APPLICATION NOTE 73885

Complete characterization of monoclonal antibodies under native and denaturing conditions

Kristina Srzentić[1], Eugen Damoc[2], Kai Scheffler[3], Tom Buchanan[4], Krisztina Radi[4], Jennifer Sutton[1]

Thermo Fisher Scientific,
[1]Cambridge, Massachusetts, US
[2]Bremen, Germany
[3]Germering, Germany
[4]Hemel Hempstead, UK

Keywords: Monoclonal antibody subunits, Top-Down and Middle-Down, characterization, biotherapeutics, native and denaturing conditions, IdeS, peptide mapping

Application benefits

- Operational simplicity for mass spectrometer setup and data acquisition using system templates available for all stages of analysis on the intact protein, subunit, and peptide levels
- Exceptional sensitivity and mass accuracy for intact mass analysis under denaturing and native conditions, allowing for confident analysis, even from low sample loading providing data with superb spectral quality and excellent mass accuracy
- Improved sequence coverage in Top/Middle-Down approaches, and confident identification and localization of site-specific modifications for advanced characterization of low abundance level components
- System versatility enables the most sensitive detection at all stages of analysis supported by the Intact Protein application mode with default and customizable pressure conditions

ThermoFisher SCIENTIFIC

Goals

- Demonstrate the capabilities of the Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer equipped with BioPharma Option for the full characterization of antibody samples
- Provide optimal LC-MS conditions to achieve conclusive information on the molecular weight and proteoform heterogeneity at the intact mAb, subunit, and peptide levels
- Elucidate the amino acid sequence by performing Top-Down, Middle-Down, and Bottom-Up analysis

Introduction

Analysis of therapeutic monoclonal antibodies (mAbs) can be challenging due to their macro-heterogeneity deriving from various *N*-linked glycan species and structural heterogeneity deriving from various endogenous modifications, which include the following: single point mutation (Lys to Gln substitution),[1] Asp isomerization and formation of succinimide, oxidation, alternative disulfide bond linkage, and formation of trisulfides (and similar).

All these modifications contribute to micro-heterogenous proteoform mixtures of covalently assembled molecules, which vary in size, charge, and hydrophobicity, and could impact mAb chemical properties.[2] To address and overcome biotherapeutic analysis-associated challenges and ensure product safety and efficacy, significant inroads have been taken to develop and refine mass spectrometry (MS)-based approaches, which today can be broadly classified into three major application workflows: intact mass analysis, subunit and Top/Middle-Down analysis, and peptide mapping, as depicted in Figure 1. These application workflows are critical during development and production as they can provide the required complementary information for an in-depth insight into the structure and composition of biotherapeutics.

Intact mass analysis omits digestion, entails minimal sample manipulation, and allows for confirmation of protein identity and characterization of glycoform profile. The resolving power and the mass accuracy of the mass spectrometer have a direct effect on the quality of the mass measurement.

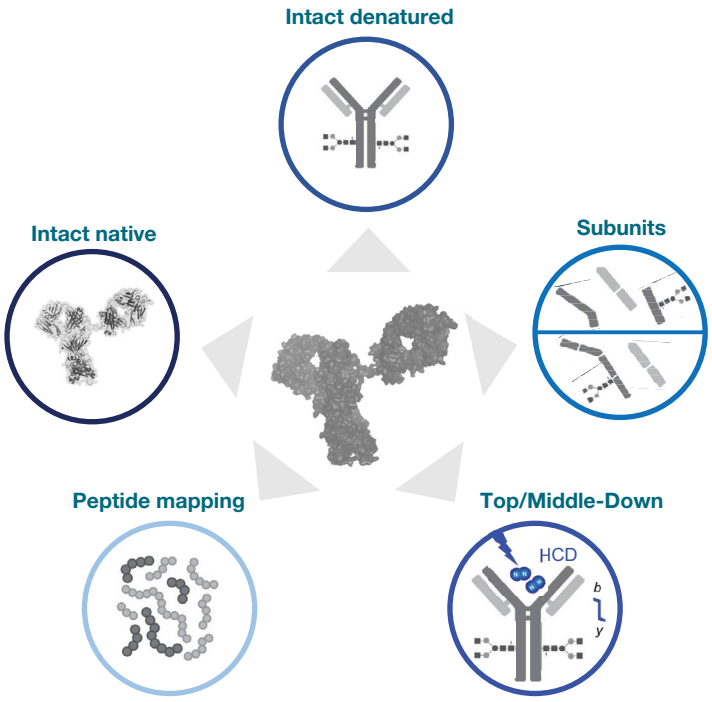


Figure 1. Complete mAb characterization. The Orbitrap Exploris 480 MS with BioPharma option supports the complete biopharmaceutical characterization by offering distinct application modes, optimized for the key protein characterization workflows: intact native and denatured, subunit, peptide mapping, and Top/Middle-Down.

MS analysis under native-like conditions allows proteins to preserve non-covalent interactions and retain high degrees of folding, unlike denaturing MS analysis, and thus enables the probing of mAb in its folded, intact, near-native state. However, in a near-native state, mAbs are less prone to protonation, and as a result, require a higher *m/z* range to be detected. Mass detection up to *m/z* 8,000, suitable for native antibody MS analysis, is provided in the Intact Protein Application mode on the Orbitrap Exploris 480 mass spectrometer equipped with BioPharma Option.

Deeper insight into mAb structure by elucidation of the amino acid sequence is achieved by fragmentation of intact or subunit species in the gas phase, as it is done by Top-Down (TD) and Middle-Down (MD) MS analysis, respectively. On the Orbitrap Exploris 480 mass spectrometer, for a TD MS experiment, intact mAb precursor ions are fragmented with Higher-energy Collisional Dissociation (HCD) in the Ion-Routing Multipole. However, the interpretation of resulting TD spectra can be challenging due to their increased complexity arising from many overlapping distributions of large fragment ions.

The MD MS approach involves the proteolytic cleavage of intact mAbs into ~23–98 kDa subunits using specific enzymes. The immunoglobulin degrading enzyme from *Streptococcus pyogenes,* IdeS is considered the gold standard to aid mAb subunit characterization.

The analysis of antibody subunits provides additional and complementary information, such as degradation products, sequence variants, and combinations of post-translational modifications, with the advantages of simple and fast sample preparation and data interpretation, making this a highly desirable approach for high throughput analysis.[3,4] This allows the analyst to perform a quick glycosylation evaluation, confirm the identity of a biosimilar compared to innovator reference biotherapeutic, as well as fast first level identification of many common PTMs.[5] Top-Down and Middle-Down approaches have received increasing attention and consequent technological development support due to their promise of providing fast and accurate answers to the study of proteins, proteoforms, and multi-proteoform complexes, including their identification, characterization, and quantification.[6,7] The technological developments provided by the Orbitrap Exploris 480 MS provide the user with an instrument capable of robust analysis of intact mAb proteoforms and subunits with high-resolution accurate mass.

MS-based protein analysis is traditionally carried out using the Bottom-Up approach of peptides obtained upon proteolytic protein digestions, which can be easily fragmented in the MS gas phase. Peptide mapping is one such Bottom-Up technique that can be used to elucidate the primary structure and the site-specific post-translational modifications (PTM) for the comprehensive characterization of biotherapeutic proteins to ensure product quality, efficacy, and safety. Peptide mapping analysis on the Orbitrap Exploris 480 MS is achieved using the Peptide application mode.

In this study, the monoclonal antibody trastuzumab was analyzed at the intact level under denaturing and native conditions, subunit level under denaturing conditions, as well as at the peptide level upon digestion with the Thermo Scientific™ SMART™ Digest kit using the three workflows outlined in Figure 2A.

Here, we demonstrate the capabilities of the Orbitrap Exploris 480 mass spectrometer equipped with BioPharma Option to comprehensively characterize trastuzumab at the intact protein, subunit, and peptide level on one single LC/MS platform, further supported by distinct application modes and predefined system templates in the method editor as depicted in Figure 2B. The system templates reduce method optimization efforts for methods that were traditionally challenging to implement routinely and have been optimized for the most commonly applied protein characterization workflows.

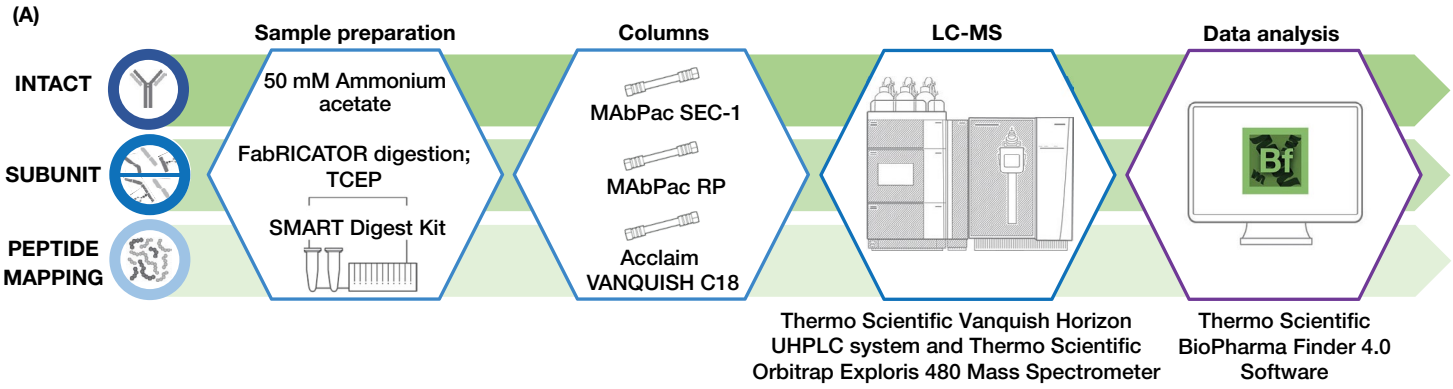


Figure 2A. mAb characterization workflows for intact mass analysis, subunit analysis, and peptide mapping comprising sample preparation, separation using suitable column chemistries in combination with liquid chromatography and mass spectrometric analysis, and concluding with software supported data processing

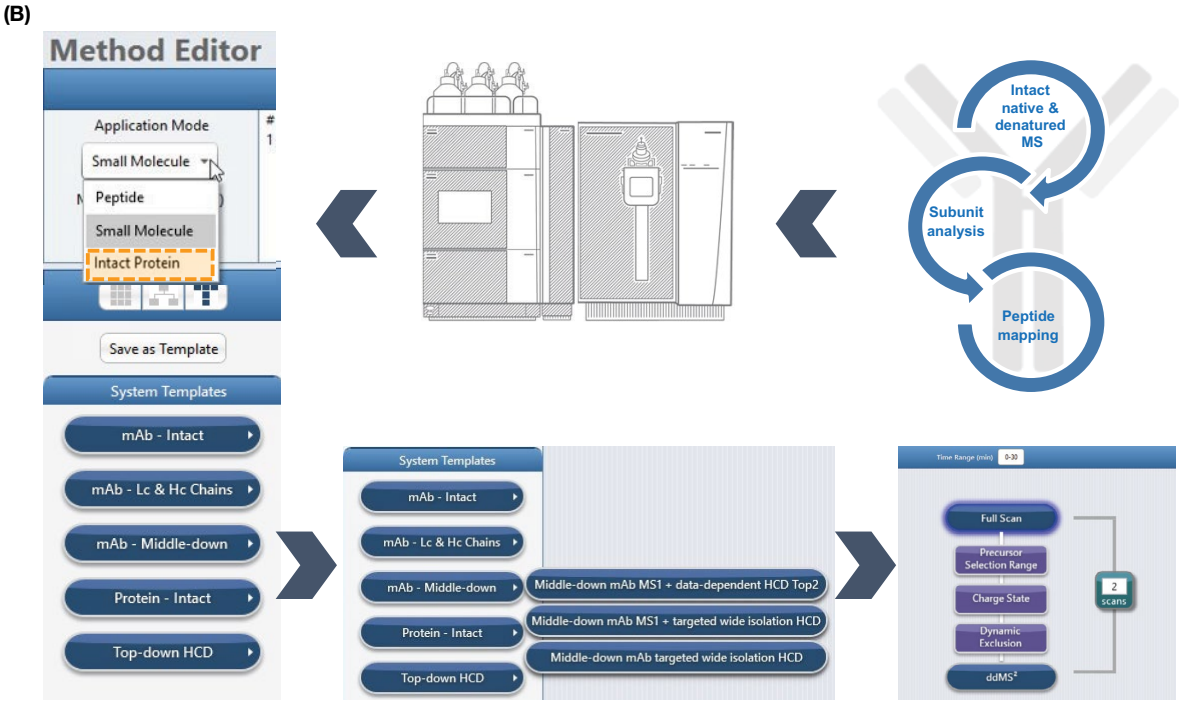


Figure 2B. Method editor and system templates available on the Orbitrap Exploris 480 mass spectrometer. A library of pre-defined method templates and an easy instrument setup is provided with the instrument software.

Experimental

Sample preparation

The commercially available mAb trastuzumab (Herceptin™, Genentech, Inc.) was used for all experiments presented in this application note.

Intact protein analysis

For intact mass analysis under native conditions, the antibody was injected in its original concentration at 21 mg/mL in a formulation buffer without any further dilution.

Subunit analysis

For subunit analysis, the sample was prepared in two ways (Figure 2): 1) the sample was denatured and reduced using 4 M guanidine hydrochloride (GdHCl/50 mM tris(2-carboxyethyl)phosphone (TCEP)) and incubated at 57 °C for 45 min. 2) the sample was treated with IdeS enzyme according to the manufacturer's protocol, followed by a reduction step by incubation with 50 mM TCEP at 57 °C for 45 min to yield LC, Fc/2, Fd', $F(ab')_2$ subunits.

Peptide mapping

For peptide mapping analysis, trastuzumab was digested at a final concentration of 0.5 mg/mL in the presence of 5 mM TCEP. Digestion was performed for 45 min at 75 °C with 15 µL SMART Digest magnetic bulk resin. The procedure was automated using a Thermo Scientific™ KingFisher™ 96 Deepwell plate with the Thermo Scientific™ KingFisher™ Duo Prime purification system, controlled by Thermo Scientific™ KingFisher™ BindIt™ 4.0 software.

Reagents and consumables

- Fisher Scientific™ Water, Optima™ LC/MS grade, (P/N W81)
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (P/N A955-1)
- Fisher Scientific™ Formic acid, Optima™ LC/MS grade (P/N A11710X1AMP)
- Fisher Scientific™ Methanol, Optima™ LC/MS grade (P/N A456-212)
- Sigma-Aldrich, Ammonium acetate, 99.999% (P/N 3723311)
- Thermo Scientific™ 8 M Guanidine-HCl Solution (P/N 24115)
- Thermo Scientific™ Bond-Breaker™ TCEP Solution, Neutral pH (P/N 77720)
- FabRICATOR™ (IdeS enzyme, Genovis)

- SMART Digest Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- Thermo Scientific™ 9 mm Target DP MacroVial 350 µL, Fused Inserts (P/N C4000-LV1)
- Thermo Scientific™ Autosampler Vial Screw Thread Caps (P/N C5000-54B)

Chromatography

For all experiments the Thermo Scientific™ Vanquish™ Horizon UHPLC system was used, consisting of:

- Thermo Scientific System Base Vanquish Flex/Horizon (P/N VF-S01-A-02)
- Thermo Scientific Vanquish Binary Pump H (P/N VH-P10-A-02)
- Thermo Scientific Vanquish Split Sampler HT (P/N VH-A10-A-02)
- Thermo Scientific Vanquish Column Compartment H (P/N VH-C10-A-02)
- MS Connection Kit Vanquish (P/N 6720.0405)

For intact mass analysis under native conditions, 1 µL corresponding to 21 µg trastuzumab sample was injected, online desalted, and eluted with 50 mM ammonium acetate under isocratic gradient using size exclusion liquid chromatography on a 4 × 150 mm Thermo Scientific™ MAbPac™ SEC-1 column with 5 µm particle size (P/N 075592).

For intact mass analysis under denaturing conditions, 1 µL corresponding to 1 µg of trastuzumab sample was injected and eluted from a 2.1 × 50 mm Thermo Scientific™ MAbPac™ RP column with 4 µm particle size (P/N 088648) applying a linear gradient.

For all mAb subunit analyses, 2 µL corresponding to 1 µg of protein were injected and separated via a 2.1 × 100 mm MAbPac RP column with 4 µm particle size (P/N 088647) applying a linear gradient.

For peptide mapping analysis, peptides obtained from a tryptic digest of trastuzumab were separated using reversed-phase liquid chromatography on a 2.1 × 250 mm Thermo Scientific™ Acclaim™ VANQUISH™ C18 UHPLC column with 2.2 µm particle size (P/N 074812-V) applying a linear gradient. 2 µL of trastuzumab digest corresponding to 1 µg of total protein were loaded on column.

Chromatographic separation details for all workflows presented here are summarized in Table 1.

Table 1. Overview of LC conditions: columns, solvents, flow rates, column temperature, and applied gradient applied used for MS analysis of A) intact protein under native conditions, B) intact protein under denaturing conditions, C) subunit analysis, D) peptide mapping

Table 1A. Intact protein analysis under native conditions		
Column	MAbPac SEC-1 5 µm, 4 × 150 mm	
Mobile phase A	Water	
Mobile phase B	100 mM ammonium acetate	
Flow rate	300 µL/min	
Column temperature	25 °C	
Gradient:	Isocratic	
	Time (min)	*%B*
	0.0	50
	10.0	50

Table 1B. Intact protein analysis under denaturing conditions		
Column	MAbPac RP, 4 µm, 2.1 × 50 mm	
Mobile phase A	Water with 0.1% formic acid (v/v)	
Mobile phase B	Acetonitrile with 0.1% formic acid (v/v)	
Flow rate	250 µL/min	
Column temperature	80 °C	
Gradient:	Linear	
	Time (min)	*%B*
	0.0	25
	1.0	25
	9.0	32
	10.0	80
	11.0	80
	12.0	25
	25.0	25

Table 1C. Subunit analysis				
Column	MAbPac RP, 4 µm, 2.1 × 100 mm			
Mobile phase A	Water with 0.1% formic acid (v/v)			
Mobile phase B	Acetonitrile with 0.1% formic acid (v/v)			
Flow rate	250 µL/min			
Column temperature	80 °C			
Gradient:	Linear			
	Reduced mAb		*Subunits (IdeS digest)*	
	Time (min)	*%B*	*Time (min)*	*%B*
	0.0	25	0.0	25
	1.0	25	1.0	25
	13.0	32	7.0	35
	14.0	80	8.0	80
	16.0	80	9.0	80
	16.5	25	9.5	25
	25.0	25	20.0	25

Table 1D. Peptide mapping		
Column	Acclaim VANQUISH C18, 2.2 µm, 2.1 × 250 mm	
Mobile phase A	Water with 0.1% formic acid (v/v)	
Mobile phase B	Acetonitrile with 0.1% formic acid (v/v)	
Flow rate	300 µL/min	
Column temperature	60 °C	
Gradient:	Linear	
	Time (min)	*%B*
	0.0	2
	40.0	40
	42.0	80
	45.0	80
	45.5	2
	60.0	2

Mass spectrometry

For all experiments, the Orbitrap Exploris 480 mass spectrometer (P/N BRE725533) equipped with the BioPharma Option (P/N BRE725539) was used. The instrument was operated with Thermo Scientific™ Xcalibur™ 4.2 SP1 software and controlled by Orbitrap Exploris Series 1.0. instrument control software. All MS parameters used in this study are summarized in Table 2.

Table 2. MS parameter settings for all experiments

MS conditions	Intact native	Intact denatured	Subunit (Reduced mAb)	IdeS digest with reduction	Top/Middle down analysis	Peptide mapping
Method type	Full MS	Full MS	Full MS [two segments]	Full MS	tMS^2	Full MS-ddTop5 HCD
Runtime (min)	10	20	25	20	20	40
Scan range (*m/z*)	2500–8000	1800–4000	600–2000	500–2000	300–2000	200–2000
Resolution	30,000	30,000	240,000/15,000	240,000	240,000	60,000/15,000
RF lens (%)	200	200	60	60	60	40
AGC target value (full MS/MS^2)	300	300	300	300	1000	300/50
Max inject time (Full MS/MS^2)	200	200	200	200	250	100/200
Isolation window (MS^2)	-	-	-	-	*m/z* 100/300	*m/z* 2
Microscans (Full MS/MS^2)	10	10	5	5	10	1/1
Source fragmentation (V)*	120*	110*	0	0	0	-
HCD collision energy (V)	-	-	-	-	12, 14, 16, 18, 20	28
MS Source Settings						
Spray voltage (+)	3800	3800	3800	3800	3800	3800
Capillary temp. (°C)	275	320	320	320	320	320
Sheath gas	25	25	25	25	25	25
Aux gas	5	5	5	5	5	5
Sweep gas	0	0	0	0	0	0
Vaporizer temp.	250	150	150	150	150	150
Application mode	Intact Protein					Peptide
Pressure mode	High		Low			Standard

*requires optimization

A library of pre-defined optimized method templates is available on the Orbitrap Exploris 480 MS for effortless method set-up and easy method transfer between instrument types. An overview of the system templates for Intact Protein application mode available in the method editor of the Orbitrap Exploris 480 mass spectrometer is provided in Table 3.

Data analysis

Raw data files were processed with Thermo Scientific™ BioPharma Finder™ 4.0 software. For intact protein and subunit spectra deconvolution as well as for Top/Middle-Down analysis, glycan structures were added as variable modifications and sequence candidates were generated in the Sequence Manager using masses listed in Table 4. Highlighted in light blue are monoisotopic masses of glycans used to generate sequence candidates for analyses of isotopically resolved subunits (reduced, digested and reduced), and average masses of mixed composition glycan structures used for deconvolution of isotopically unresolved intact trastuzumab proteoforms.

Calculated monoisotopic and average molecular masses of intact trastuzumab glycoforms and subunits are presented in Table 5. Masses represented in bold were used for deconvolution of each respective species to determine mass accuracies of experimental results.

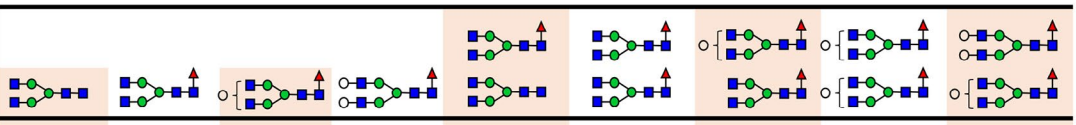
Table 3. System templates for Intact Protein application mode

Intact Protein application mode	System template
mAb – Intact	Intact mAb, denatured
	Intact mAb, native
mAb - LC & HC chains	Light (LC) and Heavy (HC) Chains targeted HCD
	Reduced mAb
mAb - Middle-Down	Middle-Down mAb MS1 + data-dependent HCD Top2
	Middle-Down mAb MS1 + targeted wide isolation HCD
	Middle-Down mAb targeted wide isolation HCD
Protein - Intact	High Resolution Survey Scan for 5–40 kDa Proteins
	Wide Mass Range High Sensitivity MS1 5 to 100 kDa
Top-Down HCD	Top-Down HCD Intact Protein 5–30 kDa
	Top-Down HCD Intact Protein greater than 30 kDa

Table 5. Theoretical monoisotopic and average masses for the LC, HC, Fc/2, and Fd´ subunits and the major glycoforms of intact trastuzumab

	Monoisotopic mass	Average
Light chain, trastuzumab (LC)	23,428.5238	23,442.93
Heavy chain, trastuzumab (HC)	49,125.4364	**49,156.14**
Heavy chain G0F, trastuzumab (HC-G0F)	50,569.966	50,600.67
Heavy chain G0F, trastuzumab (HC-G1F)	50,732.026	50,762.73
Constant fragment of trastuzumab heavy chain (Fc/2)	**23,775.9295**	23,790.76
Constant fragment of trastuzumab heavy chain G0F (Fc/2-G0F)	**25,220.460**	25,235.29
Constant fragment of trastuzumab heavy chain G0F (Fc/2-G1F)	**25,382.520**	23,597.35
Variable fragment of trastuzumab heavy chain (Fd')	**25,367.5174**	25,383.39
Intact trastuzumab	145,075.6700	**145,165.89**
Intact trastuzumab G0, G0F	147,820.2071	**147,910.43**
Intact trastuzumab G0F, G0F	147,966.3490	**148,056.57**
Intact trastuzumab G0F, G1F	148,128.4902	**148,218.71**
Intact trastuzumab G1F, G1F	148,290.6315	**148,380.85**
Intact trastuzumab G1F, G2F	148,452.7728	**148,542.99**

Table 4. Monoisotopic and average masses of elements used to calculate carbohydrate structures based on elemental composition of glycan species contributing to major trastuzumab glycoforms

Monosaccharides nomenclature: GlcNac, Gal, Man, Fuc											
Element	Monoisotopic mass	Average mass	G0	G0F	G1F	G2F	G0/G0F	G0F/G0F	G0F/G1F	G1F/G1F	G1F/G2F
			Elemental composition								
C	12	12.01079	50	56	62	68	6554	6560	6566	6572	6578
H	1.007825032	1.007968	82	92	102	112	10122	10132	10142	10152	10162
N	14.00307401	14.00669	4	4	4	4	1728	1728	1728	1728	1728
O	15.99491462	15.99937	35	39	44	49	2086	2090	2095	2100	2105
S	31.97207069	32.0639	0	0	0	0	0	0	0	0	0
Monoisotopic mass			1298.47596	1444.533869	1606.58669	1768.63952	2743.00983	2889.067738	3051.120562	3213.173385	3375.226209
Average mass			1299.19759	1445.339486	1607.48076	1769.62203	2744.537072	2890.678972	3052.820242	3214.961512	3377.102782

Deconvolution of isotopically unresolved mass spectra obtained from analysis of trastuzumab under native conditions was performed using the Thermo Scientific™ ReSpect™ and Sliding Windows algorithms. The retention time range was set to 3 to 5 minutes, capturing the elution of the entire chromatographic peak, and the sliding window width was set to 0.1 minute. It is important to note how the sliding window width is determined by calculating the autocorrelation function of the chromatogram and examining this to determine the characteristic scale width of peaks in the chromatogram. This approach is proved to be significantly more robust, less sensitive to parameter choices, and less sensitive to the peculiarities of individual features in the chromatogram. Target average spectrum offset was set to a scan numerical value (percentage option available, and if selected will offset retention time), which means that each window is offset from its predecessor by user-defined number of scans. Detailed parameters for ReSpect Sliding Window deconvolution are summarized in Table 6.

Table 6. Intact native deconvolution parameters

Parameters	Intact native MS
Algorithm	ReSpect
Output mass range (Da)*	140,000 to 160,000
Deconvolution mass tolerance (ppm)	15
Mass range (*m/z*)	2500–8000
Model mass range (Da)	10,000–160,000
Charge state range	5 to 100
Min adjacent charges (low & high model mass)	4-4
Deconvolution quality score threshold (AU)	30
Target mass (Da)	160,000
Number of peak models	1
Left/right peak shape	2
Peak detection minimum significance measure	1
Peak detection quality measure (%)	95
Peak model width factor	1
Intensity threshold scale	0.01
Noise compensation	Checked
Charge carrier	H^+ (1.00727663)
Sliding Windows	
RT range (min)*	3–5
Target average spectrum width (min)*	0.1
Scan offset (numeric)	1
Merge tolerance*	15 ppm
Max RT gap (min)	1
Min number of detected intervals*	10

*Parameters modified from the Default Native processing method with Sliding Windows algorithm.

For data processing of subunit and Top/Middle-Down spectra, the *Default ReSpect* and *Default Xtract* as well as *Top-Down Default* deconvolution methods provided in BioPharma Finder software were used with their respective parameters listed in Tables 7 and 8. For subunits carrying *N*-linked glycan modification (HC, Fc/2), only the two most abundant glycoforms (G0F and G1F) were considered for assignments of fragment ions.

For peptide mapping analysis, default peptide mapping parameters in the BioPharma Finder software template were used with the *Enable Automatic Parameter Values* selected. Using this feature facilitates the software to determine values for key parameters based on the raw mass spectrometry data. When this parameter is not selected, the software will use the customized user-defined values. It is recommended to enable this parameter. The default variable modifications were used for the database searching with CHO selected for the *N*-glycosylation search.

Table 7. Deconvolution parameters for all subunit analysis data

Parameters	LC, Fc/2, Fd'	HC*
Algorithm	Xtract (For isotopically resolved spectra)	ReSpect (For isotopically unresolved spectra)
Charge	10 to 50	20 to 70
Mass range (*m/z*)	600–2000	600–2000
Output mass range (Da)	24,000 to 26,000	45,000 to 55,000
Deconvolution mass tolerance (ppm)	10	10

* Parameters modified from the Default ReSpect processing method.

Table 8. Top/Middle-Down analysis parameters

Parameters	Intact native MS
Parameters	LC, HC, Fc/2 and Fd'
RT time	Automatically specified by .raw file segment
Activation type	HCD
Fragmentation mass tolerance (ppm)	10
m/z range*	300–2000
Output mass range	50–60,000
S/N threshold	7
Charge range	1–25
Fit factor (%)*	80
Remainder threshold (%)	25
Consider overlaps	Checked

* Parameters modified from the Top-Down Default Method processing method.

Results and discussion

New improved hardware

The Orbitrap Exploris 480 mass spectrometer consists of a Thermo Scientific™ OptaMax™ NG ion source with an adjustable heated electrospray ionization (H-ESI II) probe, a high-capacity transfer tube (HCTT), an electrodynamic ion funnel (EDIF), an S-shaped bent flatapole, a hyperbolic-rod segmented quadrupole mass filter, an Independent Charge Detector (ICD) module, a C-trap/Orbitrap block with an ultra-high-field Orbitrap mass analyzer, and finally an Ion-Routing Multipole (IRM) module. The electrodynamic ion funnel efficiently captures ions as they leave the HCTT, supporting improved sensitivity and desolvation efficiency with increased ion flux into the vacuum system through reduced ion losses. The horizontally aligned and symmetrically supported ultra-high-field Orbitrap analyzer is open to pumping on both ends and combines improved mechanical and electrical balancing with a better vacuum inside the trap, enabling higher-resolution analysis with longer transients, and improved signal-to-noise performance for intact protein analysis compared to previous platforms. In addition, the trapping path of ions in this instrument applied in all application modes routes ions via the IRM prior to mass analysis (Figure 3A). These improvements extend the lifetime of protein ions resulting in superior data obtained for intact protein analysis.

The Orbitrap Exploris 480 mass spectrometer with BioPharma Option provides the Intact Protein application mode in addition to the Small Molecule and Peptide application modes. The Intact Protein application mode enables operation of the instrument at different IRM pressure settings (Figure 3B) to provide optimal conditions for two application scenarios. First, for the analysis of large proteins like intact monoclonal antibodies under denaturing and native conditions, the pressure in the IRM can be raised to optimize the trapping and sensitivity of these compounds. Second, to obtain isotopically resolved spectra of small- and medium-size intact proteins like light and heavy chains of monoclonal antibodies, the pressure can be lowered to achieve the best possible vacuum inside the Orbitrap analyzer. The IRM pressure is measured directly by a Pirani gauge and can be regulated in the range from 3×10^{-3} to 18×10^{-3} mbar in the Diagnostics of the Orbitrap Exploris 480 MS Tune Application. For the analysis of mAb derived peptides, the Peptide application mode is applicable, which maintains the pressure in the IRM at the optimal instrument operational level.

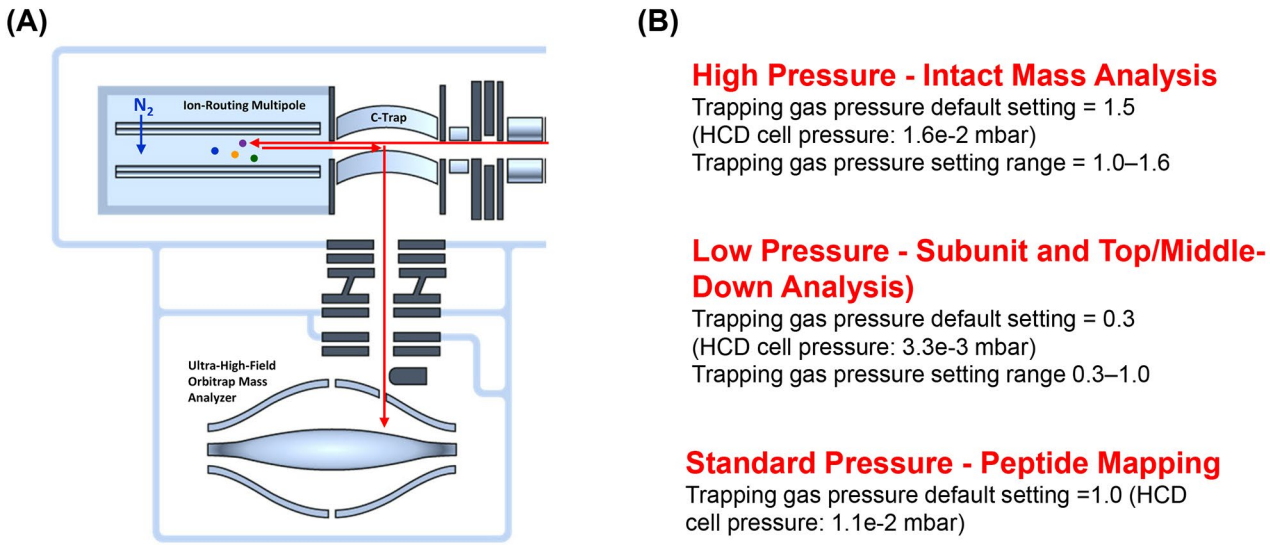


Figure 3. Schematic of the C-trap-HCD cell and Orbitrap region of the Orbitrap Exploris 480 mass spectrometer depicting the trapping gas path and options of pressure settings. (A) One trapping path mode is used for all analysis types on the Orbitrap Exploris 480 sending the ions via the HCD cell for ion trapping and back via the C-trap into the Orbitrap mass analyzer for detection. (B) The BioPharma Option enables an extended mass range up to *m/z* 8000 and Intact Protein application mode, which allows for additional selection of high- and low-pressure settings.

Intact mAb analysis under native and denaturing conditions

For the analysis of mAbs under native conditions, the parameters used for analysis were aiming to preserve the native folded state of the mAb as much as possible by using an aqueous solvent containing volatile ammonium acetate salt with near-neutral pH. LC-MS analysis was performed by isocratic separation via a MAbPac SEC-1 column with subsequent mass detection on the Orbitrap Exploris 480 mass spectrometer equipped with the BioPharma Option that enables mass detection up to *m/z* 8,000. The observed envelope for trastuzumab represents charge states from +23 to +29 detected over a mass range of *m/z* 5,000–6,500 (Figure 4A). Both raw and deconvoluted spectra show a baseline resolved pattern representing the mAb's various glycoforms with achieved mass accuracies between 2.2 and 6.2 ppm (Figure 4A insert).

Performing the LC-MS analysis under denaturing conditions using the same trastuzumab sample with separation via a MAbPac RP column results in the detection of significantly higher charge states represented in the obtained mass spectra at a lower *m/z* range by a broader charge envelope spanning from +39 to +70 (Figure 4B). A highly similar spectrum is obtained upon deconvolution with mass accuracies between 1.7 and 4.8 ppm compared to the analysis under native conditions (Figure 4B insert).

Analysis of trastuzumab subunits

Reduction of mAb inter- and intra-molecular (or inter/intra-chain) disulfide bonds with chemical agents, such as DTT or TCEP, generates light and heavy chains (25 and 50 kDa, respectively). Alternatively, smaller mAb subunits can be obtained by proteolytic digestion with the commonly used IdeS enzyme (immunoglobulin-degrading enzyme from *Streptococcus pyogenes*) that specifically cleaves below the hinge region to yield $F(ab')_2$ and Fc fragments (~98 and ~50 kDa, respectively) followed by chemical reduction yielding the three subunits Fc/2, LC and Fd´ with 23–25 kDa in molecular weight (Figure 5).

The instrument method described here comprised a full MS scan acquisition at both high- and low-resolution settings during the same analytical run. The analysis starts with the application of a higher resolution setting of 240,000, resulting in an isotopically resolved spectrum and the monoisotopic mass upon deconvolution with an achieved mass accuracy of 1.7 ppm for the earlier eluting light chain. (Figure 6). The second segment in the instrument method applies a lower resolution setting of 7500 (at *m/z* 200) for the acquisition of the heavy chain (50 kDa), resulting in an isotopically unresolved spectrum providing average masses upon deconvolution applying the ReSpect algorithm for the two glycoforms G0F and G1F with mass accuracies of 1.8 and 2.8 ppm, respectively. Furthermore, with the

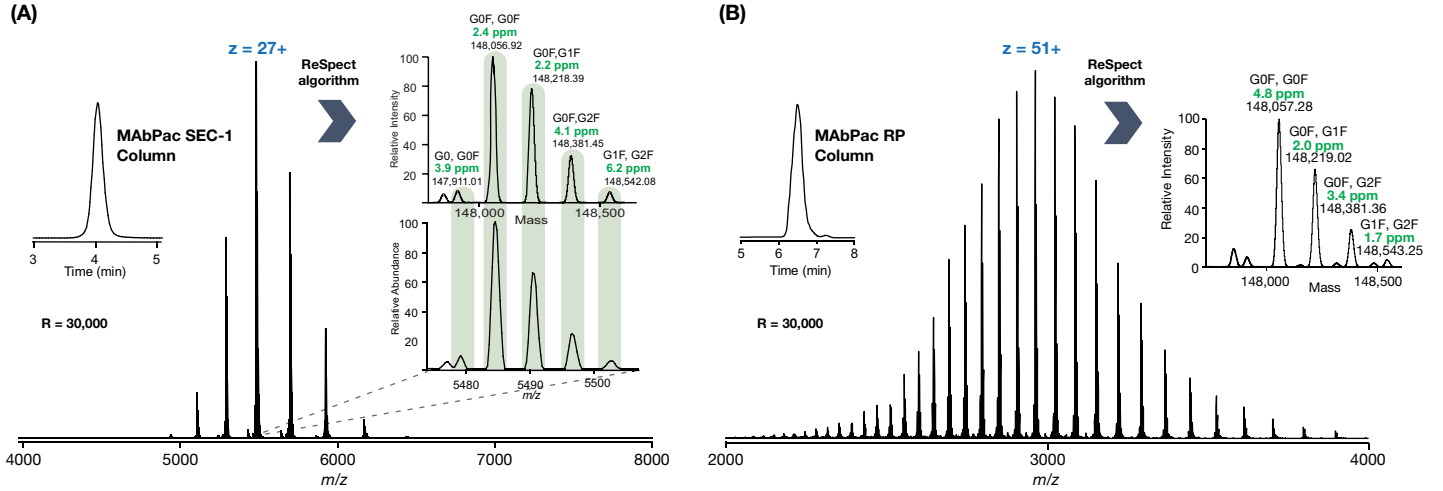


Figure 4. A) Full MS spectrum representing a single scan acquired with 10 µscans obtained from intact monoclonal antibody trastuzumab under native conditions, acquired at a resolution setting of 30,000 (at *m/z* 200). The insert represents an expanded view of the most abundant charge state (z = 27+) with baseline resolved glycoform pattern (top), and the mass spectrum obtained upon using the Sliding Windows and ReSpect algorithms (bottom). B) Full MS spectrum representing an average of six scans acquired at 10 µscans each from the intact monoclonal antibody trastuzumab (IgG1) under denaturing conditions, acquired at a resolution setting of 30,000 (at *m/z* 200). The inset represents the spectrum obtained upon deconvolution providing mass accuracies below 5 ppm for all trastuzumab glycoforms.

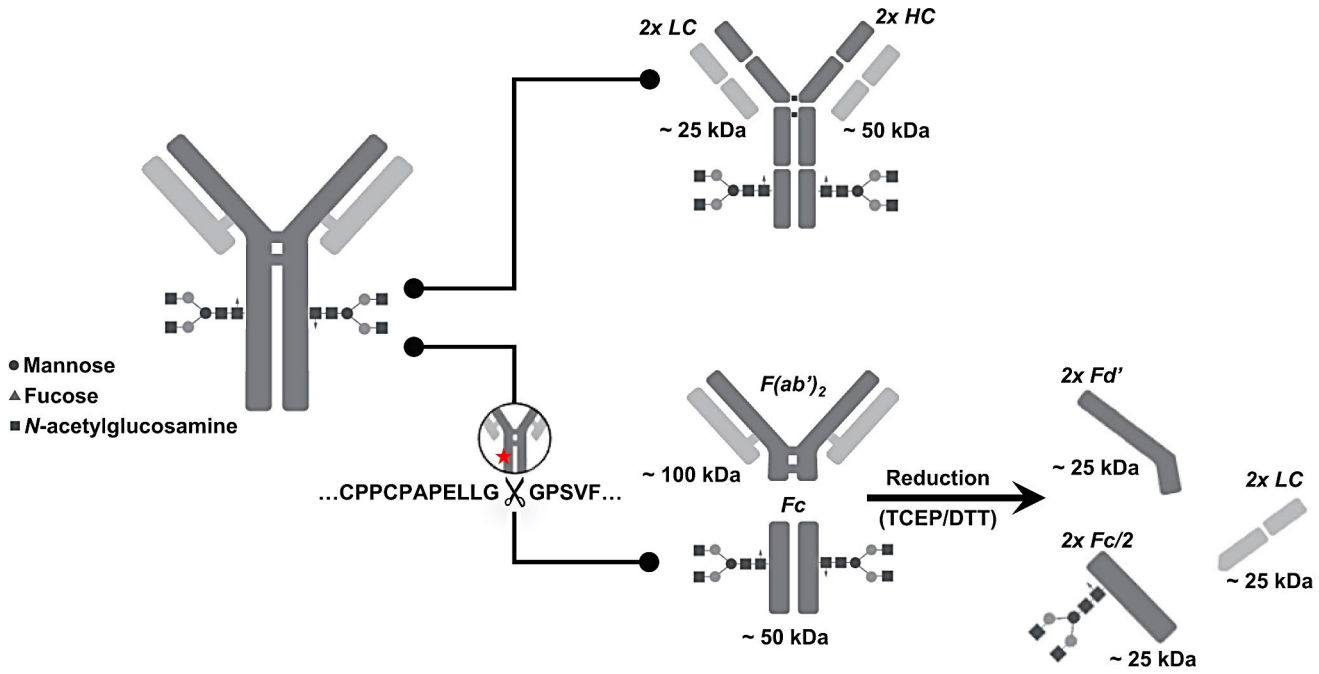
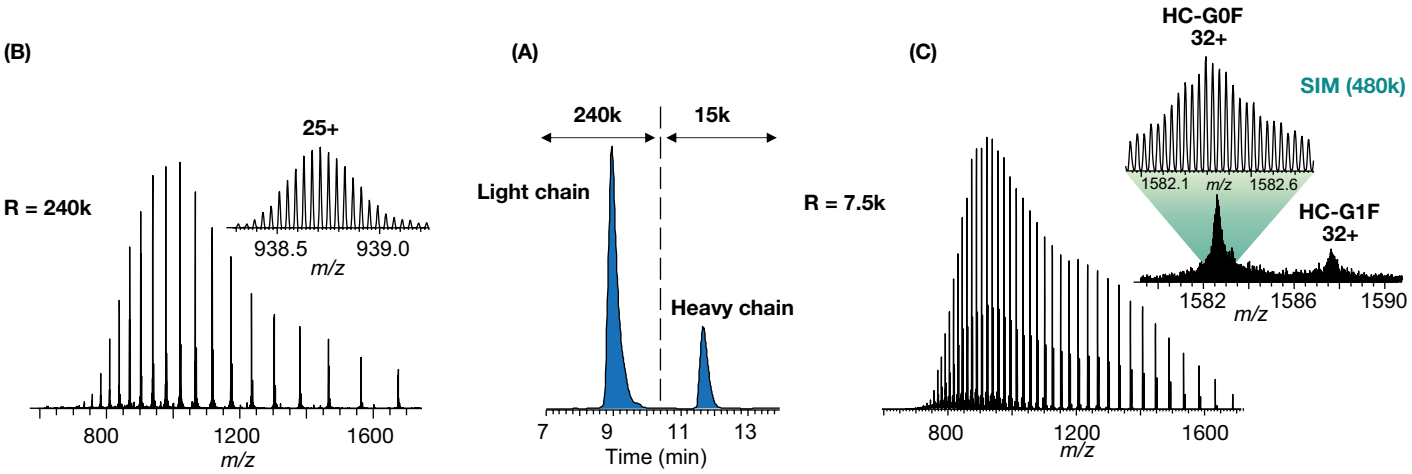


Figure 5. Subunit generation. Representation of an IgG1 class antibody structure and generated subunits via chemical reduction (top) or IdeS digestion with subsequent chemical reduction (bottom)



(D)

Subunit	Monoisotopic mass (theoretical)	Monoisotopic mass (experimental)	ΔM (ppm)
LC	23,428.5238	23,428.5698	+1.7

Xtract algorithm

ReSpect algorithm

Subunit	Average mass (theoretical)	Average mass (experimental)	ΔM (ppm)
HC - G0F	50600.67	50601.26	+1.8
HC - G1F	50762.73	50763.64	+2.8

Figure 6. Intact mass measurement of trastuzumab subunits obtained upon chemical reduction. (A) Total ion chromatogram (TIC) of the separated light and heavy chains. Full MS spectra representing (B) Full MS spectrum of the light chain acquired at a resolution setting of 240,000. (C) Full MS spectrum of the heavy chain acquired at a resolution setting of 7,500. The inserts represent the isotopically resolved +25 charge state of the light chain and a SIM scan acquired at a resolution setting of 480,000 of the +32-charge state of the heavy chain's G0F. Excellent mass accuracies of 1.7–2.8 ppm were obtained upon deconvolution (D).

Orbitrap Exploris 480 mass spectrometer, the highest resolution settings of 480,000 may be leveraged to provide baseline resolved isotopic spectra also for the heavy chain as depicted in the inset of Figure 6C, showcasing the 32+ charge state of the HC-G0F glycoform.

Top-Down and Middle-Down MS analyses of all subunits (HC/LC and LC/Fd'/Fc/2) were performed as an additional orthogonal approach for confirmation of the mAb primary structure. All targeted tandem mass spectra (tMS^2) were acquired with a resolution setting of 240,000. LC and HC subunits were fragmented with HCD in duplicate runs with HCD collision energies varying in individual experiments from 12 to 20 V, applying an isolation window of *m/z* 100 in all runs. The precursor ion isolation window was centered at *m/z* 900 for both subunits. Representative tMS^2 mass spectra for each HCD energy setting are shown in Figure 7A. As expected, increasing the HCD energy from 12 to 20 V results in mass spectra with a broadening distribution of fragment ions across the *m/z* range. In the case of the light chain, low mass ions matching *N*-terminal *b*-ion sequence tags start to form with an applied HCD energy of 16 V, while for the heavy chain terminal fragment ions are observed already at an energy setting of 12 V, indicating a difference in fragmentation susceptibility between the two subunits. Cumulative graphical fragmentation maps are presented in Figure 7B and show 62% amino acid sequence assignment of the LC, and 36.5% for the HC, which exceed previously reported reduced mAb subunit sequence coverage by ~19% and 13%, respectively.[8] The highest percentage of residues assigned in a single HCD tMS^2 analysis for LC was 39% with HCD energy set to 18 V and for HC 15% with HCD energy set to 14 V.

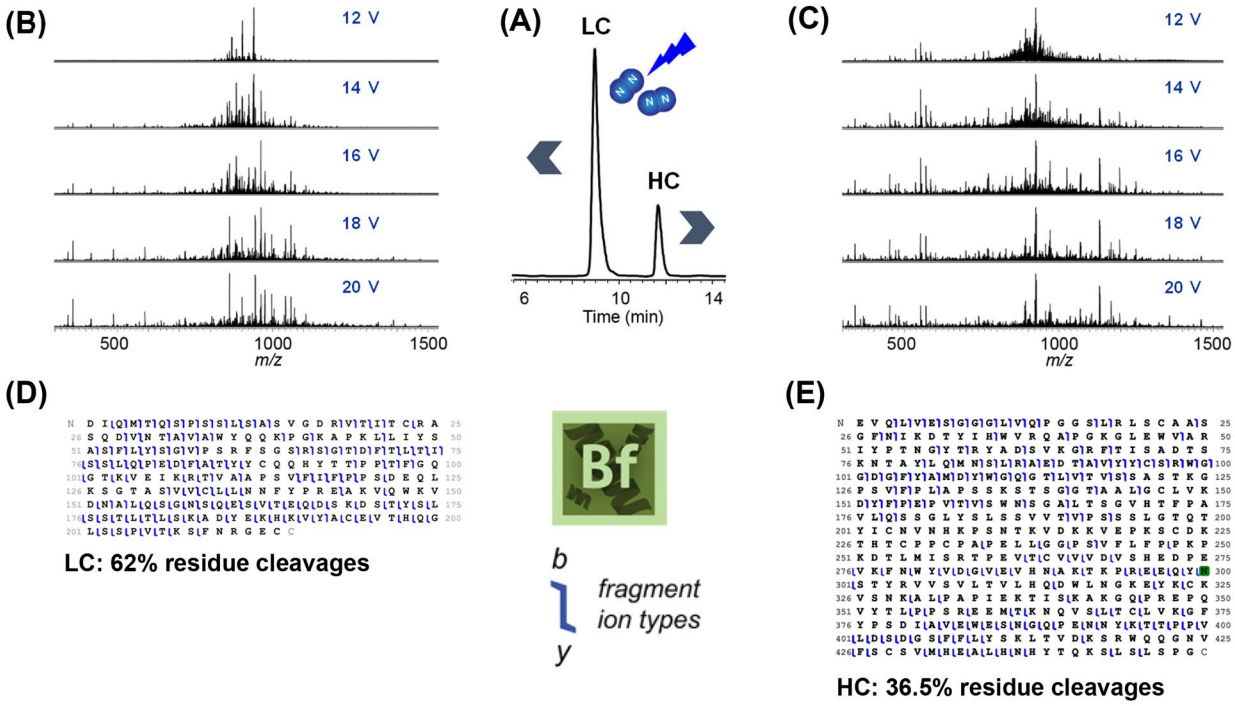
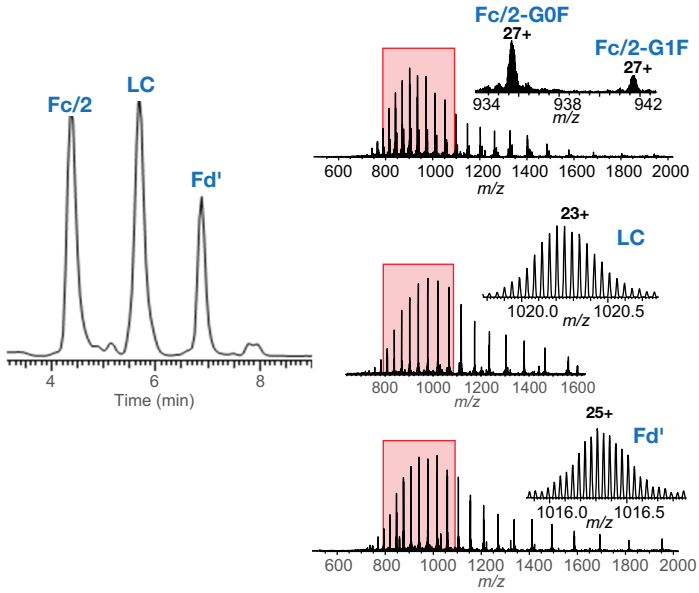


Figure 7. Top-Down analysis of reduced trastuzumab. (A) TIC of separated subunits with tandem mass spectra obtained for the light (B) and heavy chain (C) with applied collision energies increasing from 12 to 20 V. Cumulative graphical fragmentation maps obtained from TD MS of the light chain (D) and heavy chain (E) with the green rectangle in the heavy chain sequence representing the *N*-glycosylation site N300.

Subunit analysis of IdeS digested and reduced trastuzumab

The obtained total ion chromatogram shows a baseline separation of Fc/2, LC, and Fd' subunits of trastuzumab (Figure 8A). Full MS scans were acquired on the Orbitrap Exploris 480 mass spectrometer at a resolution setting of 240,000, resulting in isotopically resolved spectra for all ~23–25 kDa subunits, as highlighted in the insets with an expanded view of most abundant charge states (z = 23+, 25+ and 27+ for LC, Fd' and Fc/2, respectively). The deconvolution of the raw data using the Xtract algorithm provided monoisotopic masses matching the theoretical masses with mass deviations between 0.7 and 2.1 ppm for the three subunits (Figure 8B). The middle-down analysis was performed in separate experiments applying wide isolation windows of *m/z* 300 as indicated by the red boxes in Figure 8A with increasing collision energy settings ranging from 12 to 20 V, relating to the settings also used for experiments shown in Figure 7. Matching of obtained fragment ion spectra against the amino acid sequences of the individual subunits resulted in the cumulative graphical fragmentation maps representing excellent levels of residue cleavages for all subunits: Fc/2: 52%, LC: 58%, and Fd': 52%.

(A)



(B)

Subunit	Monoisotopic mass (theoretical)	Monoisotopic mass (experimental)	ΔM (ppm)
Fc/2 - G0F	25,220.46338	25,220.50382	+1.6
Fc/2 - G1F	25,382.51621	25,382.53286	+0.7
LC	23,428.52384	23,428.56981	+2.0
Fd'	25,367.51740	25,367.54613	+1.1

(C)

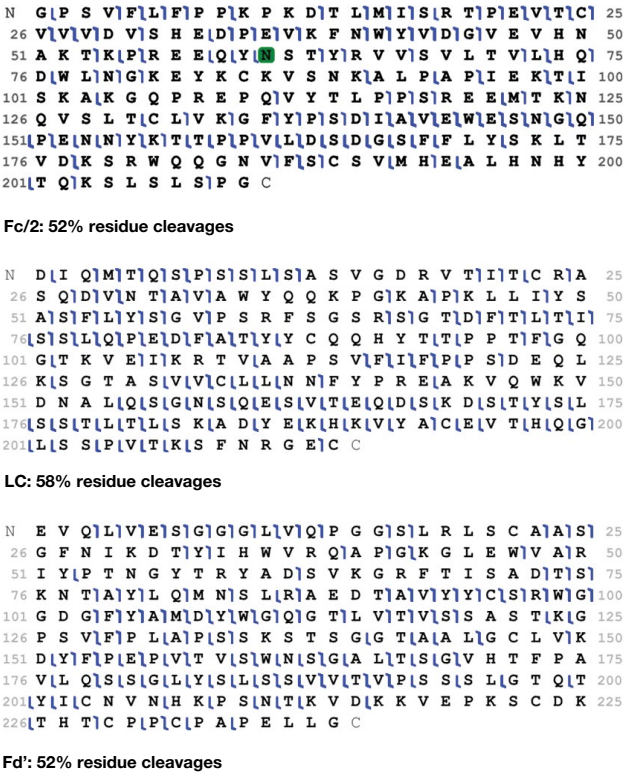


Figure 8. IdeS digested and reduced subunit analysis for trastuzumab. (A) TIC of the three separated ~23–25 kDa subunits Fc/2, LC, and Fd', obtained from an IdeS digest of trastuzumab followed by chemical reduction as well as Full MS spectra and zoom into an individual charge state for each of the subunits. Red rectangles indicate isolation windows (*m/z* 300) used for subsequent targeted HCD fragmentation (spectra not shown). (B) Comparison of theoretical and experimental masses resulting from deconvolution of the isotopically resolved mass spectra providing monoisotopic masses with ≤2 ppm mass accuracy for all subunits. (C) Cumulative graphical fragmentation maps represent excellent % residue cleavages for all subunits: Fc/2: 52%, LC: 58%, and Fd': 52%.

Peptide mapping

Trastuzumab was analyzed at the peptide level after performing protein digestion using SMART Digest Trypsin kits. Figure 9 displays the peptide mapping results obtained after processing the raw data with BioPharma Finder software. The base peak chromatogram displays the tryptic peptides separated by RP-LC over a 40 minute linear gradient and represents the identified peptides associated with the light (pink) and heavy (green) chains with color shading (Figure 9a). Obtained sequence coverage for the trastuzumab light and heavy chains was 100% (Figure 9B).

Figure 9C highlights an example of a methionine present in the trastuzumab HC, which is particularly susceptible to oxidation as it is solvent-exposed. Examples taken from BioPharma Finder software show the excellent mass accuracy obtained on the Orbitrap Exploris 480 mass spectrometer for both modified and unmodified DTLMISR peptides. The MS^2 fragmentation example shows the molecular weight increase of +15.995 Da for y4 and y5 fragment ions, providing further confidence in the identification. Moreover, both y_4 and y_5 fragment ions represent the loss of methane sulfenic acid (CH_3SOH) with an indicative mass shift of -63.9983 resp. -63.9980 Da.[9,10] A more detailed PTM assessment on mAb peptides performed on the Orbitrap Exploris 480 mass spectrometer has been described previously.[11]

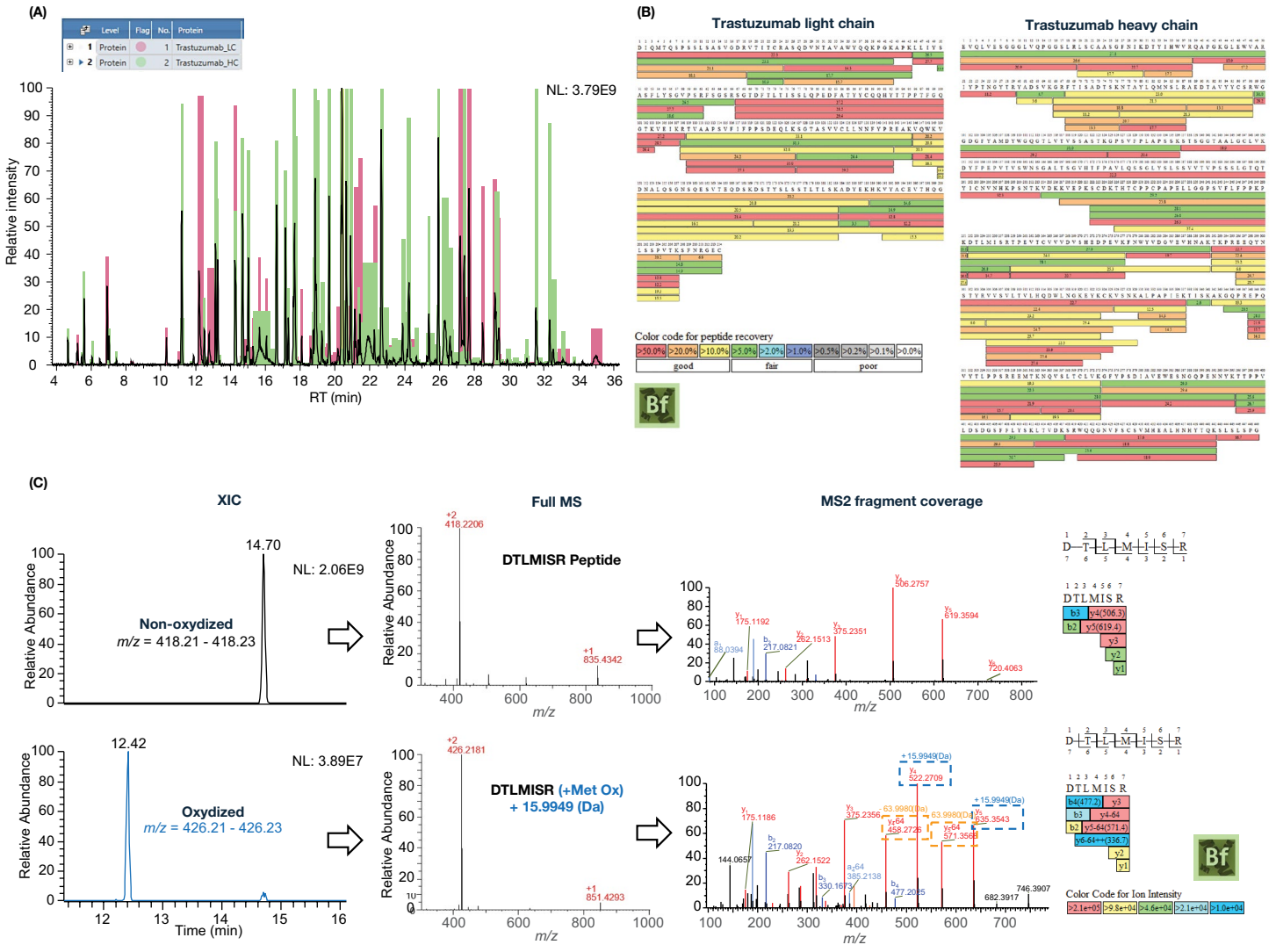


Figure 9. Peptide mapping analysis of trastuzumab. (A) Base peak chromatogram (BPC) with colored shading indicating identified peptides associated with the light (pink) and heavy (green) chains. (B) Trastuzumab sequence coverage map. (C) XICs for the peptide DTLMISR from the heavy chain with and without methionine oxidation. Indicative shifts of +15.9952 were observed for the y_4 and y_5 fragment ions.

thermo scientific

Conclusions

- Here we present data obtained from the three major workflows for characterizing biopharmaceuticals on a new LC-MS platform: intact mass analysis under native and denaturing conditions, subunit analysis complemented with Middle-Down analysis, and peptide mapping.
- Data acquisition was supported by using method templates with optimized default settings for the individual analysis workflows that are provided with the MS instrument software, allowing for easy instrument setup as well as method transfer across platforms and even across different biopharma analytical labs.
- The improved vacuum conditions compared to previous generation Orbitrap mass spectrometers support superior performance for intact protein analysis under native conditions and provide significantly improved results obtained from Top/Middle-Down analyses.
- Moreover, the new hardware design of the Orbitrap Exploris 480 mass spectrometer provides a robust, easily serviceable instrument with extended uptime and reduced footprint requiring significantly less bench space.
- The unmatched performance and application benefits provided by the Orbitrap Exploris 480 MS make it the ideal instrument for full characterization of biopharmaceuticals that require the highest sensitivity, scan speed, and resolution to achieve results with the highest confidence.

Acknowledgments

The authors would like to thank the large team at Thermo Fisher Scientific who supported the work in this application note, and in particular Angela Criscuolo, Marc Guender, David Munoz, and Jenny Ho.

References

1. Srzentić, K. et al. Advantages of Extended Bottom-Up Proteomics Using Sap9 for Analysis of Monoclonal Antibodies. *Anal. Chem.* **2014,** *86* (19) 9945–9953.
2. Beck, A.; Liu, H. Macro- and Micro-Heterogeneity of Natural and Recombinant IgG Antibodies. *Antibodies* (Basel). **2019,** *8* (1) 18.
3. Babaly, B. Protocols for the analytical characterization of therapeutic monoclonal antibodies. II – Enzymatic and chemical sample preparation. *J. Chrom. B* **2017,** *1060,* 325–335.
4. Fornelli, L. Accurate Sequence Analysis of a Monoclonal Antibody by Top-Down and Middle-Down Orbitrap Mass Spectrometry Applying Multiple Ion Activation Techniques. *Anal. Chem.* **2018,** *90* (14) 8421–8429.
5. Sjogren, J. et al. Rapid and improved characterization of therapeutic antibodies and antibody related products using IdeS digestion and subunit analysis. *Analyst* **2016,** *141,* 3114–3125.
6. Srzentić, K.; Fornelli, L. *et al.* Interlaboratory Study for Characterizing Monoclonal Antibodies by Top-Down and Middle-Down Mass Spectrometry *J. Am. Soc. Mass Spectrom.* **2020,** *31* (9) 1783–1802.
7. Toby, T. et al, Progress in Top-Down Proteomics and the Analysis of Proteoforms. *Anal. Chem.* **2016,** *9* (1) 499–519.
8. Scheffler, K.; Damoc, E. Full characterization of heterogeneous antibody samples under denaturing and native conditions on the Q Exactive BioPharma mass spectrometer. Thermo Scientific Application Note 72348 (2017). https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/AN-72348-LC-MS-Heterogeneous-Antibody-Samples-AN72348-EN.pdf
9. Pan, H. et al. Methionine oxidation in human IgG2 Fc decreases binding affinities to protein A and FcRn. *Protein science: a publication of the Protein Society* **2009,** *18* (2) 424–33.
10. Guan, Z. et al. Detection and characterization of methionine oxidation in peptides by collision-induced dissociation and electron capture dissociation. *J. Am. Soc. Mass Spectrom.* **2003,** *14,* 605–613
11. Widdowson, P.; Claydon, A.; Buchanan, T. Accelerating monoclonal antibody peptide mapping with high acquisition speed Orbitrap-based MS. Thermo Scientific Application Note 73672 (2020). https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-73672-lc-ms-peptide-mapping-accelerating-throughput-an73672-en.pdf

Find out more at **thermofisher.com/biopharma**

ThermoFisher SCIENTIFIC