A Complete Workflow Solution for Intact Monoclonal Antibody Characterization Using a New High-Performance Benchtop Quadrupole-Orbitrap LC-MS/MS

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Overview

Purpose: A LC/MS-based workflow solution was developed for robust, accurate and comprehensive intact monoclonal antibody (mAb) characterization.

Methods: Thermo Scientific Q Exactive quadrupole-Orbitrap mass spectrometers were used for intact mass measurement and top-down sequencing. Full MS spectra of intact or reduced mAb were analyzed using Protein Deconvolution 1.0 that utilizes the ReSpect™ algorithm for molecular mass determination. The top-down msx HCD spectra were analyzed using Thermo Scientific ProSightPC software 2.0.

Results: A mass error of less than 10 ppm was routinely achieved for intact mAb mass measurement. Using an on-line high resolution top-down MSMS approach, over 40% of the fragmentation site was achieved for intact light chain that covers 100% sequence. Results from this study indicates that both precise mass measurement and extensive, high confident sequence information can be obtained for intact mAb using this workflow solution that combines high resolution MS, fast chromatography, high throughput msx HCD and accurate data analysis.

Introduction

Monoclonal antibodies (mAbs) are increasingly developed and utilized for the diagnostic and therapeutic treatment of diseases including cancer. Due to the heterogeneity of mAb products, thorough characterization is necessary for their reproducible as well as safe production. Among the analytical tools used for the analysis of therapeutic mAb, mass spectrometry has become more and more important in providing valuable information on various protein properties, such as intact mass, amino acid sequence, post-translational modification including glycosylation form distribution, minor impurities due to sample processing and handling and high order structure, etc. In this study, a high resolution LC-MS based workflow solution was developed for robust, accurate and comprehensive intact mAb characterization. The fast chromatography, the superior resolution and mass accuracy provided by the Q Exactive™ Orbitrap™ MS, and accurate data analysis of this workflow provides high-confident screening tool to accelerate biopharmaceutical product development cycles.

Methods

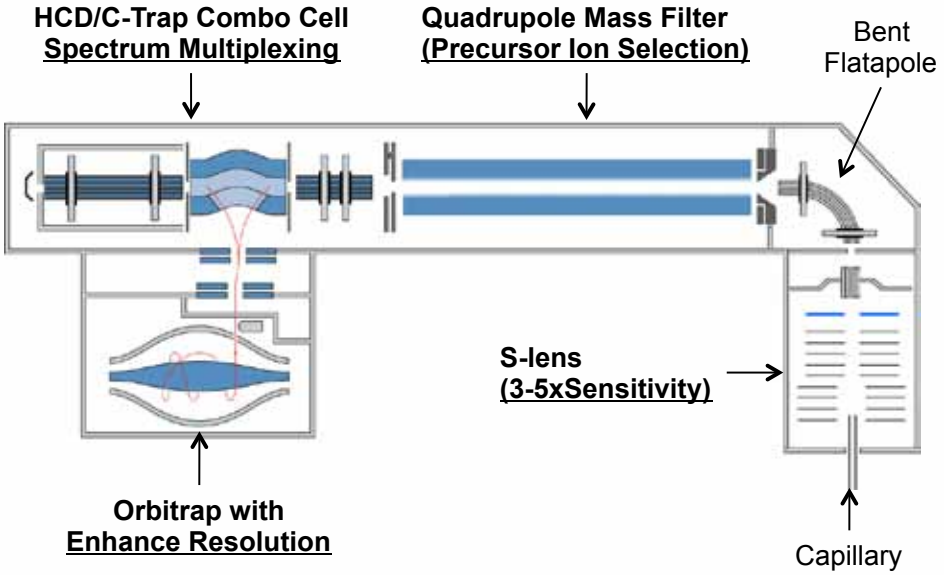
Samples: Four intact mAbs were used in this study. To reduce intact mAb, the sample was incubated for one hour at 60 C in 6 M guanidine-HCl containing 5 mM DDT.

HPLC: Thermo Scientific ProSwift RP-10R monolithic column (1 x 50mm) was used for desalting and separation of light and heavy chain. LC solvents are 0.1% formic acid in H2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 80°C during analysis. Flow rate was 60 µL/min. After injection of 5 µg mAb, a 15 min gradient was used to elute mAbs from the column (0.0min, 20%B; 1.0min, 35%B; 3.0min, 55%B; 4.0min, 98%B; 7.0min, 98% B; 7.1min, 20%B; 15.0min, 20%B).

Mass Spectrometry: Q Exactive Orbitrap instruments (Figure 1) were used for this study. Intact and reduced mAbs were analyzed by ESI-MS for intact molecular mass. Top-down MSMS was performed using high energy collision dissociation with a unique spectrum multiplexing feature (msx HCD). In this data acquisition mode, fragment ions produced from several individual HCD events, each on a precursor of a different charge state of the reduced mAb, were detected together in the Orbitrap mass analyzer. The spray voltage was 4kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275°C. S-lens level was set at 55. In-source CID was set at 45 eV. Resolution was 17,500 or 140,000 for full MS and 140,000 for top-down MSMS. The AGC target was set at 1E6 for full scan and 2E5 for MSMS. Maximum IT was set at 250 ms.

Data Processing: Full MS spectra of intact or reduced mAbs were analyzed using Protein Deconvolution 1.0 (Figure 2) that utilizes the ReSpect algorithm for molecular mass determination. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the mAb. The averaged spectra were subsequently deconvoluted using an input m/z range of 2000 to 4000 m/z, an output mass range of 140000 to 160000 Da, a target mass of 150000 Da, and minimum of at least 8 consecutive charge states from the input m/z spectrum to produce a deconvoluted peak. To identify glycoforms, the masses were compared to the expected masses with the various combinations of commonly found glycoforms. The top-down msx HCD spectra were analyzed using ProSightPC™ software 2.0 under the single protein mode with a fragment ion tolerance of 5 ppm.

FIGURE 1. Schematics of Q Exactive Mass Spectromete

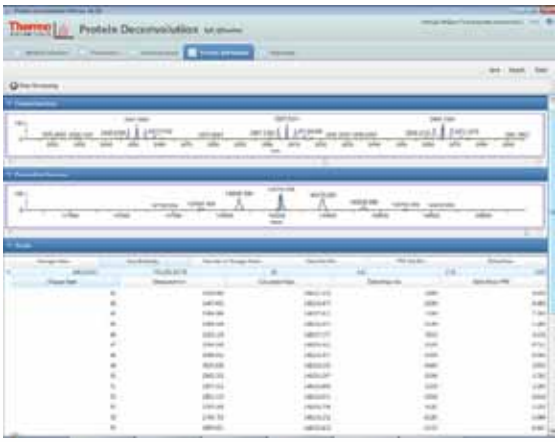


Key Features of The Q Exactive instrument:

- The incorporation of S-lens at the source dramatically enhanced its sensitivity.
- Quadrupole mass filter enables precursor selection for data-dependent MS2 and selected ion monitoring.
- Advanced signal processing increased resolution by two folds, which results in a maximum resolution of 140,000 and a maximum scan speed of 12Hz at a resolution of 17,500.
- Spectrum multiplexing and parallel ion injection/orbitrap detection significantly improved duty cycle.

FIGURE 2. Protein Deconvolution 1.0

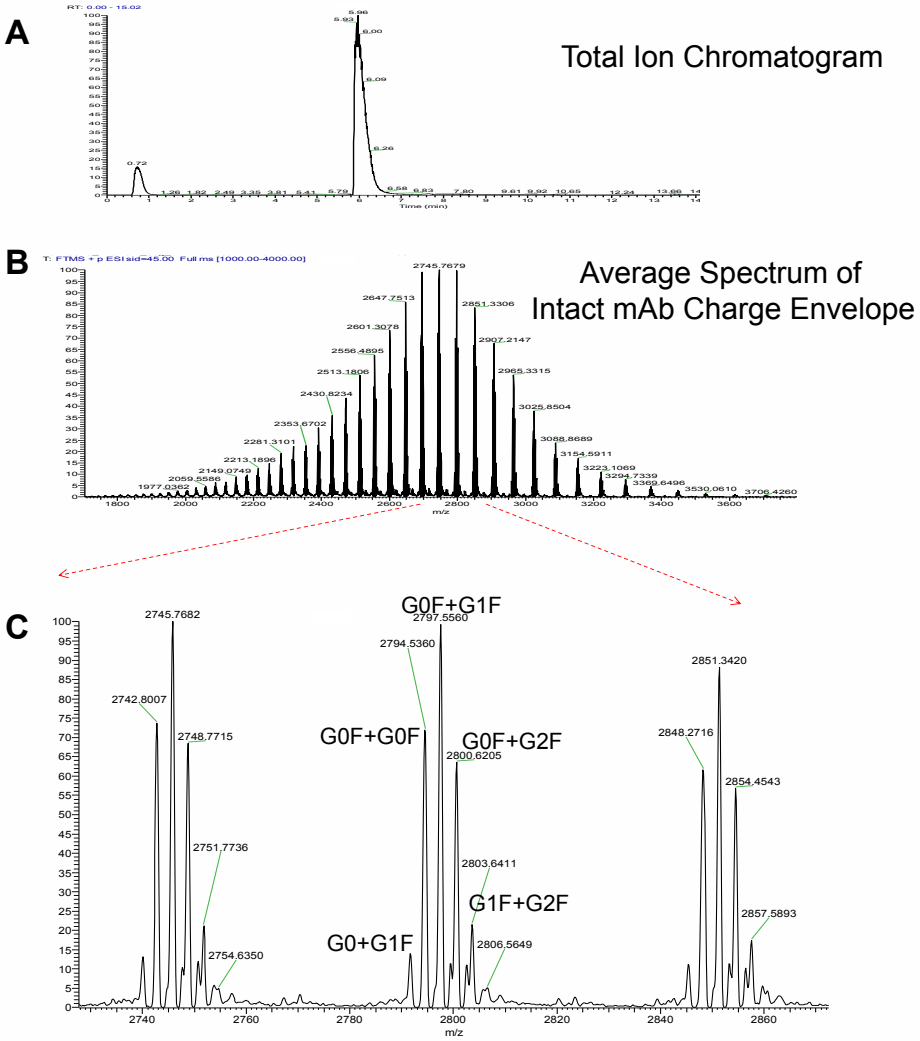
A screenshot of the software with the source and deconvoluted spectrum and a list of the components for that protein at the bottom of the page. In this application, the user has a choice from two algorithms, Xtract or ReSpect, depending on whether or not the target protein is isotopically resolved.



1) Select data file
2) Choose appropriate deconvolution parameters
3) Create an averaged spectrum from a chromatographic peak
4) Perform deconvolution
5) Print or save report

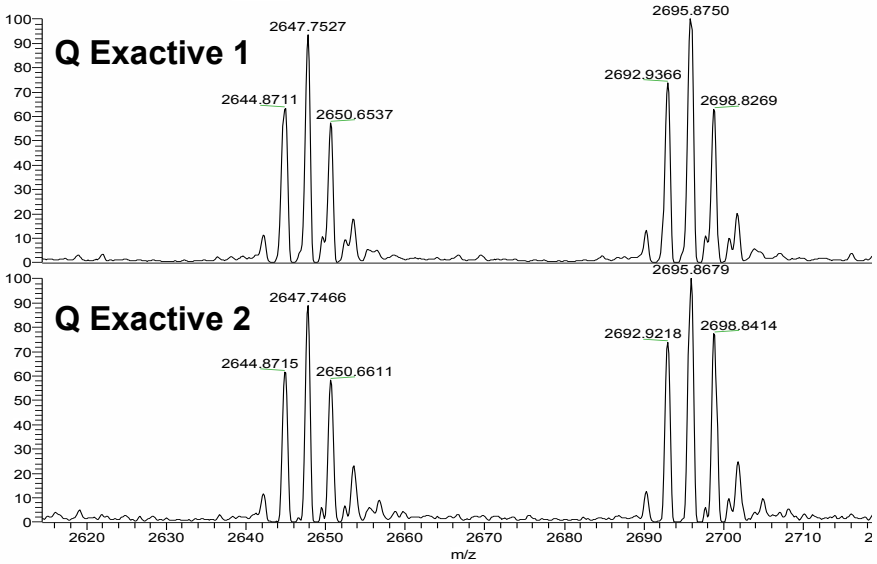
Results

FIGURE 3. LC-MS Result of mAb



Five micrograms of mAb were desalted and eluted from a ProSwift™ RP-10R monolithic column using a 15min gradient and analyzed using ESI-MS on the Q-Exactive. The was mAb eluted over one minute as shown in (A). The average spectrum over the elution time shows a nicely distributed complete charge envelope of the mAb (B). A zoom-in view of each charge state reveals five major glycosylation forms that are baseline separated (C).

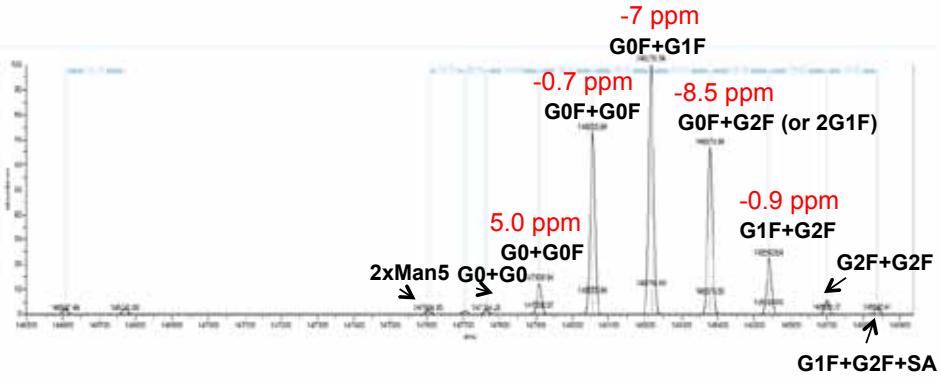
FIGURE 4. Consistency of instrument performance



Mass difference of major components is **< 6 ppm** between instruments

After each of the mAb datasets were analyzed using the Protein Deconvolution software, the masses were compared to the masses expected for the known amino acid sequence with the various combinations of glycoforms commonly found on mAbs. One such result is shown below in Figure 5.

FIGURE 5. Deconvoluted spectrum for a mAb with known composition and mass errors



To measure the mass accuracy and reproducibility of mAb samples on the Q Exactive in conjunction with ReSpect, the mAb sample was analyzed several times using two different instruments over three different days. The results for ppm mass accuracy are shown in Table 1 and the results for relative abundance of the various glycoforms are shown in Table 2.

Table 1: ppm mass deviations from expected target masses for the 5 most abundant glycoforms

		ppm mass measurement errors				
RAW file	**Q Exactive**	**G0+G0F**	**G0F+G0F**	**G0F+G1F**	**G0F+G2F**	**G1F+G2F**
1	1	-10.5	0.7	-10.5	-13.8	-18.0
2	1	-3.2	-4.3	-6.9	3.2	N/A
3	1	-11.6	-1.1	-8.8	-11.2	-12.0
4	1	5.1	-5.0	-2.6	5.1	5.6
5	2	-14.3	3.0	-6.9	-5.4	-5.9
6	2	-8.6	-2.2	-12.2	-12.5	-12.9
7	2	-14.3	-6.6	-12.3	-14.8	-10.1

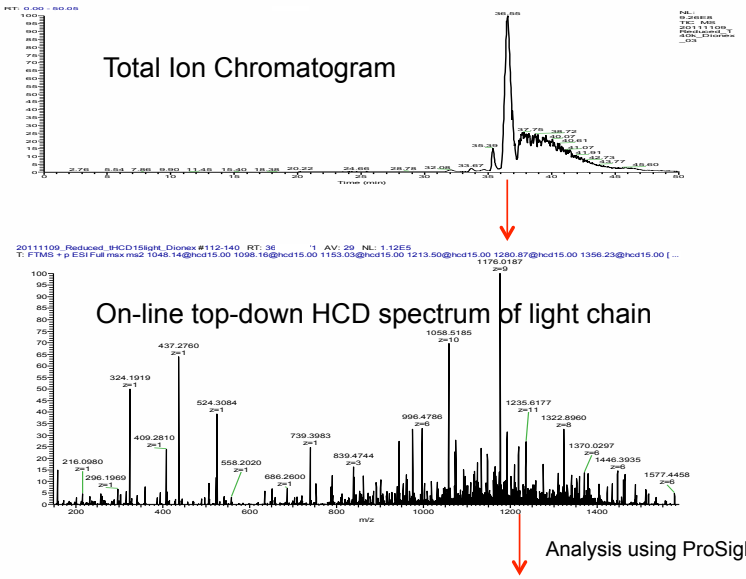
The average ppm error for all 34 measurements was 6.9 ppm with a standard deviation of 6.4 ppm. This indicates that the Q Exactive is a very powerful platform for confirmation of protein primary structure.

		Relative abundances				
RAW file	Q Exactive	G0+G0F	G0F+G0F	G0F+G1F	G0F+G2F	G1F+G2F
1	1	12.9	74.1	100.0	67.0	23.4
2	1	12.3	76.0	100.0	71.4	29.8
3	1	12.0	72.8	100.0	66.2	22.0
4	1	12.2	75.0	100.0	67.0	23.6
5	2	12.7	75.7	100.0	63.6	21.6
6	2	13.2	75.4	100.0	64.8	21.0
7	2	12.9	76.6	100.0	64.7	21.6

For the top 5 glycoforms, the relative intensity reproducibility is within a few percent.

To obtain amino acid sequence, on-line, top-down MS/MS was applied to the reduced mAb samples using msx HCD. Besides the improved throughput from spectrum multiplexing, the advanced signal processing provides improved resolution and higher Orbitrap scan speeds, which is critical for on-line protein top-down sequencing. As a result, high resolution, information rich spectra were generated on the one minute LC elution time for reduced mAb samples. For the light chain, over 40% sequence coverage was achieved, including the N-terminal variable region, with a mass error of less than 5 ppm for fragment ions. The result is shown below in Figure 6.

FIGURE 6. On-line top-down sequencing of light chain



- 100% sequence coverage
- <5 ppm fragment mass error
- ProSightPC E-value= 5.71e-69

Conclusion

- ProSwift RP-10R monolithic column provides robust and efficient separation of mAbs.
- Q Exactive MS produces accurate and reproducible mass analysis for intact mAb analysis.
- Q Exactive on-line top-down analysis generates extensive sequence information for reduced mAb, offering a fast way to confirm sequence identity.
- Protein deconvolution suite enables fast and accurate calculation of the intact mass of mAbs.
- ProSightPC software offers confident sequence assignment for high resolution top-down spectrum generated by Q Exactive instrument.
- Both precise mass measurement and extensive, high confident sequence information can be obtained for intact mAb using this workflow solution that combines high resolution MS, fast chromatography, high throughput msx HCD and accurate data analysis.

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