peptide



Glycosylation site occupancy

Signature HCD fragment ions for glycans (Gn) and glycopeptides (Y1, Y2) are identified in BioPharma Finder software.

Protein	Residue #	Modification	Category	Peptides	Sequence
Herceptin Heavy Chain	300	N300+A1G0	Glycoform	1:E296-R304 = 1188.50473m(N300+A1G0)	EEQYNSTYR
Herceptin Heavy Chain	300	N300+A1G0F	Glycoform	1:E296-R304 = 1188.50473m(N300+A1G0F)	EEQYNSTYR
Herceptin Heavy Chain	300	N300+A1G1F	Glycoform	1:E296-R304 = 1188.50473m(N300+A1G1F)	EEQYNSTYR
Herceptin Heavy Chain	300	N300+A2G0	Glycoform	1:E296-R304 = 1188.50473m(N300+A2G0)	EEQYNSTYR
Herceptin Heavy Chain	300	N300+A2G0F	Glycoform	1:E296-R304 = 1188.50473m(N300+A2G0F)	EEQYNSTYR
Herceptin Heavy Chain	300	N300+A2G1F	Glycoform	1:E296-R304 = 1188.50473m(N300+A2G1F)	EEQYNSTYR

ThermoFisher SCIENTIFIC

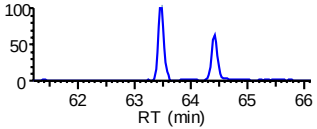
Trastuzumab Emtansine
Top 15 Most Abundant Glycoforms Measured by Peptide Mapping

Protein	Residue #	Modification	Category	Sequence	Confidence	Average % Abundance	% Abundance Run 1	% Abundance Run 2	% Abundance Run 3
Heavy Chain	300	N300+A2G0F	Glycoform	EEQYNSTYR	100	35.22	34.70	34.70	36.24
Heavy Chain	300	N300+A2G1F	Glycoform	EEQYNSTYR	100	31.77	31.22	31.53	32.55
Heavy Chain	300	N300+A1G0F	Glycoform	EEQYNSTYR	100	9.31	10.83	9.53	7.59
Heavy Chain	300	N300+A2G2F	Glycoform	EEQYNSTYR	100	5.54	5.50	5.56	5.58
Heavy Chain	300	N300+A2G0	Glycoform	EEQYNSTYR	100	4.79	4.66	4.85	4.85
Heavy Chain	300	N300+M5	Glycoform	EEQYNSTYR	100	4.72	4.78	4.83	4.53
Heavy Chain	300	N300+A1G1F	Glycoform	EEQYNSTYR	100	3.53	4.06	3.65	2.87
Heavy Chain	300	N300+A1G0	Glycoform	EEQYNSTYR	100	2.35	2.54	2.45	2.05
Heavy Chain	300	N300+A2G1	Glycoform	EEQYNSTYR	100	2.32	2.25	2.39	2.31
Heavy Chain	300	N300+Unglycosylated	Glycoform	EEQYNSTYR	100	1.97	1.94	2.00	1.97
Heavy Chain	300	N300+A2S1G1F	Glycoform	EEQYNSTYR	100	0.82	0.76	0.82	0.88
Heavy Chain	300	N300+A1G1	Glycoform	EEQYNSTYR	100	0.59	0.61	0.64	0.54
Heavy Chain	300	N300+M6	Glycoform	EEQYNSTYR	100	0.59	0.56	0.60	0.60
Heavy Chain	300	N300+A2S1G0F	Glycoform	EEQYNSTYR	100	0.40	0.39	0.41	0.41
Heavy Chain	300	N300+M4	Glycoform	EEQYNSTYR	100	0.39	0.38	0.43	0.35

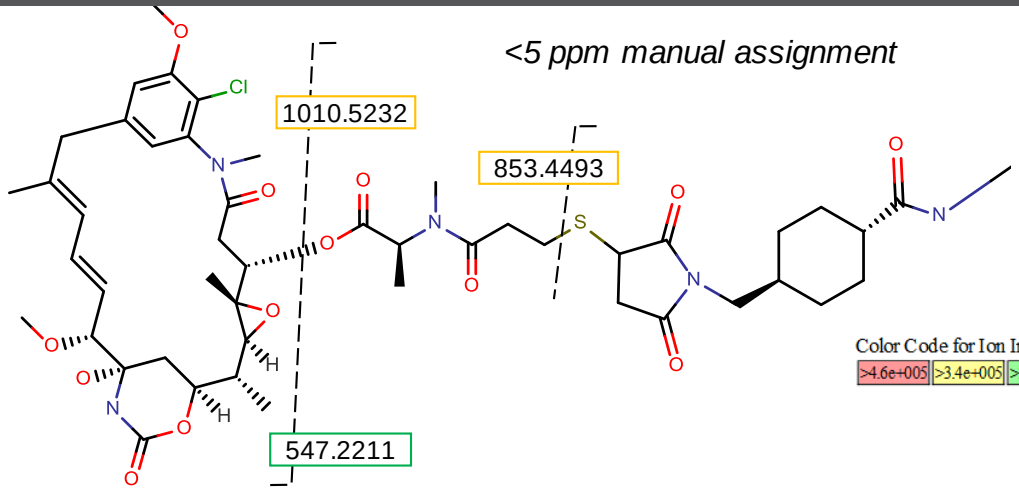
Automatic detection by BioPharma Finder

HCD Fragmentation of Heavy Chain Hinge Peptide KVEPK-(MCC-DM1)

Elutes as doublet due to stereocenter in DM1



<5 ppm manual assignment



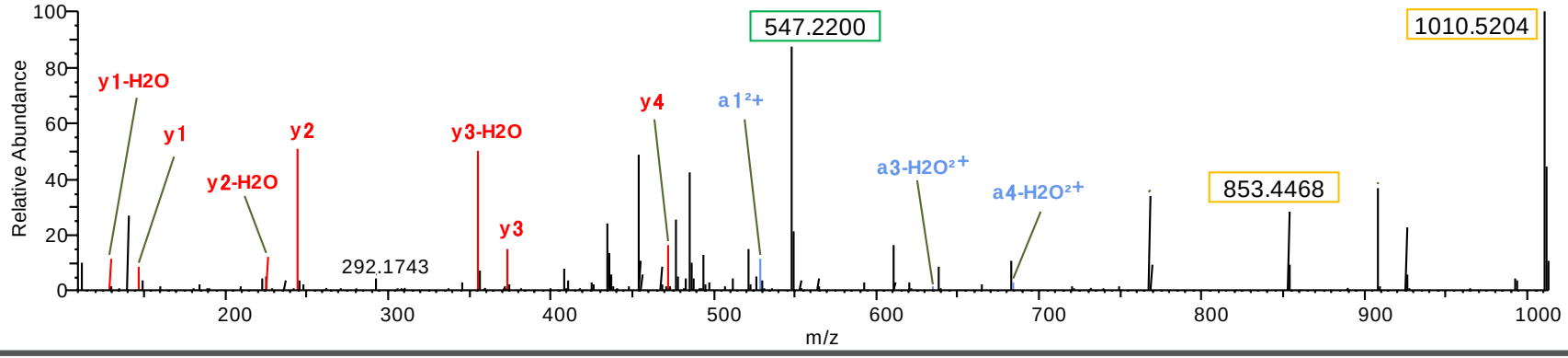
1 2 3 4 5
K V E P K

1 2 3 4 5
K V E P K

y4(472.3)
y3-H2O
y2
y1-H2O

Color Code for Ion Intensity

>4.6e+005 | >3.4e+005 | >2.5e+005 | >1.8e+005 | >1.3e+005



ThermoFisher SCIENTIFIC

Trastuzumab emtansine Lysine-conjugation mapping

Peptide Sequence	Modification	Protein	Site	Delta (ppm)	RT
ASQDVNTAVAWYQQKPGK	DM1	Light Chain	K39	-0.25	67.47
PGKAPK	DM1	Light Chain	K42	-0.31	63.21
VEIKR	DM1	Light Chain	K107	-0.46	65.96
EAKVQWK	DM1	Light Chain	K145	0.09	67.23
VQWKVDNALQSGNSQESVTEQDSK	DM1	Light Chain	K149	0.09	68.57
VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	DM1	Light Chain	K169	0.2	68.77
DSTYSLSSTLTLSKADYEK	DM1	Light Chain	K183	1.07	73.01
ADYEKHK	DM1	Light Chain	K188	-0.77	59.14
HKVYACEVTHQGLSSPVTK	DM1	Light Chain	K190	0.01	58.54
VYACEVTHQGLSSPVTKSFNR	DM1	Light Chain	K207	0.15	64.92
LSCAASGFNIKDTYIHWVR	DM1	Heavy Chain	K30	-2.37	73.12
QAPGKGLEWVAR	DM1	Heavy Chain	K43	-0.62	71.01
YADSVKGR	DM1	Heavy Chain	K65	-0.67	65.35
FTISADTSKNTAYLQMNSLR	DM1	Heavy Chain	K76	0.39	70.82
VDKK	DM1	Heavy Chain	K216	-0.74	63.99
KVEPK	DM1	Heavy Chain	K217	0.46	63.38
SCDKTHTCPPCPAPELLGGPSVFLFPPKPK	DM1	Heavy Chain	K225	-1.61	72.91
THTCPPCPAPELLGGPSVFLFPPKPK	DM1	Heavy Chain	K249	-0.4	73.5
PKDTLMISR	DM1	Heavy Chain	K251	0.01	70.58
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	DM1	Heavy Chain	K277	-0.05	71.84
FNWYVDGVEVHNAKTK	DM1	Heavy Chain	K291	0.26	67.17
TKPR	DM1	Heavy Chain	K293	0.19	64.16
EYKCK	DM1	Heavy Chain	K323	-0.04	64.44
CKVSNK	DM1	Heavy Chain	K325	-0.47	62.93
VSNKALPAPIEK	DM1	Heavy Chain	K329	-0.38	68.18
ALPAPIEKTISK	DM1	Heavy Chain	K337	-0.66	71.07
TISKAK	DM1	Heavy Chain	K341	-0.25	64.06
AKGQPR	DM1	Heavy Chain	K343	-0.07	62.99
EEMTKNQVSLTCLVK	DM1	Heavy Chain	K363	-0.07	72.43
LTVDKSR	DM1	Heavy Chain	K417	-0.6	66.04

Trastuzumab Emtansine Lysine-Conjugation Mapping

44
Lysines total in sequence

30
Lys-MCC-DM1 sites automatically detected and manually confirmed

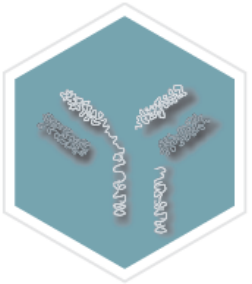
High Mass Range (HMR) Mode

Intact mAb and ADC Analysis

Optimized intact protein analysis under both native and denaturing conditions. Highest quality spectra for the widest range of therapeutic proteins.

Protein Mode

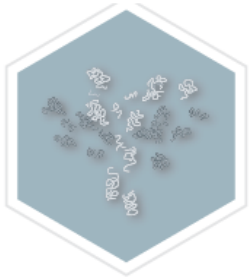
Subunit Analysis
Top/Middle-Down



Extreme resolving power of the Orbitrap mass analyzer ensures isotopic resolution of subunits and facilitates top/middle-down sequencing.

Standard Mode

Peptide Mapping



Perform peptide mapping with Orbitrap technology for unparalleled acquisition speed, mass accuracy, and spectral quality.

Summary

- Q Exactive Plus/HF with BioPharma option is a platform for complete biotherapeutic protein characterization
- BioPharma Finder software allows easy analysis of data from intact, subunit, and peptide mapping assays
- Biologics characterization is a complex endeavor which requires multiple approaches with Orbitrap mass spectrometry
- Structural fragility and complexity of ADCs can be accommodated by native MS analysis using Q Exactive BioPharma