# Middle-down Analysis of Monoclonal Antibody Middle using Nano-flow Liquid Chromatography and a Novel Tribrid Orbitrap Mass Spectrometer

*Jie Qian,[1] Keith A. Waddell,[2] Zhiqi Hao[2]*
*[1]Thermo Fisher Scientific, Somerset, NJ, [2]Thermo Fisher Scientific San Jose, CA*

Thermo SCIENTIFIC

# Overview

**Purpose:** Sensitive analysis of monoclonal antibody using middle-down approach on a novel Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer

**Methods:** A method combining nano-flow liquid chromatography and complementary higher-energy collisional dissociation (HCD) and electron transfer dissociation (ETD) fragmentation on an Orbitrap Fusion Mass Spectrometer was developed and implemented

**Results:** A monoclonal antibody was enzymatically and chemically cleaved into Fd', Fc/2 and light chain respectively. Intact masses of the monoclonal antibody and its proteolytic fragments were measured. The proteolytic fragments (Fd', Fc/2 and light chains) were directly fragmented using HCD and ETD. An average 50% amino acid coverage was achieved and glycosylation site was unambiguously identified

# Introduction

Monoclonal antibodies (mAbs) are an increasingly important line of therapeutics for the biopharmaceutical industry. The demand to better understand the biochemical and biophysical properties of mAbs has become critical. Recent developments in high resolution mass spectrometry (MS) with multiple dissociation techniques have clearly shown its distinctive power for characterization of intact proteins and in particular its subunits. The mass spectrometry–based study of mAbs in middle-down approach provides a wealth of information to interpret its structural features. Here, we describe the use of an Orbitrap Fusion mass analyzer in combination with nano liquid chromatography (LC) for characterizing a mAb protein using different dissociation techniques.

# Methods

**Sample Preparation**

The intact mAb protein was enzymatically cleaved below the hinge region into a F(ab')2 fragment and two Fc fragments. Aliquot of candidate NIST RM 8670 mAb lot #3F1b was treated with FabRICATOR® (Genovis, Sweden) at 37 ºC for 1 hour. The resulting F(ab')2 and Fc fragments were further denatured and reduced in 50mM dithiothreitol (Sigma, Saint Louis, MO) at 56 ºC for 1 hour. The proteolytically fragmented mAb was diluted to 1.5 µg/µL using 0.1% formic acid in water.

**Liquid Chromatography**

Fd' and Fc/2 fragments after reduction were chromatographically eluted from a Thermo Scientific™ PepSwift™ column, Monolithic easy spray column (200 µm x 25 cm, Thermo Fisher Scientific, Amsterdam, the Netherlands). One µL of the stock was loaded per injection. Nano flow reverse phase chromatography was performed with a 800 nl/minute gradient of 5-60% in 32minutes using the Thermo Scientific™ EASY-nLC™ 1000 system. The proteins were directly detected by a standard Orbitrap fusion mass spectrometer. Liquid chromatography solvents used include the aqueous as 0.1% formic acid in water (Fisher Scientific, Fair Lawn, New Jersey) and the organic as 0.1% formic acid (Fisher Scientific, Fair Lawn, New Jersey) in acetronitirile (Fisher Scientific, Fair Lawn, New Jersey).

**Mass Spectrometry**

The Fd', Fc/2 and light chain eluted were directly detected by a standard Orbitrap Fusion MS under both full scan mode and tandem scan mode using higher-energy collisional dissociation (HCD) and electron transfer dissociation (ETD). Full mass spectra of mAb fragments were acquired at 240,000 resolution at *m/z* 200 with mass range *m/z* 400-2000. AGC setting for full MS spectrum was at 1e5 with 100ms maximum injection. Ion transferring temperature was set at 300 ºC. Full mass spectra of intact mAb were acquired at 15,000 resolution at *m/z* 200 with mass range *m/z* 1000-6000.

**Data Analysis**

The full mass spectra were analyzed with Thermo Scientific™ Protein Deconvolution™ 3.0 software for molecular mass determination . The tandem mass spectra of Fd', Fc/2 and light chain were deconvoluted by Xtract. The deconvoluted spectra were processed by ProSightPC 3.0 software for middle-down data interpretation.

# Results

### Full Mass Spectrum of Intact mAb

Intact mAb (candidate NIST RM 8670 mAb lot #3F1b) was surveyed by LC-MS. Data shown below was acquired at 15,000 resolution on Orbitrap Fusion MS with source CID at 70. The application of 40-80 source CID is beneficial for most mAb proteins to help remove the adducts and thus promote a cleaner signal. The optimal SID setting is protein dependent.

**FIGURE 1. Full MS spectrum of the intact mAb (candidate NIST RM 8670 mAb lot #3F1b) obtained from LC-MS analysis.**

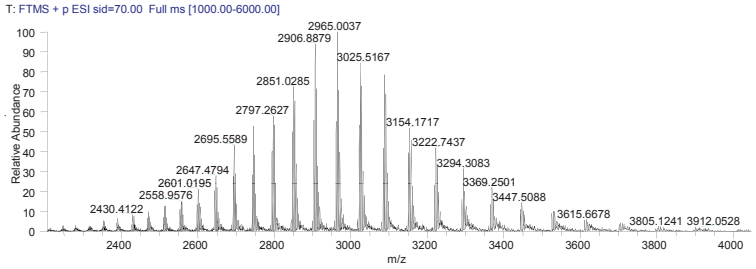
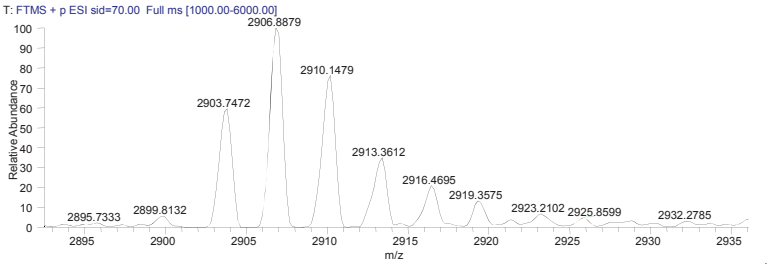



**FIGURE 2. Expanded view of full MS spectrum of the intact mAb. Different glycoforms at charge +51 was shown.**




### Intact LC-MS Analysis of Proteolytic Fragmented mAb

A mixture including approximately 20 pmol of Fd', Fc/2 and light chain, respectively, was eluted at 800 nl/min and directly analyzed at 240,000 resolution on Orbitrap Fusion MS. The mAb fragments were separated by PepSwift monolithic column. For a 800 nl/min gradient of 5-60% in 32 minutes, different glycoforms of Fc/2 were first eluted at 20.67minute, followed by light chain at 22.34minute. Eluted last was Fd' chain at 24.76min.

Full MS spectra acquired at 240,000 resolution provides base line resolution of the isotope distribution of the ~25,000Da mAb fragments. The isotopically resolved spectra were deconvoluted for monoisotopic masses. Figure 6 shows the deconvoluted monoisotopic mass of the light chain is 23113.3568Da, which suggests a mass accuracy of 2.2ppm comparing to its theoretical monoisotopic mass at 23113.3041Da.

**FIGURE 3. Total Ion Current Chromatogram of MAb Fragments Eluted from a PepSwift Monolithic Nano Column.**

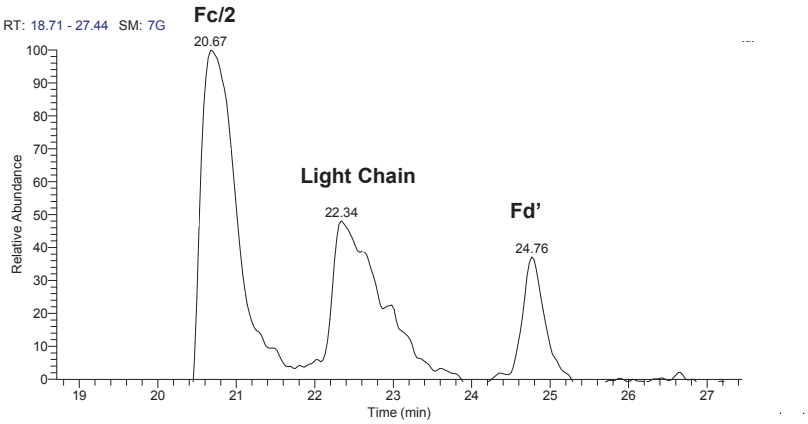


**FIGURE 4. Full MS spectrum of mAb fragments at 240,000 resolution. (A) Full MS spectrum of Fc/2; (B) Full MS spectrum of light chain; (C) Full MS spectrum of Fd'.**

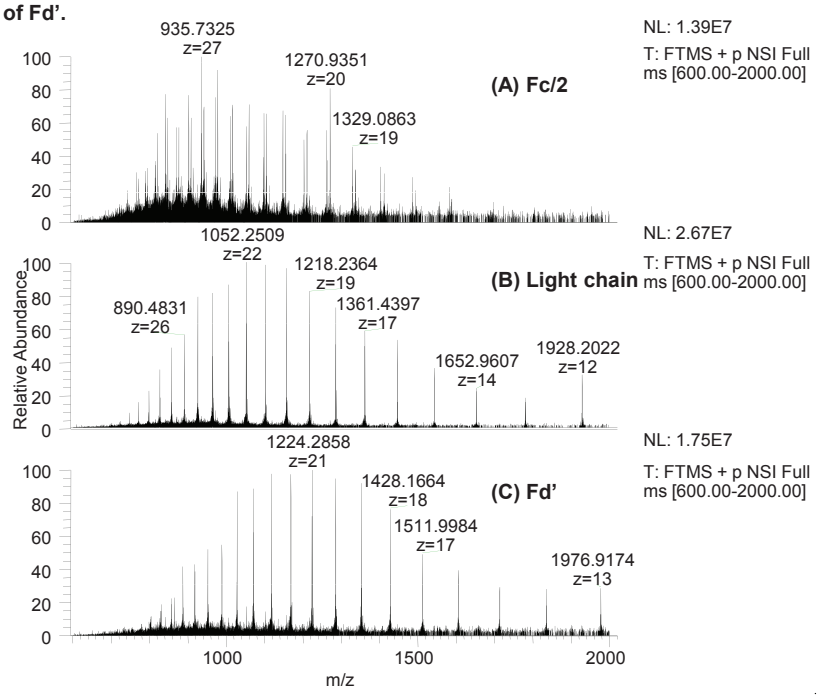



**FIGURE 5. Isotopically resolved mAb fragments at 240,000 resolution. (A) Fc/2+G1F, charge +28; (B) Light Chain, charge 21; (C) Fd', charge 21.**

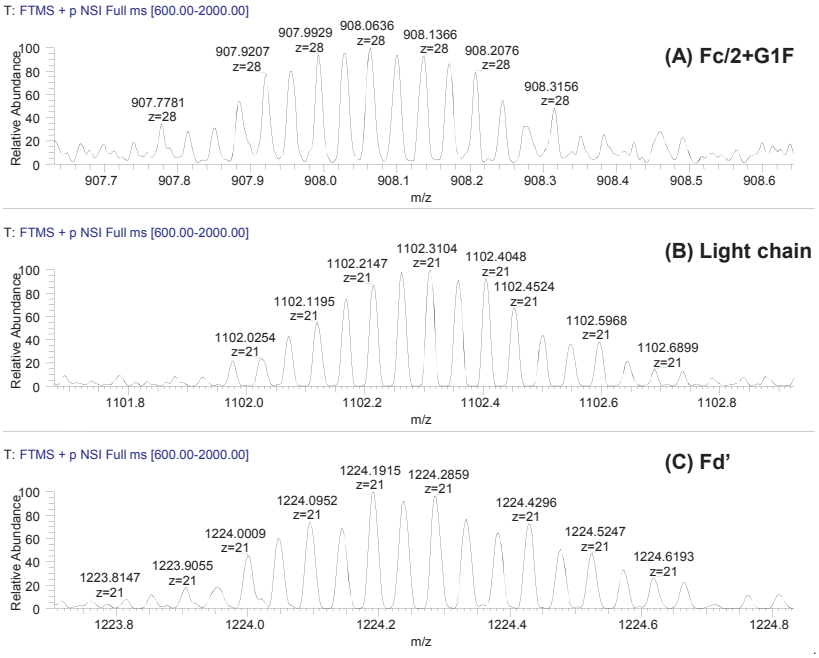
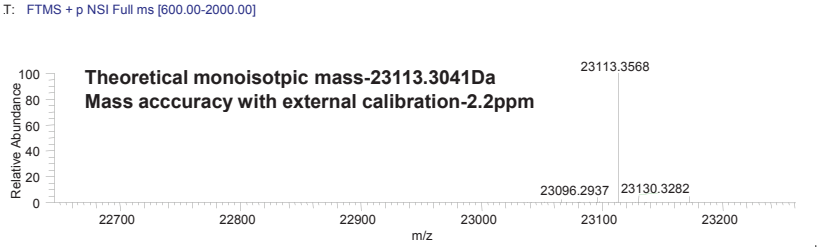



**FIGURE 6. Deconvoluted monoisotopic mass of light chain.**



**Top Down Analysis of Proteolytic Fragmented MAb**

Middle down analysis of mAb protein was performed by LC-MS analysis of the proteolytic fragments. Precursor ions at *m/z* 964.65 of light chain, *m/z* 1117.87 of Fd' and *m/z* 902.20 of the G0F glycoform of Fc/2 were selected respectively for ETD and HCD MS/MS at 120,000 resolution. Reaction time of 3-5 ms was applied for ETD fragmentation. The normalized collision energy for HCD was 15-25 %. As shown in Figure 7, tandem spectra generated contain well resolved, multiply charged fragment ions. Interpretation of these ions based on the mAb protein sequence was performed using ProSightPC 3.0. The combined results of ETD and HCD suggest 50% sequence coverage for light chain, 52% coverage for Fc/2 with G0F and 32% coverage for Fd' respectively (Figure 8, 10, 11). N-terminal modification of pyroglutamate of Fd'chain was confirmed based on fragment ions from both ETD and HCD. Both intact and tandem spectra identified the Lys loss at C-terminus of Fc/2.

ETD is widely known for its advantage in preferentially fragmenting the peptide back bone and keeping the labile modifications attached. It has been recognized as the method of choice for locating the sites of such labile PTMs including glycosylation. Multiple identified ETD fragments between Asn61 and Asn79 unambiguously located the glycan G0F on Asn61 of Fc/2 chain. In the highly complex tandem spectrum, the high resolution and accurate mass allows and is also necessary for confident identification of low abundance glycan-containing fragment ions in the presence of interference. For example, shown in Figure 7 insert is the identification of the +9 charged $C_{61}$ ion with G0F; Figure 9 presents the unambiguous identification of $C_{57}$ without G0F. Although not all the isotopic peaks of this c ion were observed due to background interference, all seven isotopes identified were within 3 ppm mass error (external mass calibration). Both ETD ions, $C_{61}$ and $C_{57}$, have played critical roles in identification and localization of G0F on Asn61 residue.

**FIGURE 7. ETD spectrum of Fc/2 with G0F with precursor ion as *m/z* 902.20, charge +28. The insert is an expanded view of the spectrum covering C61 ion at charge +9 with 3ppm mass accuracy, which confirms the addition of G0F glycan.**

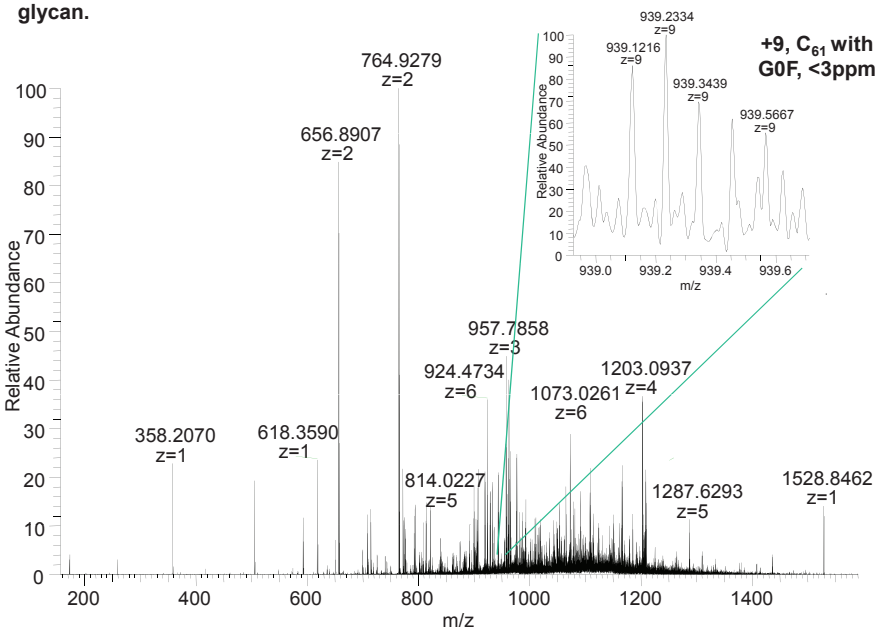



**FIGURE 8. ETD (blue) and HCD (red) coverage of Fc/2+G0F chain. Asn61 was highlighted for addition of G0F.**

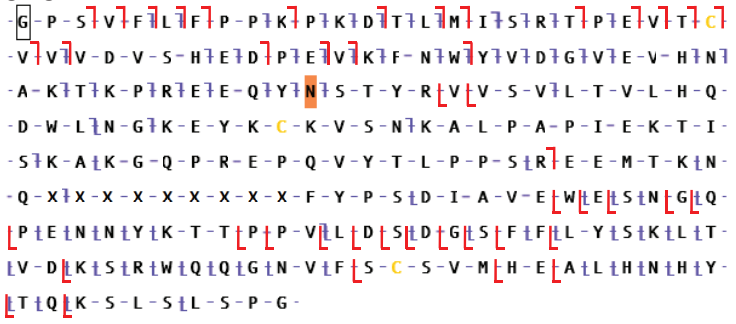


**FIGURE 9. Comparison of experimental $C_{57}$ ion of Fc/2+G0F chain to theoretically predicted isotope distribution. This ion is critical for identifying and localizing G0F on Asn61 residue.**

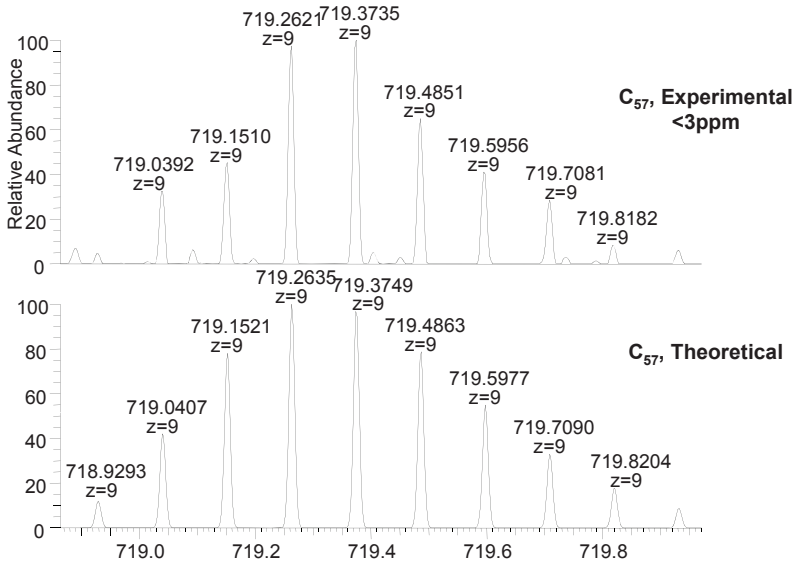



**FIGURE 10. ETD (blue) and HCD (red) coverage of light chain.**

**FIGURE 11. ETD (blue) and HCD (red) coverage of Fd' chain.**

# Conclusion

The middle-down study indicated that complementary fragmentation mechanisms allow extensive sequence coverage as well as identification and localization of PTMs including labile glycosylation. The middle-down approach produced detailed structure information deep into the middle of the mAb chains especially in the epitope region.

# Acknowledgements

We would like to thank National Institute of Standards and Technology for providing the intact mAb protein sample (candidate NIST RM 8670 mAb lot #3F1b).

**www.thermofisher.com**



**Africa** +43 1 333 50 34 0
**Australia** +61 3 9757 4300
**Austria** +43 810 282 206
**Belgium** +32 53 73 42 41
**Canada** +1 800 530 8447
**China** 800 810 5118 (free call domestic)
400 650 5118
**Denmark** +45 70 23 62 60
**Europe-Other** +43 1 333 50 34 0
**Finland** +358 9 3291 0200
**France** +33 1 60 92 48 00
**Germany** +49 6103 408 1014
**India** +91 22 6742 9494
**Italy** +39 02 950 591
**Japan** +81 45 453 9100
**Latin America** +1 561 688 8700
**Middle East** +43 1 333 50 34 0
**Netherlands** +31 76 579 55 55
**New Zealand** +64 9 980 6700
**Norway** +46 8 556 468 00
**Russia/CIS** +43 1 333 50 34 0
**Singapore** +65 6289 1190
**Spain** +34 914 845 965
**Sweden** +46 8 556 468 00
**Switzerland** +41 61 716 77 00
**UK** +44 1442 233555
**USA** +1 800 532 4752

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand

PN-64147-EN-0716S