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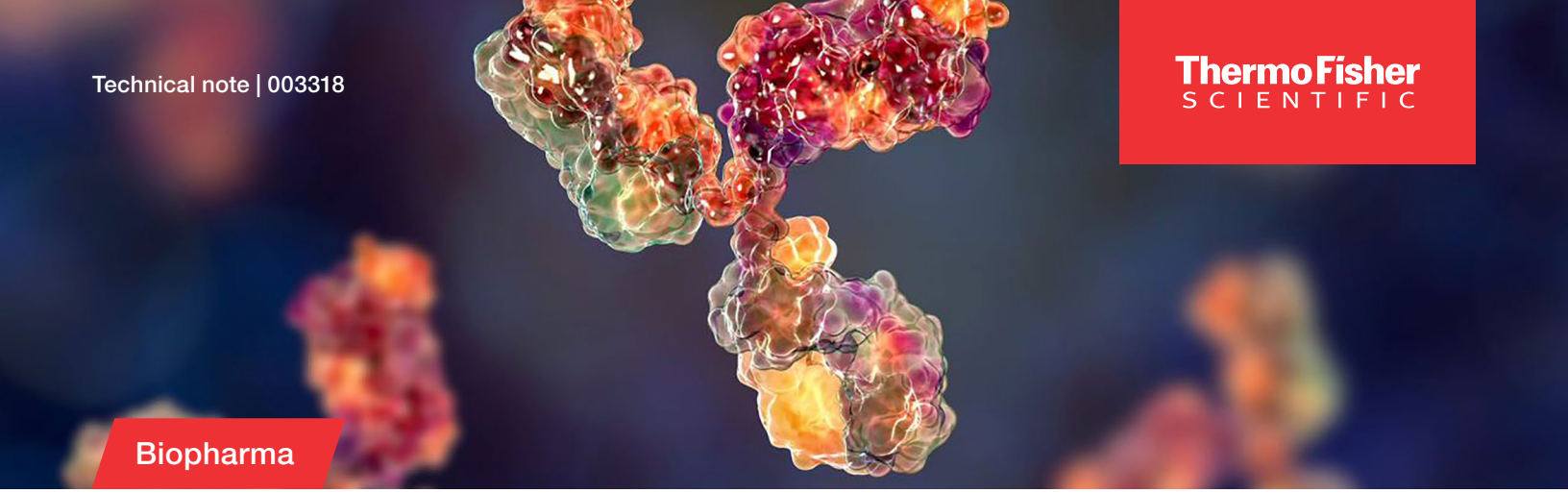

Biopharma

In-depth characterization of monoclonal antibodies

Intact mass analysis and middle-down mass spectrometry approaches on an Orbitrap Ascend BioPharma Tribrid mass spectrometer

Authors

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Keywords

Orbitrap Ascend BioPharma Tribrid mass spectrometer, Tribrid, monoclonal antibodies, mAbs, mAb subunits, EThcD, UVPD, PTCR, high mass range, native and denaturing conditions, intact mass analysis, middle-down mass spectrometry, trastuzumab

Goal

To assess the performance of the Thermo Scientific™ Orbitrap™ Ascend BioPharma Tribrid™ mass spectrometer for comprehensive monoclonal antibody (mAb) characterization using intact mass analysis and middle-down mass spectrometry (MD-MS) approaches. The MD-MS methods were developed using a combination of powerful ion activation techniques including electron transfer dissociation (ETD), electron transfer higher energy collision dissociation (EThcD), ultraviolet photodissociation (UVPD), and ion manipulation by proton-transfer charge reduction (PTCR).

Introduction

mAbs can display structural heterogeneity due to the presence of various post-translational modifications (PTMs), such as glycosylation, oxidation, and deamidation. These modifications can affect their structure, stability, and function. Therefore, in-depth characterization of mAb heterogeneity is critical to ensure the safety, efficacy, and quality of biotherapeutics. The middle-down mass spectrometry (MD-MS) approach has emerged as a promising tool for therapeutic characterization.[1-4] It involves the formation of mAb subunits by reduction and/or digestion, followed by tandem MS (MS/MS) fragmentation of these subunits, offering high sequence coverage while involving a straightforward sample preparation.

The Orbitrap Ascend BioPharma Tribrid mass spectrometer offers many benefits for biopharmaceutical characterizations. The high mass accuracy and high resolution offered by the Orbitrap technology enable precise mass measurement of intact mAbs or subunits. The Orbitrap Ascend BioPharma MS provides multiple MS/MS fragmentation

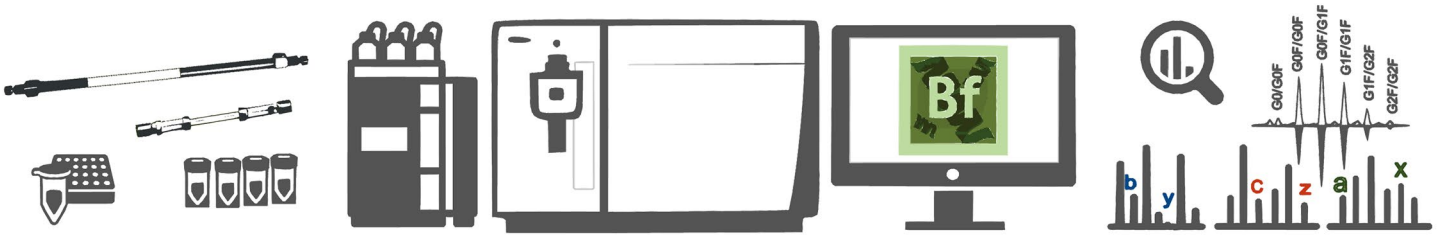


Figure 1. Major LC-MS components used for biopharmaceutical characterization. Experiments were using the Thermo Scientific Vanquish Flex UHPLC system and Orbitrap Ascend Tribrid BioPharma mass spectrometer, together with the MAbPac reverse phase (P/N 088648) and SEC-1 size exclusion (P/N 077592) columns for LC separation, and BioPharma Finder software for data processing.

techniques, including collision-induced dissociation (CID), higher-energy collisional dissociation (HCD), ETD, EThcD, electron transfer and collision-induced dissociation (ETciD), and UVPD, to achieve a full characterization of mAbs. In addition, this powerful instrument offers the PTCR technology with extended mass ranges (up to 16,000 *m/z* with Native MS option enabled) that can be employed alone or combined with different fragmentation techniques for the characterization of complex biotherapeutics. The Orbitrap Ascend BioPharma MS also benefits from its unique revolutionary architecture by having a second ion-routing multipole (front HCD cell) in the system which improves the transmission of large molecules and increases the sensitivity to detect low-abundance species in complex samples.

In this work, intact mass analysis and MD-MS approaches were employed to characterize trastuzumab and its subunits on an Orbitrap Ascend BioPharma Tribrid MS. All the analyses were performed in the intact protein mode at various pressure modes, and for intact trastuzumab the Native MS option was used. Figure 1 shows the main LC-MS workflow employed for intact and subunit analyses of trastuzumab.

Experimental

Consumables

- Water, UHPLC-MS grade, Thermo Scientific™ (P/N W81)
- Acetonitrile, 100%, UHPLC-MS grade, Thermo Scientific™ (P/N A9561)
- Formic acid, Optima™ LC-MS grade, Fisher Chemical™ (P/N A11710X1-AMP)
- Ammonium acetate, 99.999%, Sigma-Aldrich (P/N 3723311)
- 8 M Guanidine-HCl solution, Thermo Scientific™ (P/N 24115)
- Dithiothreitol (DTT) No-Weight™ Format, Thermo Scientific™ Pierce™ (P/N 20291)
- Promega™ IdeS™ Protease (P/N PRV7511)
- Eppendorf™ Protein LoBind™ Tubes (P/N 05-414-205)
- Herceptin™ (trastuzumab) was obtained from Genentech (South San Francisco, CA)

Chromatography

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

- System Base Vanquish Flex/Horizon (P/N VF-S01-A-02)
- Vanquish Binary Pump F (P/N VF-P10-A)
- Vanquish Autosampler (P/N VF-A10-A)
- Vanquish Column Compartment (P/N VH-C10-A)
- MS Connection Kit for Vanquish LC systems (P/N 6720.0405)

For intact mass analysis under native conditions, proteins were desalted online using a size exclusion column (Thermo Scientific™ MabPac™ SEC-1 column, 5 µm, 4 × 150 mm, P/N 077592) and isocratic elution with 50 mM ammonium acetate at 300 µL/min flow rate.

For intact mass and subunit analysis under denaturing conditions, the Thermo Scientific™ MabPac™ RP column, 4 µm, 2.1 × 50 mm (P/N 088648) was used with a gradient of solvent A consisting of water/0.1% formic acid and solvent B consisting of acetonitrile/0.1% formic acid.

All column specifications and LC gradients used for the different analyses are summarized in Tables 1A-1D

Table 1A. LC method for native intact trastuzumab analysis (8 min method duration)		
Column	MabPac SEC-1, 5 µm, 4 × 150 mm	
Gradient	**Time**	**%B**
	0	50
	8	50
LC parameters	Flow rate (mL/min)	0.300
	Column temperature (°C)	25
	Mobile phase A	Water
	Mobile phase B	100 mM Ammonium acetate
	Sampler temperature (°C)	7

Table 1B. LC method for intact denaturing trastuzumab analysis (8 min method duration)		
Column	MabPac RP, 4 µm, 2.1 × 50 mm	
Gradient	**Time**	**%B**
	0	25
	1.0	25
	3.5	50
	4.5	80
	5.5	80
	5.6	25
	8	50
LC parameters	Flow rate (mL/min)	0.250
	Column temperature (°C)	80
	Mobile phase A	Water with 0.1% formic acid
	Mobile phase B	Acetonitrile with 0.1% formic acid
	Sampler temperature (°C)	7

Table 1C. Denaturing LC method for separating Fc/2, LC, and Fd' subunits (16 min method duration)		
Column	MabPac RP, 4 µm, 2.1 × 50 mm	
Gradient	**Time**	**%B**
	0	5
	1.5	20
	10.0	40
	10.5	90
	13.0	90
	13.5	5
	16	5
LC parameters	Flow rate (mL/min)	0.250
	Column temperature (°C)	80
	Mobile phase A	Water with 0.1% formic acid
	Mobile phase B	Acetonitrile with 0.1% formic acid
	Sampler temperature (°C)	7

Table 1D. Denaturing LC method for separating LC and HC subunits (16 min method duration)		
Column	MabPac RP, 4 µm, 2.1 × 50 mm	
Gradient	**Time**	**%B**
	0	25
	1.0	25
	10.0	41
	10.5	90
	12.5	90
	13.0	25
	16	25
LC parameters	Flow rate (mL/min)	0.250
	Column temperature (°C)	80
	Mobile phase A	Water with 0.1% formic acid
	Mobile phase B	Acetonitrile with 0.1% formic acid
	Sampler temperature (°C)	7

Sample preparation

Trastuzumab monoclonal antibody was aliquoted into 10 µg/µL stock solution for all experiments.

Intact mAb analysis

For intact mass analysis under native conditions, 1 µL of antibody (10 µg/µL) was analyzed using a size exclusion column in SEC-LC-MS experiments.

For intact mass analysis under denaturing conditions, the antibody was diluted with 0.1% formic acid in water to a series of concentrations at 10 ng/µL, 50 ng/µL, 100 ng/µL, 500 ng/µL, and 1 µg/µL. 1 µL of each sample was analyzed using a reverse phase column in RP-LC-MS experiments.

Subunit analysis

For subunit analysis of light chain (LC) and heavy chain (HC), the antibody was denatured and reduced using ≈4 M guanidine hydrochloride (GdHCl)/50 mM DTT, followed by incubation at 57 °C for 45 min.

For subunit analysis of LC, Fc/2, and Fd', the antibody was digested with the IdeS protease according to the manufacturer's protocol. The sample was incubated at 37 °C for 60 min to obtain the Fc/2 and the F(ab')2 subunits. Further reduction at 57 °C for 45 min using 4 M GdHCl/50 mM DTT led to the formation of LC, Fc/2, and Fd' subunits (Figure 2).

Mass spectrometry

The mass spectrometer used for all experiments was an Orbitrap Ascend BioPharma Tribrid mass spectrometer equipped with EASY-IC, EASY-ETD, PTCR, UVPD, and Native MS options and controlled by Orbitrap Tribrid series Tune 4.0 software. All the MS parameter settings are summarized in Tables 2A-2E.

Intact native trastuzumab was analyzed using a Full MS method with the instrument in the intact protein high pressure mode (front HCD pressure at 15 mTorr and back HCD pressure at 20 mTorr), at an Orbitrap resolution setting of 30,000 with the mass range set to 4,000–12,000 *m/z*, RF lens at 150%, normalized AGC target at 250%, maximum injection time at 200 ms, source fragmentation voltage at 200 V, source CID compensation scaling factor at 0.02, and 10 µscans.

Intact denaturing trastuzumab was analyzed using a Full MS method with the instrument in the intact protein standard pressure mode (front HCD pressure at 8 mTorr and back HCD pressure at 8 mTorr), at an Orbitrap resolution setting of 30,000 with the mass range set to 2,000–5,000 *m/z*, RF lens at 60%, normalized AGC target at 50%, maximum injection time at 200 ms, source fragmentation voltage at 150 V, and 3 µscans.

The Fc/2, LC, and Fd' subunits of trastuzumab were analyzed using a Full MS method with the instrument in the intact protein low pressure mode (front HCD pressure at 2 mTorr and back HCD pressure at 2 mTorr), at an Orbitrap resolution setting of 240,000 with the mass range set to 600–2,000 *m/z*, RF lens at 60%, normalized AGC target at 1,000%, maximum injection time at 507 ms, source fragmentation voltage at 15 V, and 2 µscans.

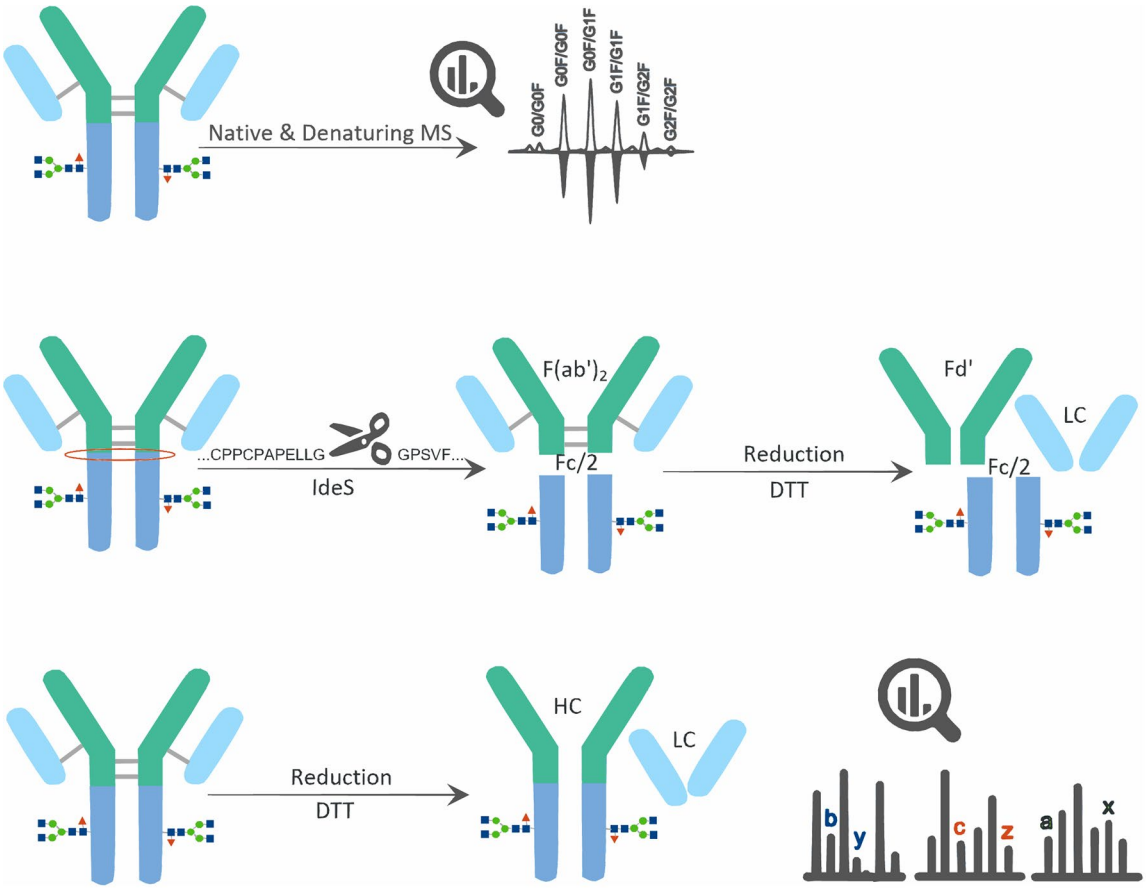


Figure 2. Illustration of trastuzumab sample preparation for intact mass and middle-down analyses.
Top – intact mAb was diluted and analyzed under native and denaturing conditions; Middle – IdeS digestion and DTT reduction to yield Fc/2, LC, and Fd' subunits; Bottom – DTT reduction to generate LC and HC subunits.

The LC and HC subunits of trastuzumab were analyzed using a Full MS method consisting of two experiments with the instrument in the intact protein low pressure mode. The first experiment covered the elution window of the LC subunit, using an Orbitrap resolution setting of 240,000 with the mass range set to 600–2,000 *m/z*, RF lens at 60%, normalized AGC target at 1,000%, maximum injection time at 507 ms, source fragmentation voltage at 15 V, and 2 µscans. The second experiment covered the elution window of the HC subunit, using an Orbitrap resolution setting of 7,500 with the mass range set to 600–2,000 *m/z*, RF lens at 60%, normalized AGC target at 1,000%, maximum injection time at 50 ms, source fragmentation voltage at 15 V, and 10 µscans.

For middle-down fragmentation of all subunits (Fc/2, LC, Fd', and HC), targeted MS^2 experiments were conducted with the instrument in the intact protein low pressure mode using an Orbitrap resolution setting of 240,000 with the mass range set to 300–2,000 *m/z*, RF lens at 60%, normalized AGC target at 2,000%, maximum injection time at 507 ms, source fragmentation voltage at 15 V, and 10 µscans. A quadrupole isolation window of 100 *m/z* was used with a center mass representing a single selected charge state of a subunit during the scheduled start/end retention time window in which the targeted subunit was eluting. The data type was set to full profile by using the "Toggle full profile data to ON" diagnostic in the Tune diagnostics menu. Three fragmentation techniques were used including ETD, EThcD, and UVPD. For ETD/EThcD fragmentation, the ETD reagent target was set at 2e6, with the reaction time set to 1.5 ms, 3.0 ms, 5.0 ms, 10.0 ms, and 15.0 ms. For EThcD fragmentation, the HCD supplemental activation energy was set at 12%. For UVPD fragmentation, the activation time of the UVPD laser was set at 10 ms, 20 ms, 30 ms, 40 ms, and 50 ms.

For targeted MS^3 middle-down experiments of all subunits (Fc/2, LC, Fd', and HC) with PTCR reactions, experiments were performed based on a targeted EThcD MS^2 method with 5 ms ETD reaction time and a targeted UVPD MS^2 method with 20 ms reaction time. The EThcD or UVPD MS^2 experiment was followed by PTCR using a PTCR reagent target of 2e6, PTCR reaction time of 30 ms, PTCR isolation window (*m/z*) that covered the scan range of interest for MS^2 product ions, and an extended mass range of 500–8,000 *m/z*.

Two replicate data sets were acquired for each condition to ensure the reproducibility and to improve the sequence coverage with search results combined.

Certain source parameters, including spray voltage, gas settings, ion transfer tube temperature, vaporizer temperature, and source fragmentation voltage (marked "*" in Table 2), are subjected to user optimization for individual instrument setup.

Table 2A. MS method for intact native trastuzumab analysis (8 min method duration)

Global parameters	Application mode	Intact Protein
	Pressure mode	High Pressure
	Expected peak width	9
	Advanced peak determination	True
	Default charge state	25
	Ion source type	H-ESI
	Positive spray voltage (V)*	3,500
	Sheath gas (arb unit)*	25
	Aux gas (arb unit)*	7
	Ion transfer tube temperature (°C)*	275
	Vaporizer temperature (°C)*	150
MS scan parameters	Detector type	Orbitrap
	Scan type	Full MS
	Resolution	30,000
	Scan range (*m/z*)	4,000–8000
	RF lens (%)	150
	Normalized AGC target (%)	250
	Maximum injection time mode (ms)	200
	Micro scans	10
	Source fragmentation voltage (V)*	250
	Source CID compensation scaling factor	0.02
	Data type	Profile
	**Parameters are subjected to optimization*	

Table 2B. MS method for intact denaturing trastuzumab analysis (8 min method duration)

Global parameters	Application mode	Intact Protein
	Pressure mode	Std. Pressure
	Expected peak width	9
	Advanced peak determination	True
	Default charge state	10
	Ion source type	H-ESI
	Positive spray voltage (V)*	3,500
	Sheath gas (arb unit)*	25
	Aux gas (arb unit)*	7
	Ion transfer tube temperature (°C)*	275
	Vaporizer temperature (°C)*	150
MS scan parameters	Detector type	Orbitrap
	Scan type	Full MS
	Resolution	30,000
	Scan range (*m/z*)	2,150–5,000
	RF lens (%)	60
	Normalized AGC target (%)	50/250**
	Maximum injection time mode (ms)	200
	Micro scans	3
	Source fragmentation voltage (V)*	150
	Source CID compensation scaling	None
	Data type	Profile

**Parameters are subjected to optimization*
***AGC target as 250% for sample load of 10 ng and 50 ng*

Table 2C. MS method for denaturing trastuzumab Fc/2, LC, Fd', HC subunits analysis (16 min method duration)

Global parameters	Application mode	Intact Protein
	Pressure mode	Low Pressure
	Expected peak width	9
	Advanced peak determination	True
	Default charge state	20
	Ion source type	H-ESI
	Positive spray voltage (V)*	3,800
	Sheath gas (arb unit)*	25
	Aux gas (arb unit)*	10
	Ion transfer tube temperature (°C)*	275
	Vaporizer temperature (°C)	50
MS scan parameters for subunits at MS^1 level	Detector type	Orbitrap
	Scan type	Full MS
	Resolution	240,000/7,500**
	Scan range (*m/z*)	600–2,000
	RF lens (%)	60
	Normalized AGC target (%)**	1,000/100**
	Maximum injection time mode (ms)	507/50**
	Micro scans	2/10**
	Source fragmentation voltage (V)	15
	Data type	Profile

**Parameters are subjected to optimization*
***Settings specifically for HC subunit*

Table 2D. MS method for denaturing trastuzumab subunits analysis in Figure 7 (16 min method duration)

Global parameters	Application mode	Intact Protein
	Pressure mode	Low Pressure
	Expected peak width	9
	Advanced peak determination	True
	Default charge state	20
	Ion source type	H-ESI
	Positive spray voltage (V)*	3,800
	Sheath gas (arb unit)*	25
	Aux gas (arb unit)*	10
	Ion transfer tube temperature (°C)*	275
	Vaporizer temperature (°C)*	50
MS scan parameters for targeted MS^2 subunit fragmentations	Detector type	Orbitrap
	Scan type	Targeted MS^2
	Resolution	240,000
	Isolation mode	Quadrupole
	Isolation window (*m/z*)	100
	Scan range (*m/z*)	300–2,000
	RF lens (%)	60
	Normalized AGC target (%)	2,000
	Maximum injection time (ms)	507
	Micro scans	10
	Source fragmentation voltage (V)	15
	Source CID compensation scaling	None
	Data type	Profile**
Targeted MS^2 ETD fragmentation	**Activation type**	**ETD**
	Reaction time (ms)	1.5/3.0/5.0/ 10.0/15.0
	Reagent target*	2e6
Targeted MS^2 EThcD fragmentation	Activation type	EThcD
	Reaction time (ms)	1.5/3.0/5.0/ 10.0/15.0
	Reagent target*	2e6
	ETD supplemental activation	True
	Is EThcD active	True
	SA collision energy (%)	12
Targeted MS^2 UVPD fragmentation	**Activation type**	**UVPD**
	UVPD activation time (ms)	10/20/ 30/40/50

**Parameters subject to optimization*
***Toggle full profile ON in Tune diagnostic file*

Table 2E. MS method for denaturing trastuzumab subunits analysis in Figure 8 (16 min method duration)		
Global parameters	Application mode	Intact Protein
	Pressure mode	Low Pressure
	Expected peak width	9
	Advanced peak determination	True
	Default charge state	20
	Ion source type	H-ESI
	Positive spray voltage (V)*	3,800
	Sheath gas (arb unit)*	25
	Aux gas (arb unit)*	10
	Ion transfer tube temperature (°C)*	275
	Vaporizer temperature (°C)*	50
MS scan parameters for targeted MS^2 fragmentation and targeted MS^2 fragmentation plus PTCR MS^3	Detector type	Orbitrap
	Scan type	Targeted MS^2
	Resolution	240,000
	Isolation mode	Quadrupole
	Isolation window (*m/z*)	100
	MS^2 activation type	PTR
	MS^2 PTR reagent target	2e6
	Scan range (*m/z*)	300–2,000/500-8,000**
	RF lens (%)	60
	Normalized AGC target (%)	2,000
	Maximum injection time (ms)	507
	Micro scans	10
	Source fragmentation voltage (V)	15
	Source CID compensation scaling	None
	Data type	Profile***
Targeted MS^2 EThcD fragmentation	**Activation type**	**EThcD**
	Reaction time (ms)	5
	Reagent target	1e6
	ETD supplemental activation	True
	Is EThcD active	True
	SA collision energy (%)	12
Targeted MS^2 EThcD fragmentation plus PTCR MS^3	**Activation type**	**EThcD**
	Reaction time (ms)	4
	Reagent target	1e6
	ETD supplemental activation	True
	Is EThcD active	True
	SA collision energy (%)	12
	MS^2 PTR isolation window (*m/z*)	1,700 (centered at 1,150 *m/z*)
	MS^2 PTR reaction time (ms)	30
Targeted MS^2 UVPD fragmentation	**Activation type**	**UVPD**
	UVPD activation time (ms)	20
Targeted MS^2 UVPD fragmentation plus PTCR MS^3	**Activation type**	**UVPD**
	UVPD activation time (ms)	15
	MS^2 PTR isolation window (*m/z*)	1,700 (centered at 1,150 *m/z*)
	MS^2 PTR reaction time (ms)	30
	**Parameters subject to optimization* ***Scan range 500–8,000* m/z *for PTCR MS^3 experiments* ****Toggle Full Profile Data to ON in tune diagnostics menu*	

Data analysis and post-processing

Data analysis was performed using Thermo Scientific™ BioPharma Finder™ 5.2 software.

The MS full scan spectra for intact trastuzumab (native and denaturing) and the subunits (Fc/2, LC, Fd', and HC) were deconvoluted with the intact mass analysis workflow within BioPharma Finder software. The Thermo Scientific™ ReSpect™ and Sliding Window algorithm was used for isotopically unresolved data, while the Xtract and Sliding Window algorithm was utilized for isotopically resolved data. The mass tolerance for deconvolution was 20 ppm; the sliding window merge tolerance was 15 ppm; and the minimum numbers of detected intervals was 10.

The targeted MS^2 and MS^3 spectra for MD-MS experiments were processed using the top-down analysis workflow with the Xtract and Sliding Window algorithm. The S/N threshold for deconvolution was 7 and the fragmentation mass tolerance was 10 ppm.

Glycan structures were added as variable modifications to generate sequence candidates for major glycoforms in the Sequence Manager. For subunits carrying N-linked glycan modification, only the two most abundant glycoforms (A2G0F and A2G1F) were considered for the assignment of fragment ions.

The expected theoretical monoisotopic and average masses in Daltons for the common intact trastuzumab glycoforms and subunits are presented in Tables 3A-3B.

Table 3A. Theoretical monoisotopic and average masses for trastuzumab subunits

	Monoisotopic mass (Da)	Average mass (Da)
Light chain (LC)	23,428.524	23,443.23
Heavy chain (HC)	49,125.436	49,156.14
Heavy chain G0F (HC-G0F)	50,569.970	50,601.48
Heavy chain G1F (HC-G1F)	50,732.023	50,763.62
Heavy chain G2F (HC-G2F)	50,894.076	50,925.76
Fc/2 (-Lys)	23,775.930	23,790.75
Fc/2 (-Lys) G0F	25,220.464	25,236.26
Fc/2 (-Lys) G1F	25,382.517	25,398.40
Fc/2 (-Lys) G2F	25,544.570	25,560.54
Fd'	25,367.517	25,383.42

Table 3B. Average masses for major trastuzumab glycoforms

Glycoform	Average MW (Da)
Trastuzumab, aglycosylated	145,165.89
Trastuzumab, G0/G0F	147,910.43
Trastuzumab, G0F/G0F	148,056.57
Trastuzumab, G0F/G1F	148,218.71
Trastuzumab, G1F/G1F (or G0F/G2F)	148,380.85
Trastuzumab, G1F/G2F	148,542.99
Trastuzumab, G2F/G2F	148,705.13

Results and discussion

Intact mAb analysis under the native and denaturing conditions

For intact mAb analysis under the native conditions, an ESI compatible volatile solution of 50 mM ammonium acetate (pH 6.9) was used to preserve the native folding state of the mAb. For intact mAb analysis under the denaturing condition, the mAb sample was exposed to acid and organic solvent (e.g., formic acid in water and acetonitrile) so that protein structure was more unfolded. As a result, the surface to accept protons during ionization is smaller for the mAb under the native conditions compared to the mAb under denaturing conditions, leading to fewer charge states and higher *m/z* detected for trastuzumab under the native conditions (z = 19–30 in the *m/z* range of 5,000–10,000) compared to the mAb in the denaturing conditions (z = 30–70 in the *m/z* range of 2,000–5,000) (Figure 3A).

Figure 3A displays the Full MS spectra acquired from intact trastuzumab under the denaturing (top) and native (bottom) conditions, with insets showing an expanded view of the most abundant charge state with baseline resolved glycoforms. The mirror plots in Figure 3B compare the deconvoluted results obtained using the ReSpect and Sliding Window algorithm for intact denaturing trastuzumab (top) and intact native trastuzumab (bottom). High mass accuracies were obtained for major glycoforms of trastuzumab under both the denaturing and native conditions (Figure 3B).

The instrument front end conditions play an important role for intact protein analysis, as they have great impact on the de-clustering and de-solvation of the protein sample ions, which would then affect the spectral quality.[5] The parameter settings for source fragmentation, vaporizer temperature, ion transfer tube temperature and gases are subjected to optimization for individual instruments. The parameters provided in Table 2 of the experimental section serve as a good starting point.

Intact mass analysis of trastuzumab dilution series under the denaturing conditions was performed to demonstrate the sensitivity of the Orbitrap Ascend BioParma MS. Figure 4 shows the Full MS and deconvoluted results of 10 ng – 1 µg of trastuzumab loaded onto the column. A good signal of trastuzumab was obtained for the concentration as low as 10 ng (Figures 4A and 4B). The mass accuracy measured for the major glycoforms remained consistently within 11 ppm at all concentration levels (Figure 4C).

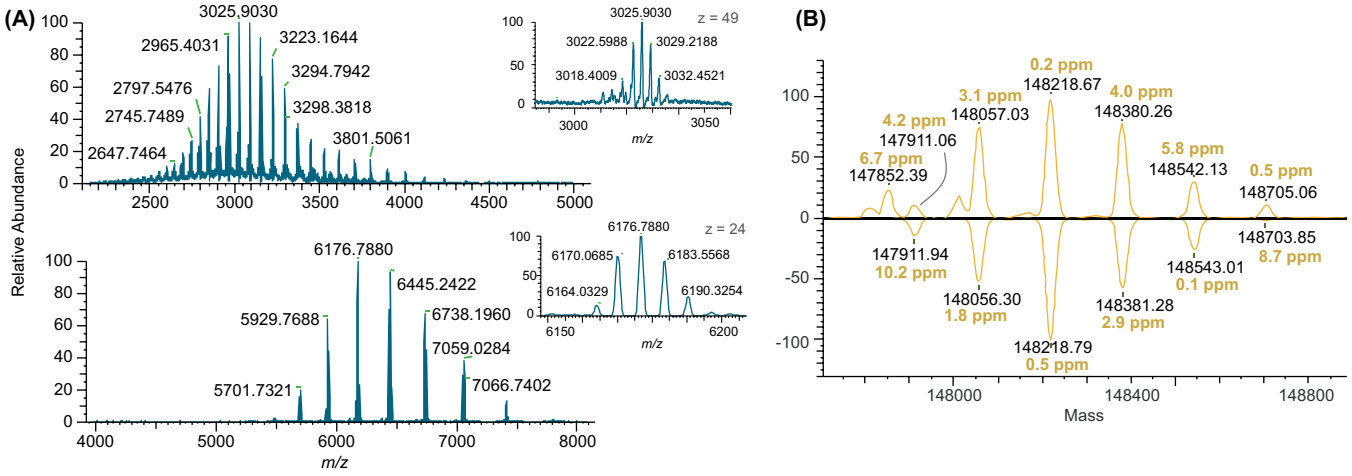


Figure 3. Intact mass analysis of trastuzumab under the denaturing and native conditions. (A) Full MS spectra acquired from intact trastuzumab under the denaturing (top) and native (bottom) conditions. The insets show an expanded view of the most abundant charge state with baseline resolved glycoforms. (B) Deconvoluted masses measured for intact trastuzumab under the denaturing (top) and native (bottom) conditions using the ReSpect and Sliding Window algorithm.

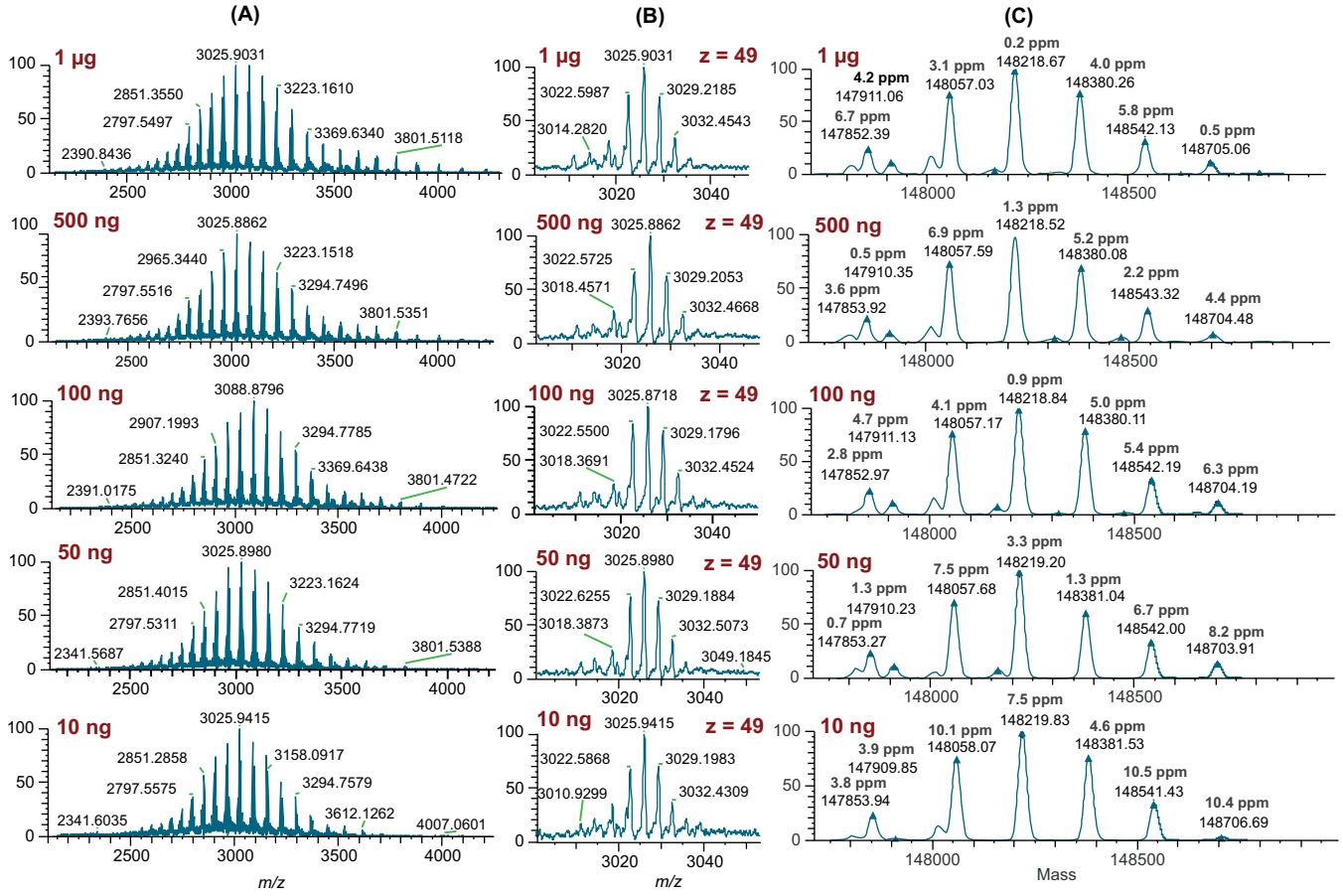


Figure 4. Intact mass analysis of trastuzumab dilution series under the denaturing conditions. (A) Full MS spectra of denatured trastuzumab with 10 ng to 1 µg loaded on column. (B) Zoomed in spectra of the 49+ charge state with baseline resolved glycoforms. (C) Deconvoluted results of trastuzumab obtained using the ReSpect and Sliding Window algorithm in BioPharma Finder software.

Intact mass analysis of trastuzumab subunits

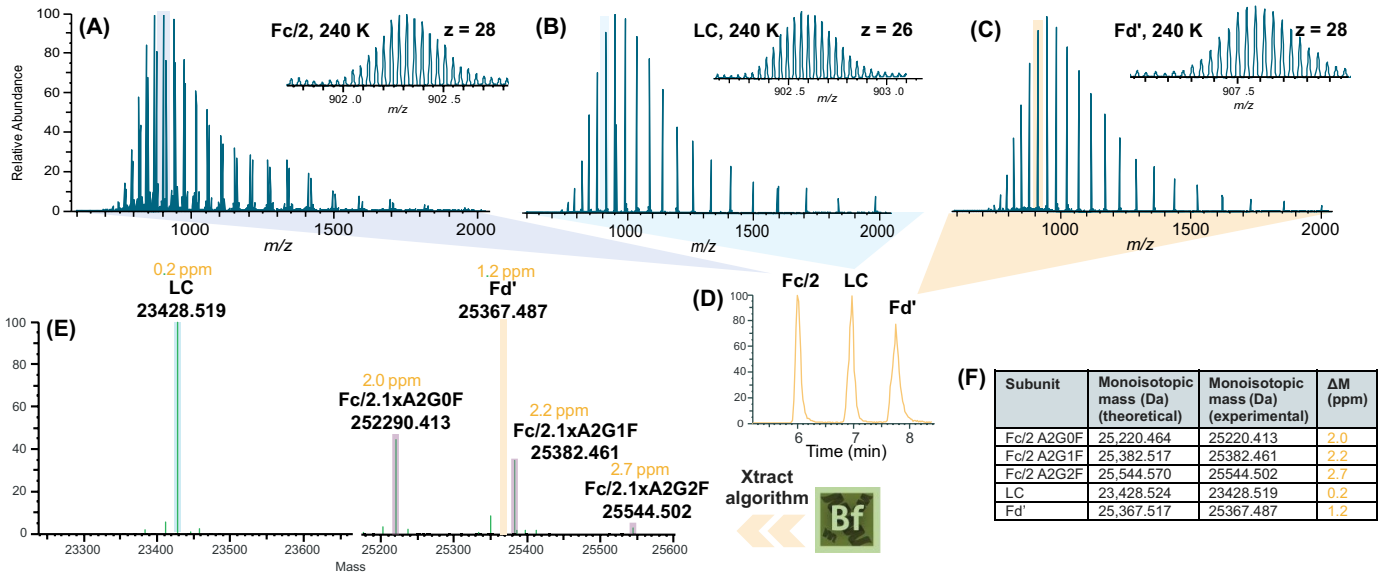
Two sets of trastuzumab subunits were prepared as described in the sample preparation section.

Treatment of trastuzumab with DTT at high temperature (57 °C) led to a complete reduction of inter- and intra-chain disulfide bonds to generate two subunits: LC (≈23 kDa) and HC (≈50 kDa). The digestion of trastuzumab with IdeS protease produced F(ab')2 (≈98 kDa) and Fc/2 (≈25 kDa) subunits. Further reduction of F(ab')2 by DTT resulted in the formation of LC (≈23 kDa) and Fd' (≈25 kDa) subunits.

To measure the mass of each trastuzumab subunit and obtain its retention time information for targeted MS^2 experiments, Full MS acquisitions were performed using RP-LC-MS. Figures 5 and 6 display the Full MS spectra and deconvoluted results for two sets of trastuzumab subunits, respectively.

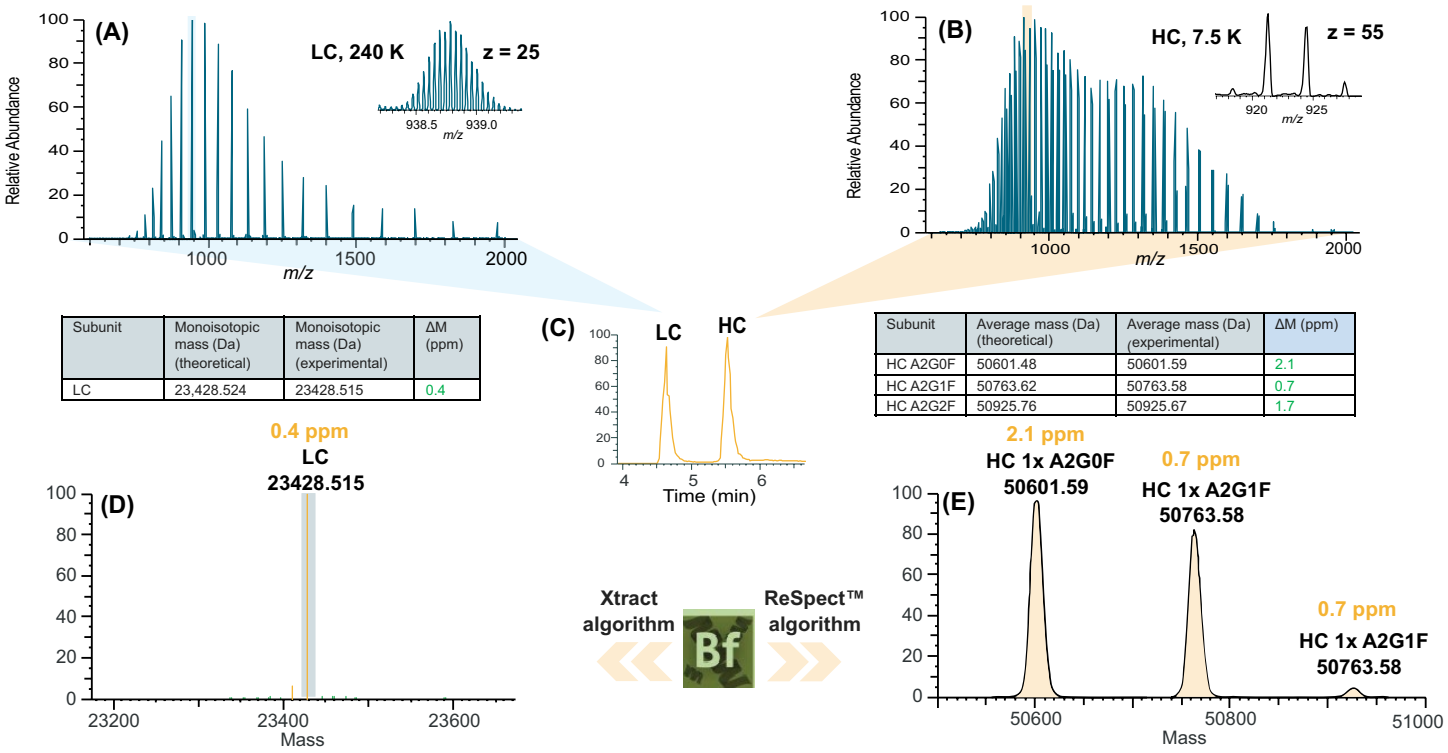
A high Orbitrap resolution setting of 240,000 was used to acquire the isotopically resolved Full MS spectra of the Fc/2, LC, and Fd' subunits from IdeS digestion of trastuzumab (Figures 5A-5C). Three subunits can be baseline separated chromatographically (Figure 5D) using the gradient shown in Table 1C, enabling targeted MS^2 fragmentation of these subunits. The monoisotopic masses of three subunits were deconvoluted using the Xtract and Sliding Window algorithm. Excellent mass accuracies (<3 ppm) were obtained for all the subunits (Figures 5E and 5F).

The data of the LC and HC subunits from DTT reduction of trastuzumab were acquired with the gradient shown in Table 1D. A high Orbitrap resolution setting of 240,000 was employed to obtain isotopically resolved data of the LC subunit, while a lower resolution setting of 7,500 was used to acquire the data of the HC subunit. Figure 6 shows the Full MS spectra and deconvoluted results of the LC and HC subunits. Deconvolution of the isotopically resolved MS spectrum of the LC subunit (Figure 6A) using the Xtract and Sliding Window algorithm gave an excellent mass accuracy of <3 ppm (Figure 6C). Similarly, excellent mass accuracies (<3 ppm) were obtained for three major glycoforms (A2G0F, A2G1F, and A2G2F) of the HC subunit from the deconvolution of the isotopically unresolved spectrum using the ReSpect and Sliding Window algorithm (Figures 6B and 6E).



Subunit	Monoisotopic mass (Da) (theoretical)	Monoisotopic mass (Da) (experimental)	ΔM (ppm)
Fc/2 A2G0F	25,220.464	25220.413	2.0
Fc/2 A2G1F	25,382.517	25382.461	2.2
Fc/2 A2G2F	25,544.570	25544.502	2.7
LC	23,428.524	23428.519	0.2
Fd'	25,367.517	25367.487	1.2

Figure 5. Subunit analysis of Fc/2, LC, Fd' subunits from IdeS digestion of trastuzumab. (A)-(C) Full MS spectra of the Fc/2, LC, and Fd' subunits acquired at a resolution setting of 240,000 and 2 µscans. The insets show the isotopically resolved charge state around *m/z* ≈900 (z = 28 for Fc/2 and Fd' and z = 26 for LC). (D) Total ion chromatogram of Fc/2, LC, and Fd' subunits. (E)-(F) Deconvoluted results of three trastuzumab subunits obtained using the Xtract and Sliding Window algorithm with BioPharma Finder software. Excellent mass accuracies (<3 ppm) were obtained for all three subunits.



Subunit	Monoisotopic mass (Da) (theoretical)	Monoisotopic mass (Da) (experimental)	ΔM (ppm)
LC	23,428.524	23428.515	0.4

Subunit	Average mass (Da) (theoretical)	Average mass (Da) (experimental)	ΔM (ppm)
HC A2G0F	50601.48	50601.59	2.1
HC A2G1F	50763.62	50763.58	0.7
HC A2G2F	50925.76	50925.67	1.7

Figure 6. Subunit analysis of the LC and HC subunits from trastuzumab. (A) Full MS spectrum of the LC subunit acquired at a resolution setting of 240,000 and 2 µscans. The inset shows the isotopically resolved charge state (z = 25) around *m/z* 939. (B) Full MS spectrum of HC subunit acquired at a resolution setting of 7,500 and 10 µscan. The inset shows the isotopically unresolved charge state (z = 55) around *m/z* 922. (C) Total ion chromatogram of the LC and HC subunit showing baseline separation of two subunits. (D) Excellent mass accuracy of <3 ppm was obtained for the LC subunit from the deconvolution of isotopically resolved data using the Xtract algorithm within BioPharma Finder software. (E) Excellent mass accuracies of <3 ppm were obtained for three major glycoforms of the HC subunit from the deconvolution of the isotopically unresolved data using the ReSpect and Sliding Window algorithm within BioPharma Finder software.

Middle-down analysis of trastuzumab subunits using ETD, EThcD, and UVPD

For all subunits of trastuzumab (Fc/2, LC, Fd', and HC), a quadrupole isolation window of 100 *m/z* centered around *m/z* 900 was used to cover the precursors with high charge state and high abundance, which are in favor of ETD/EThcD/UVPD fragmentations. Replicate ETD data were acquired using five different reaction times of 1.5 ms, 3.0 ms, 5.0 ms, 10 ms, and 15 ms to generate c/z fragments. In replicate EThcD experiments, the same ETD reaction times mentioned above were used in combination with a supplemental HCD collision energy of 12%. Supplemental HCD can generate b/y ions in addition to c/z ion fragments from ETD. For UVPD fragmentation, the UVPD activation times were set at 10 ms, 20 ms, 30 ms, 40 ms, and 50 ms to generate a/x, b/y, and c/z ion series for sequence matching. Figure 7 shows the sequence coverage and fragmentation maps of trastuzumab subunits from middle-down analyses using ETD, EThcD, and UVPD.

For Fc/2, LC, and Fd' subunits of trastuzumab, the combined middle-down data from five ETD experiments led to high sequence coverage of three subunits (80% for Fc/2, 75% for LC, and 75% for Fd'; Figures 7A and 7C). The combined data from five EThcD experiments provided an improved sequence coverage of three subunits (90% for Fc/2, 84% for LC, and 84% for Fd'; Figures 7A and 7D) due to the formation of additional *b/y* ions from supplemental HCD fragmentation. While ETD spectra are dominated by *c/z* fragments, UVPD can generate a diverse series of *a/x, b/y,* and *c/z* fragments. This property of UVPD resulted in even higher sequence coverage of three trastuzumab subunits (93% for Fc/2, 91% for LC, and 87% for Fd'; Figures 7A and 7E) when the results from five UVPD conditions were combined.

For LC and HC subunits generated directly from DTT reduction of trastuzumab, a similar trend of improvement in sequence coverage was observed for three fragmentation techniques. Specifically, the combined ETD data led to a sequence coverage of 78% and 48% for HC and LC, respectively (Figures 7B and 7F). The sequence coverage of two subunits was improved to 89% for LC and 58% for HC using the combined data from five EThcD experiments (Figures 7B and 7G). UVPD provided an additional increase in sequence coverage of two subunits (91% for LC and 71% HC; Figures 7B and 7H).

Figure 7. Middle-down analyses of trastuzumab subunits using ETD, EThcD, and UVPD. (A) Bar chart showing sequence coverage of the Fc/2, LC, and Fd subunits from IdeS digestion of trastuzumab using ETD, EThcD, and UVPD. (B) Bar chart showing sequence coverage of the LC and HC subunits from reduction of trastuzumab using ETD, EThcD, and UVPD. (C)-(E) Fragmentation maps of the Fc/2, LC and Fd subunits obtained from five experiments using ETD, EThcD, and UVPD; F-H) Fragmentation maps of the LC and HC subunits obtained from five experiments using ETD, EThcD, and UVPD. High sequence coverage was obtained using all fragmentation techniques. EThcD and UVPD provided an improved sequence coverage than ETD due to the formation of additional fragment types.

Middle-down analysis of trastuzumab subunits using EThcD and UVPD coupled with PTCR

PTCR offers the ability to declutter congested MS^2 spectra by utilizing perfluoroperhydrophenanthrene (PFPP) reagent to abstract protons from multiply protonated fragment ions, thereby reducing the charge state and dispersing the ion population to higher *m/z* area for better data interpretation.[4]

In this set of experiments, PTCR was coupled with EThcD and UVPD to enhance middle-down characterization of trastuzumab subunits. In the EThcD only method, ETD reaction time was set to 5 ms with ETD reagent target set at 1e6; in the UVPD only method, UVPD reaction time was set to 20 ms. For comparison, in the EThcD+PTCR method, ETD reaction time was set to 4 ms, while a UVPD activation time of 15 ms was used in the UVPD+PTCR method. In both PTCR methods, the mass range was set to *m/z* 500–8,000. For the PTCR reaction, the reagent target was set to 2e6, the reaction time was set to 30 ms, and a wide isolation window of 1,700 *m/z* centered at 1,150 *m/z* was defined to cover the scan range of interest for MS^2 fragmentation. Two replicate experiments were performed on trastuzumab subunits using each method to assess the improvements from additional PTCR reactions. A sequence coverage of 72% was obtained for the Fc/2 subunit by combining the data from two replicate EThcD experiments without PTCR (Figures 8A and 8B). By comparison, the combined search of EThcD+PTCR data led to an improved sequence coverage 80% for the Fc/2 subunit (Figures 8A and 8C). When combining all the replicate results from the experiments with and without PTCR, EThcD experiments achieved 87% coverage for Fc/2 subunit (Figures 8A and 8D). A similar trend was also observed for UVPD vs. UVPD+PTCR methods. While UVPD alone provided a sequence coverage of 66% for the Fc/2 subunit, the additional PTCR reactions led to an increase of sequence coverage to 75%, and combining all the replicate results from the UVPD experiments with and without PTCR led to a sequence coverage of 89% for the Fc/2 subunit (Figures 8A, 8E, 8F and 8G).

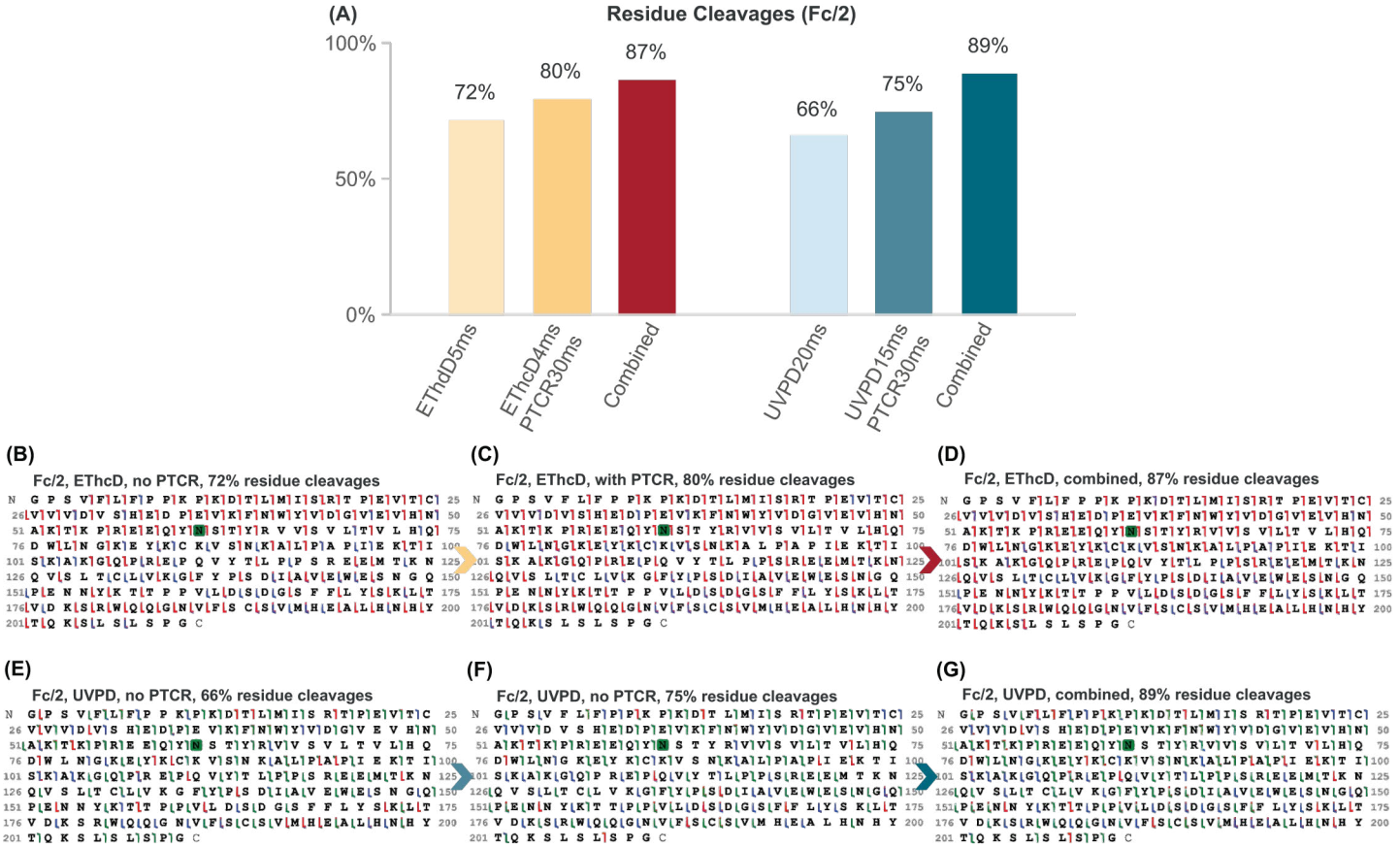


Figure 8. Combined PTCR technique with other fragmentation methods benefits residue coverage by decluttering the congested MS^2 spectra. (A) Bar chart representing the improved residue coverage for the Fc/2 subunit from 72% to 80% for EThcD fragmentations, and 66% to 75% for UVPD fragmentations, from two replicates of the selected experiment condition as labeled in the figure. When combined with all the replicate results from experiments with and without PTCR, EThcD experiments achieved 87% coverage for Fc/2 subunit and UVPD experiments achieved 89% coverage. (B) Fragmentation map of Fc/2 subunit for EThcD fragmentation with 5 ms reagent reaction time with two replicates (left). (C) Fragmentation map of Fc/2 subunit from EThcD 4 ms plus PTCR 30 ms method with two replicates (middle) and (D) cumulative fragmentation map of Fc/2 subunit from (B) and (C) (right). (E) Fragmentation map of Fc/2 subunit for UVPD fragmentation with 20 ms activation time with two replicates (left). (F) Fragmentation map of Fc/2 subunit from UVPD 15 ms plus PTCR 30 ms method with two replicates (middle) and (G) cumulative fragmentation map of Fc/2 subunit from (E) and (F) (right).

The advantages of EThcD, UVPD, and PTCR techniques can be leveraged together to provide a nearly complete sequence coverage of mAb subunits. For Fc/2, LC, Fd' subunits from IdeS digestion of trastuzumab, the combined results from the raw files of EThcD, UVPD, and PTCR (10 in total) resulted in >90% sequence coverage for three subunits (98% for Fc/2, 95% for LC, and 92% for Fd'; Figures 9A and 9C). For LC and HC subunits from the DTT reduction of trastuzumab, the combined results from EThcD, UVPD, and PTCR raw files (10 in total) provided 97% for LC and 73% for HC (Figures 9B and 9D).

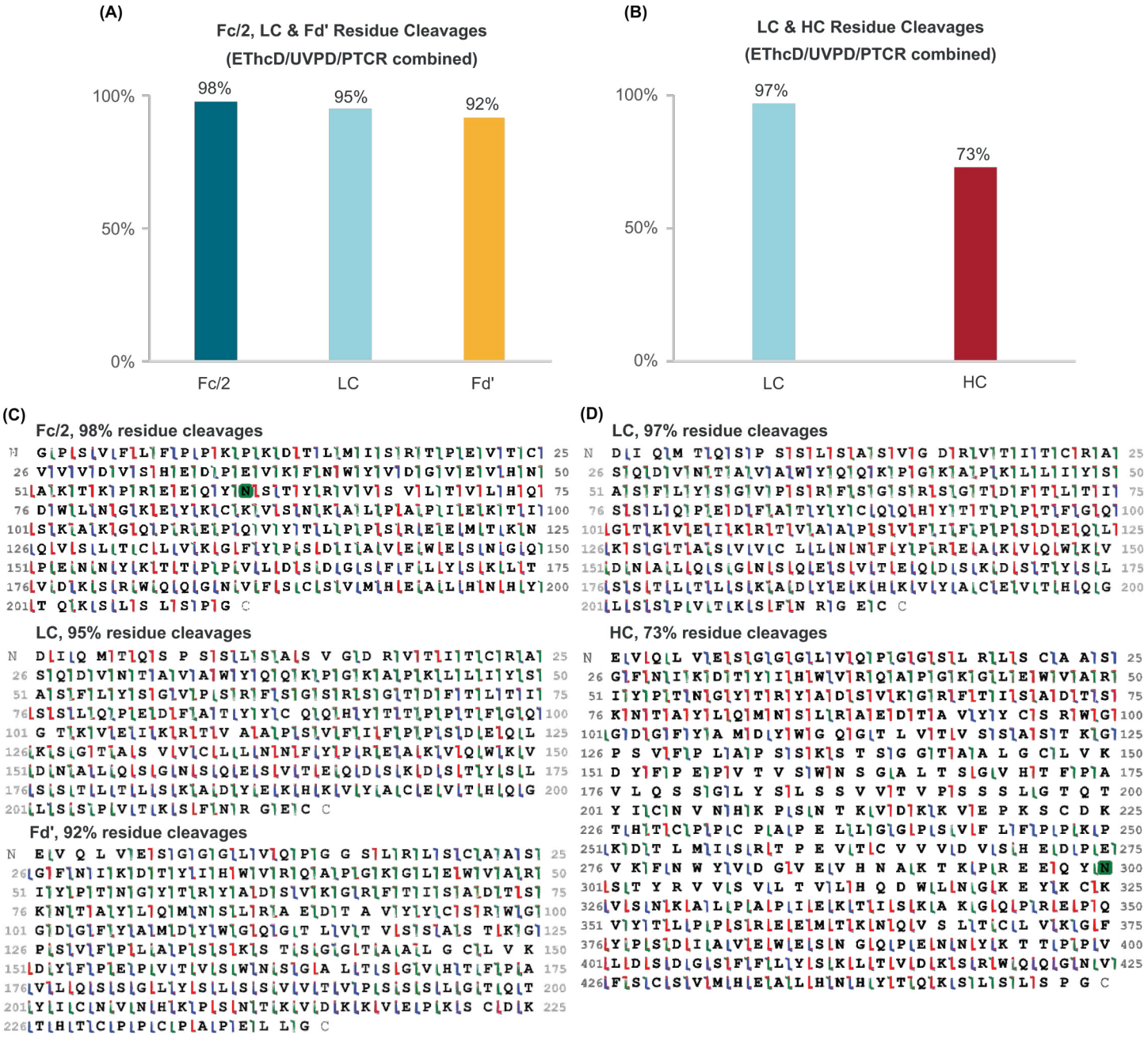


Figure 9. Combined results of EThcD, UVPD, and PTCR middle-down analyses provided excellent sequence coverage of trastuzumab subunits. (A) Bar chart showing a nearly complete sequence coverage of Fc2, LC and Fd' subunits obtained from the combined results of EThcD, UVPD, and PTCR raw files (10 in total). (B) Bar chart showing a nearly complete sequence coverage of the LC subunit (97%) and high sequence coverage of the HC subunit (73%) when combining the raw files of EThcD, UVPD, and PTCR (10 in total). (C) Fragmentation maps of the Fc/2, LC, and Fd' subunits from the combined results of 10 raw files. (D) Fragmentation maps of the LC and HC subunits from the combined results of 10 raw files.

Conclusion

In this work, intact and middle-down workflows were employed to obtain precise intact mass and high sequence coverage of mAb subunits using the Vanquish Flex UHPLC system and Orbitrap Ascend BioPharma Tribrid mass spectrometer. The Orbitrap Ascend BioPharma MS provides the following advantages for comprehensive biopharmaceutical characterization.

- The high mass accuracy and high resolution offered by the Orbitrap technology provides precise mass measurement for both intact mAbs (native and denaturing) and their subunits.
- The system provides multiple fragmentation techniques, such as ETD, EThcD, and UVPD, for achieving high sequence coverage for various biopharmaceutical molecules.
- Compared to ETD, EThcD and UVPD provide an improved sequence coverage of mAb subunits due to the formation of additional fragment types.
- The combination of PTCR with EThcD and UVPD led to further increase in sequence coverage of mAb subunits and the number of complementary ion pairs.
- The combination of EThcD, UVPD, and PTCR offers a nearly complete sequence coverage (>90%) for subunits in the size of 20–25 kDa (Fc/2, LC, and Fd' subunits) and high sequence coverage (>70%) for the HC subunit (≈50 kDa).

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