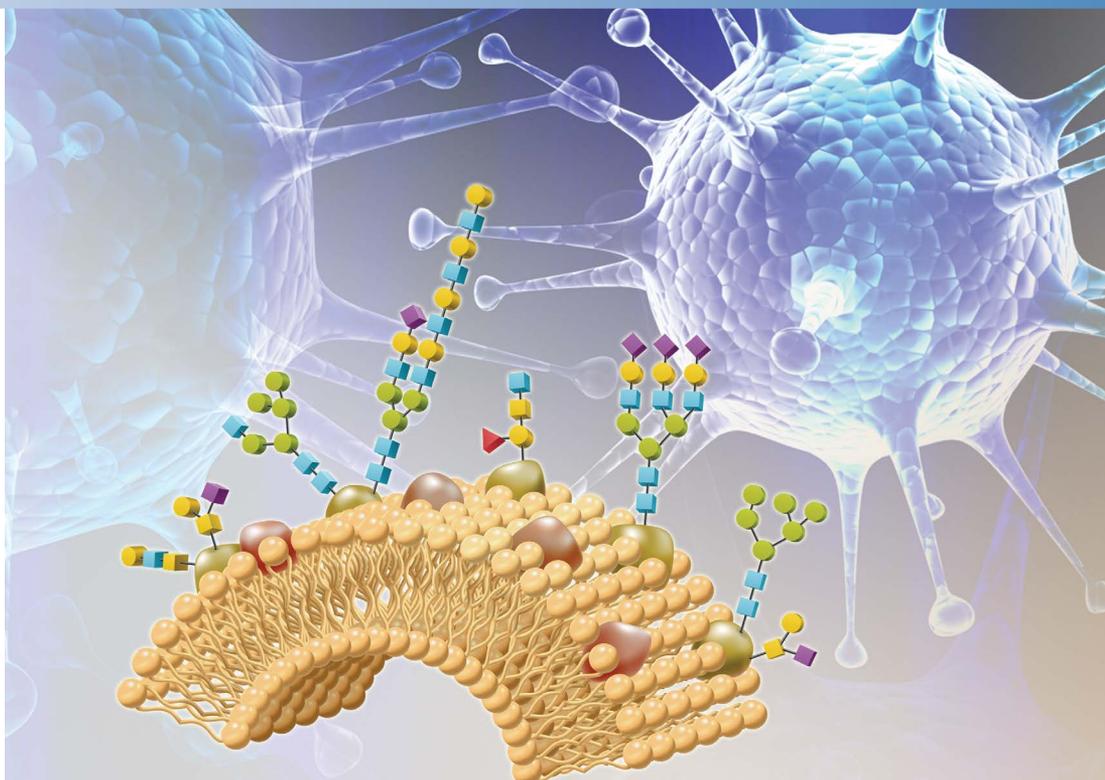


Orbitrap Fusion MS for Glycan and Glycopeptide Analysis

Key Words

Glycomics, proteomics, biopharma, glycan analysis



Orbitrap Fusion Mass Spectrometer for Glycan and Glycopeptide Analysis

Carbohydrate analysis, also known as glycosylation analysis, glycan analysis, or sometimes simply as sugar analysis, is of growing importance to sciences as diverse as pharmaceutical drug development, cancer research, stem cell research and biofuels development. Post-translational modification (PTM) of proteins with glycans to form glycoproteins is a common biological motif. Glycans in glycoproteins are involved in a wide range of biological and physiological processes including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth, and development. Aberrant glycosylation of proteins is connected to cancer progression, invasion, and metastasis. With many biological functions, glycosylation is one of the most important post-translational modifications of eukaryotic cell proteins.

Glycan functions are often dependent on the structure of the glycans attached to the protein. Glycans are covalently attached to proteins primarily through two structural motifs. They can be attached to the amide group of an asparagine, referred to as “*N*-linked glycans” or attached to proteins through the hydroxyl group on serine or threonine, referred to as “*O*-linked glycans”. The biological activity and function of *N*-linked glycans are well studied as compared to *O*-linked glycans, however, both types of glycans are investigated as biomarkers, in order to understand changes related to complex organelle development, and as part of therapeutic protein drug development with strong indication that efficacy is effected by glycosylation. The biotechnology industry has already produced a number of glycoprotein therapeutics with over 30 approved glycoprotein-based biodrugs on the market and that number is rapidly increasing.

Agencies such as the U.S. FDA and the European Medicines Agency have placed increased pressure on biopharmaceutical manufacturers to demonstrate satisfactory programs for understanding, measuring, and controlling glycosylation in glycoprotein-based drugs, with recent updated guidance from the U.S. FDA for characterization of biosimilar therapies. This guidance suggests that the oligosaccharide content of glycoprotein products should be understood and monitored to ensure that it is consistent from one lot of therapeutics to another. There are many analytical approaches that have been employed in the area of glycan and glyco-proteomics research, and can be generally summarized under the following categories:

- Glycan analysis
- Glycosylation site profiling
- Glycoform profiling

These analyses are performed to determine what type of carbohydrates are present, the quantity of the carbohydrates present, and the site(s) of the protein affected. Choosing the most appropriate mass spectrometer depends on experimental demands and instrumental capabilities. This document is intended to assist in the decision-making process and to highlight the Thermo Scientific™ Orbitrap™ Fusion Tribrid mass spectrometer – a novel mass spectrometer that significantly improves traditional glycomics and glycoproteomics experiments through advances in speed, and resolution. In addition to improving traditional experiments, Orbitrap Fusion MS offers unique acquisition strategies and workflows through its flexibility in fragmentation types and MSⁿ capabilities that enable the user to push the boundaries of what is possible in glycan and glycopeptides analyses.

Glycan Analysis

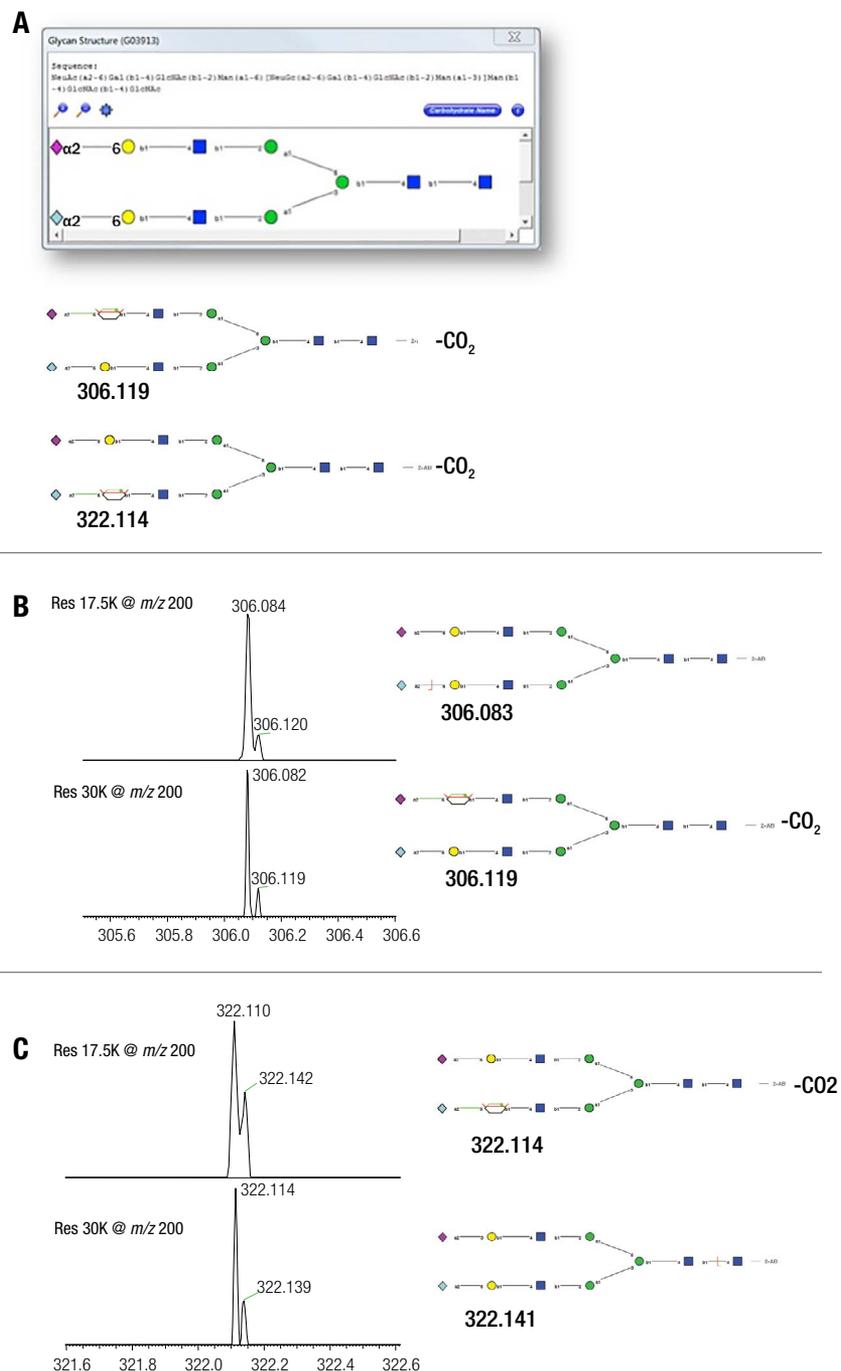
Glycan analysis requires characterization of the sugar sequence, branching, linkages between monosaccharide units, anomeric configuration, and the location of possible substituents. Mass spectrometry (MS) has emerged as one of the most powerful tools for the structural elucidation of glycans. This is due to its sensitivity of detection, the small sample requirement and its ability to analyze complex mixtures of glycans derived from a variety of organisms and cell lines. MS can provide information about sequence, branching patterns, location of possible substituents and can be quantitative. The main attractive feature of MS based methods, compared to other techniques, is the maximization of structural information relative to the amount of time, labor and sample quantity required.

One of the key mass spectrometer requirements for successful glycan analysis is the ability to generate useful fragment ions for structural elucidation. Unlike in proteomics, where peptide/protein identification can be performed from partial fragmentation of the peptide backbone owing to well defined protein databases and linearity of the peptide structure, glycan analysis requires as much fragmentation of the glycan backbone as possible. As monosaccharides can be linked to form larger structures in many branching patterns, this diversity in linkages often leads to many possible isomers which are structurally and biologically different. To fully characterize the glycan sample present, the MS must generate maximal backbone cleavages for structural elucidation.

The Orbitrap Fusion mass spectrometer has multiple fragmentation techniques—collision induced dissociation (CID), higher-energy collisional dissociation (HCD), and optional electron transfer dissociation (ETD). Each of these fragmentation techniques can be performed at any stage of MSⁿ, with detection of the fragment ions in either the ion trap or Orbitrap mass analyzer. Each of the fragmentation techniques provides unique advantages for glycan structural elucidation.

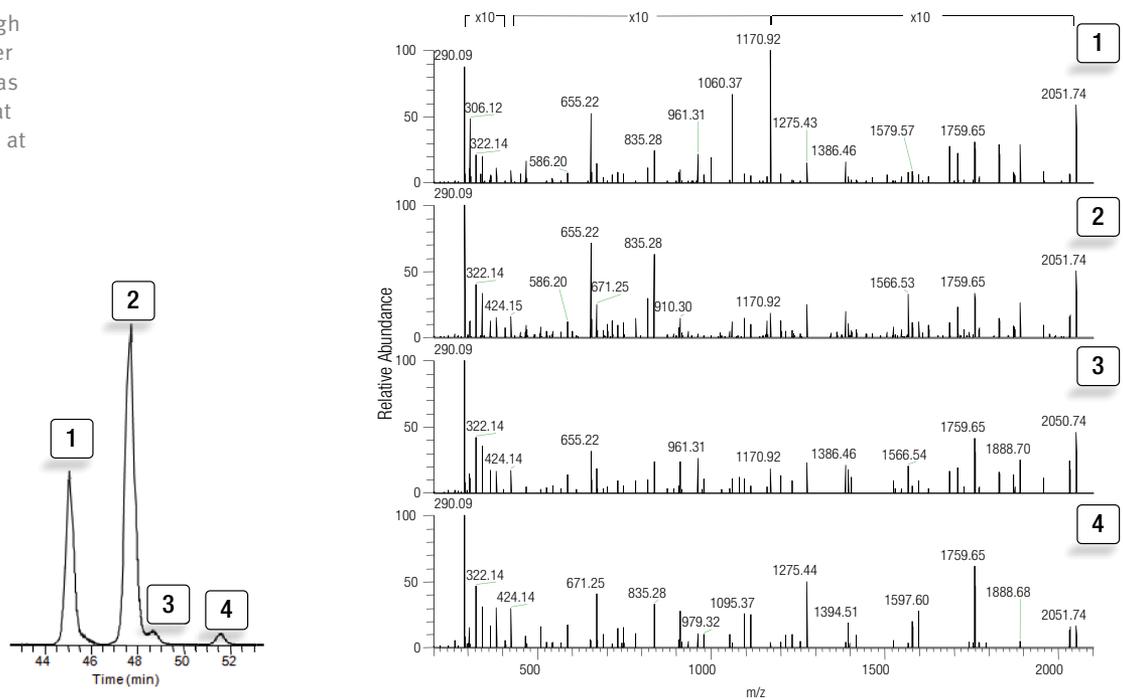
Performing HCD fragmentation with high resolution and accurate mass (HR/AM) fragment ion detection in the Orbitrap mass analyzer on the Orbitrap Fusion MS allows for differentiation of near mass fragment ions which is required for accurate assignment of branching and linkages (Figure 1). Altering the HCD collision energy on the Orbitrap Fusion MS can provide different types of fragment ions within a glycan to allow further characterization. Lower collision energy produces primarily glycosidic fragments while higher collision energy produces cross ring and internal double cleavage for the determination of branching and linkage and for the resolution of isobaric structures. Use of step collision energy (SCE), where a given precursor is fragmented with multiple HCD collision energies and scanned out in a single mass spectrum, ensures maximal detection of all types of fragments at the fastest rate.

Figure 1. A) Shows a glycan that has α -2-6 linked NeuAc and α -2-6 linked NeuGc. The α -2-6 linked NeuAc produces diagnostic fragment ion ${}^{o,4}A_2 - CO_2$ at m/z 306.119 while α -2-6 linked NeuGc produces diagnostic fragment ion ${}^{o,4}A_2 - CO_2$ at m/z 322.114. These can be used as signatures to figure out the type of sialic acid linkages that are occurring. **B)** Shows zoomed in region of the MS/MS spectrum acquired under different resolutions where the diagnostic ions appear. In this example α -2-6 linked NeuAc produces diagnostic fragment ion ${}^{o,2}A_4 - CO_2$ at m/z 306.119. But from the same glycan the loss of NeuGc results in an ion 306.083. In the zoomed region for MS/MS spectra notice that at 17.5 K resolution doesn't fully resolve these two ions, while 30 K does. **C)** For the same glycan, NeuGc produces diagnostic fragment ion ${}^{o,2}A_4 - CO_2$ at m/z 322.114. While the Z1 ion (loss of GlcNAc-2-AB) produces fragment at 322.141. Similar to previous example it took a resolution of 30 K to fully resolve the ions.



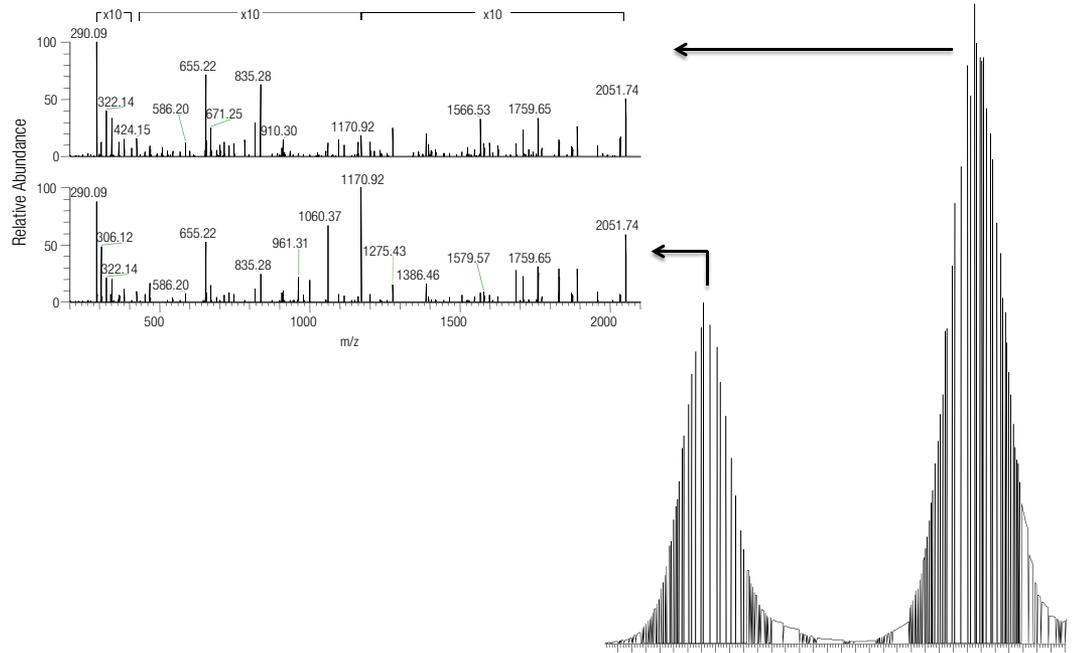
In the past, due to insufficient LC separation, chromatography profiles contained only a small number of separated peaks, where each peak (abundant) contained many glycans (contribution from multiple isomers). As the separated peaks were very abundant most commercial mass spectrometers were fast enough to obtain MS/MS spectra on a LC time scale, however, their coverage and separation were low. With the introduction of new mixed mode columns that have the ability to separate structural isomers, thereby increasing the number of separated peaks, the speed of the mass spectrometer on an LC-timescale becomes essential. Additionally, the range of peak abundance varies across the structural isomers introducing a wide dynamic range for detection for these glycans. The mass spectrometers must generate good quality MS/MS data for high and low abundance peaks at a scan rate amenable to LC separations. Instruments that are fast, sensitive, and have wide dynamic range are essential for glycomics. With the Orbitrap Fusion MS, ions are first collected in the Ion Routing Multipole (IRM) before being shuttled to the mass analyzer of choice (Orbitrap or dual cell linear ion trap). During mass analysis, the next ion of interest is accumulated in the IRM increasing the productivity of the system and ultimately enabling faster scan rate which is amenable to the LC separation described here (18 Hz MS/MS scan rate at 15,000 resolving power at m/z 200). Additionally, the new functionality allows for sensitive detection of HCD fragments in the Orbitrap or ion trap over wide mass and dynamic ranges, providing essential information for structural elucidation of glycans. Figure 3 below shows high quality MS/MS spectra generated for both abundant and low abundant glycans.

Figure 3. Orbitrap Fusion provides high quality MS/MS data (shown here) over wide dynamic range. MS spectrum was acquired at a resolution of 120,000 (at m/z 200) while MS/MS were acquired at a resolution of 30,000 (at m/z 200).



Due to the Orbitrap Fusion mass spectrometer's unique configuration, the Orbitrap mass analysis can be extensively parallelized with operation of the mass selecting quadrupole, the IRM (used for both accumulating ions and for HCD fragmentation) and the ion trap. This allows for maximal concurrent ion manipulations and mass analyses, increasing both scan rates and duty cycle of the instrument. Having fast HR/AM full scans along with fast MS/MS detection are essential for achieving enough scans across the LC peak widths for full scan quantitation and MS/MS for confirmation of identity. Figure 4 shows the separation and identification of 2 glycan structural isomers. The Orbitrap Fusion MS is fast enough to maintain more than enough full scans (>16 scans) across the peak for precursor quantitation, thereby, ensuring that both qualitative and quantitative information can be obtained within a single analysis.

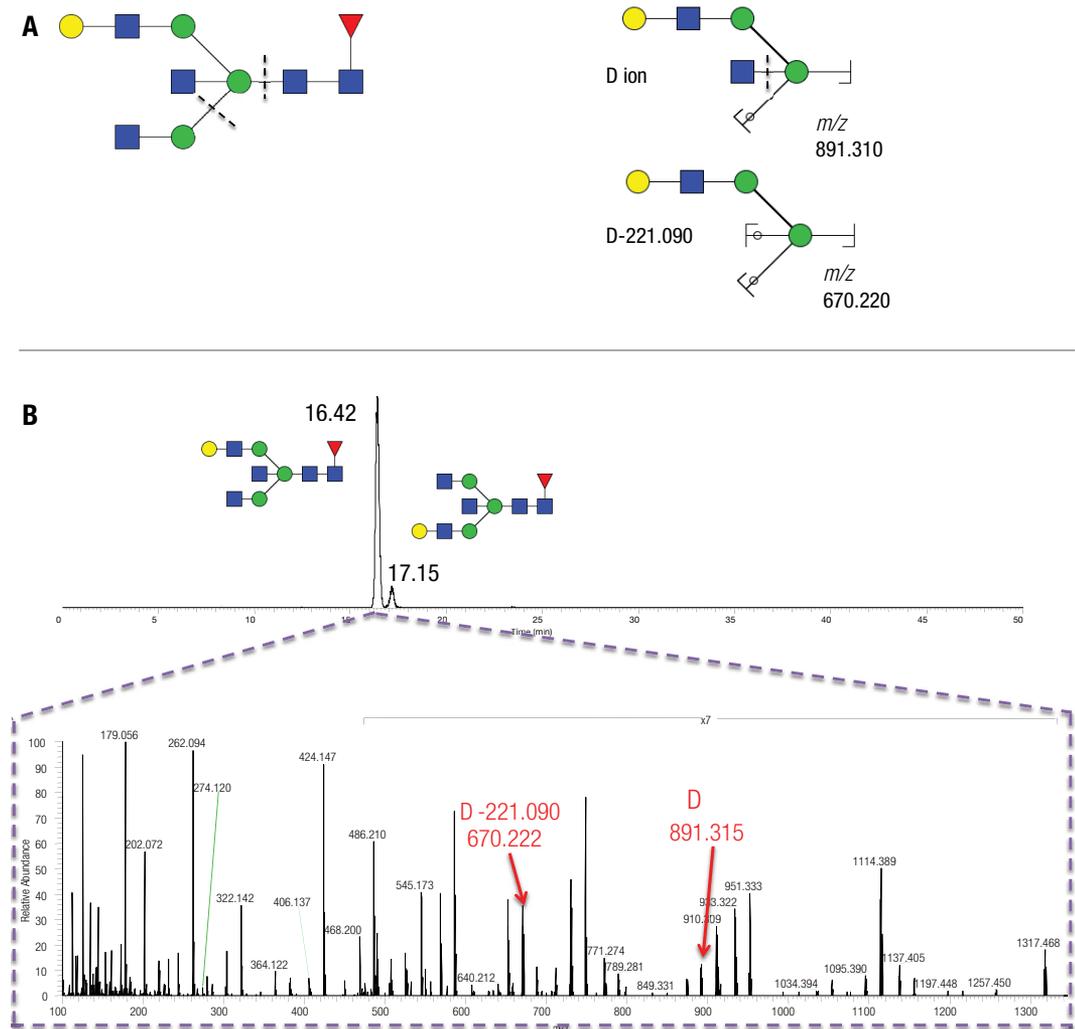
Figure 4. The Orbitrap Fusion MS performs fast MS and concurrent MS/MS for optimal qualitative/quantitative workflow. Shown here are extracted ion chromatograms (XIC) for two glycans separated by LC-MS. Notice that more than enough scans (> 16 scans) across the peak are performed for quantitation. MS spectra were acquired at a resolution of 120,000 (at m/z 200) and MS/MS were acquired at a resolution of 30,000 (at m/z 200).



The structural characterization of glycans is essential in the bio-therapeutics and bio-pharmaceutical industry. Glycans contribute to the efficacy and safety of protein based drugs, for example, recombinant proteins and monoclonal antibodies (mAbs) are often dependent on the structure and types of glycans attached to the proteins. The structures of glycans are quite diverse, complex and heterogeneous due to PTMs and physiological conditions. Minor changes in glycan structure (creation of structural isomers) can result in striking differences in biological function and clinical applications. The selection of appropriate stationary phases to separate these isomers along with mass spectrometers to generate useful fragments to help identify these resolved structures are crucial in glycan analysis. HILIC amide columns are particularly useful for the separation of *N*-linked glycans released from antibodies, for example mAbs, where the majority of glycans are neutral. The typical workflow here is to label the glycans with 2-aminobenzamide (2-AB) separate them with HILIC columns and identify with mass spectrometry. Additionally, selecting the right mode of ionization is important for this workflow as negative mode much more than positive mode will generate useful fragment ions which aids in identification of structural isomers. Unfortunately, not all commercial mass spectrometers can operate and produce useful fragmentation in the negative mode.

Figure 5 shows the separation and identification of 2 glycan structural isomers released from human IgG. The HCD MS/MS spectrum generated in negative mode on Orbitrap Fusion was able to produce useful fragment ions that aided in the assignment of correct glycan structures to the resolved peaks.

Figure 5. A) Shows schematic representation of useful fragment ions needed to assign galactose to the correct antennae. **B)** HCD MS/MS spectrum acquired in negative mode that shows the presence of the D and D-221.090 ion.

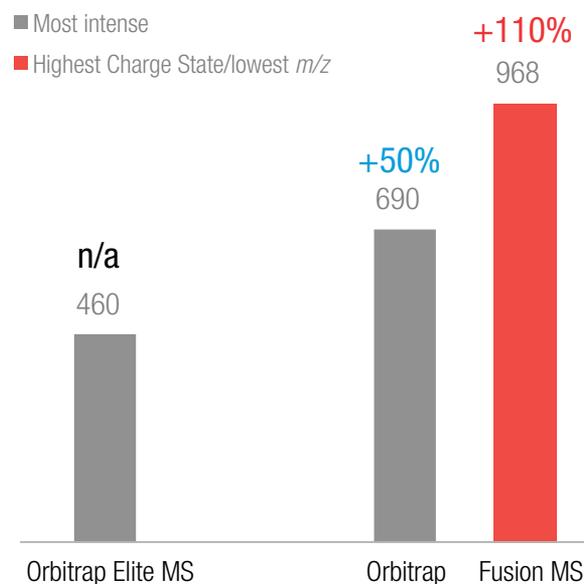


Glycoproteomics

The field of proteomics has benefited tremendously from collisional-activated dissociation (CAD) as this fragmentation technique generates abundant peptide bond cleavages resulting in large number of peptides and protein identifications. However, CAD is not ideal for glycopeptides analysis as this fragmentation does not produce the desired peptide backbone cleavages for sequencing.¹ On commercial mass spectrometers CAD fragmentation generates varying degrees of structural information for glycopeptides. Low energy CAD predominantly fragments the glycan on a glycopeptide rather than the peptide, generating spectra that are dominated by glycosidic bond cleavages rather than the desired peptide bond cleavages. Thus, making it very difficult to sequence the glycopeptide.²⁻⁵ Further complicating the issue is the cleavage of the peptide-glycan bond, resulting in the loss of information about glycosylation site. The increased collision energy on CAD can generate some peptide backbone fragmentation, but this comes with a complication. It generates mixed MS/MS spectrum where both glycan and peptide information are present making structural interpretation complicated.⁶ Regardless of whether high- or low-energy CAD is employed, fragmentation of the peptide-glycan bond still occurs limiting the ability to derive information about the site of glycosylation. To circumvent these issues, majority of researchers have abandoned the strategy of dealing with intact glycopeptides structures and trying to get complete information

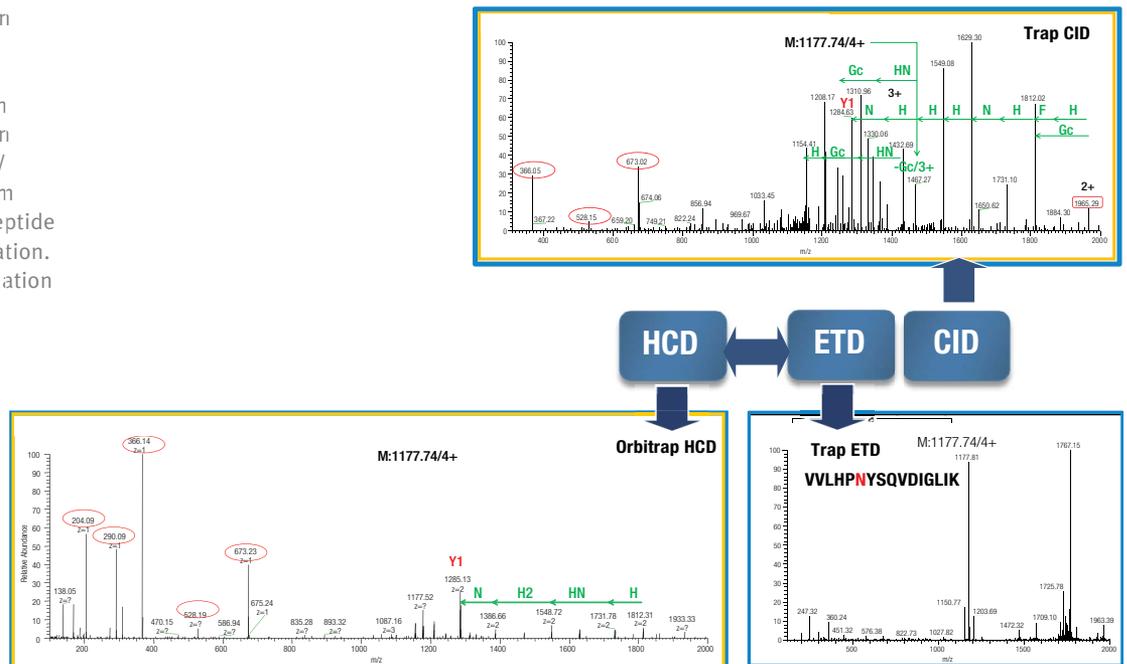
about the glycopeptide. Instead researchers are trying to obtain partial information, such as sequencing backbone of the peptide and identifying glycosylation sites. In this strategy, glycopeptides are treated with enzymes such N-glycosidase F or A (PNGase F/A) resulting in the removal of glycans. In this approach, the site of glycosylation asparagine is converted to aspartic acid. By using high resolution mass spectrometers, researchers can identify the site of glycosylation due to the mass shift of 0.9840 Da. However, no information about the glycan structure is obtained due to the removal of the glycan prior to MS analysis. Additionally, one can increase the confidence in the glycosylation site assignment by the incorporation of stable isotope labeling by performing PNGaseF/A digestion in the presence of H_2O^{18} . This approach involves the release of glycans by a deamidation reaction and the incorporation of H_2O^{18} leading to a mass shift of 2.9890 Da on the asparagine residue. Regardless of whether H_2O^{18} is incorporated or not, the process of monitoring chemical deamidation for identifying glycopeptides leads to large number of false positives as studies have shown that chemical deamidation can occur during sample preparation and not due to the release of glycans.⁷⁻¹¹ These issues highlight the importance of intact glycopeptide analyses and the search for alternative methods that enables comprehensive structural characterization at the intact level. Electron-capture dissociation (ECD)¹² and electron-transfer dissociation (ETD)^{13,14} are two fragmentation techniques that are far better suited for glycopeptide analyses due to their nonergodic type of dissociation. ETD produces extensive fragmentation of the peptide backbone enabling sequencing of the peptide while preserving glycans on the peptide backbone. This allows for unambiguous assignment of the glycosylation sites, thus providing complementary information to CAD fragmentation. The Orbitrap Fusion mass spectrometer has multiple fragmentation techniques available such as CID, HCD and the much desired ETD for glycoproteomics. The ETD ion source used in Orbitrap Fusion MS is based on Townsend discharge ion source which generates a highly stable reagent ion flux with minimal user input for optimization and tuning as was required on previous ETD sources. Additionally, the Orbitrap Fusion MS has been implemented with intelligent, automated precursor ion sorting routines, reagent filtering using the quadrupole mass filter, and charge-state-specific calibration of ETD reaction times that maximize the quality of ETD spectra and increase the number of glycopeptides identified compared to previous generation mass spectrometers (Figure 6).

Figure 6. Comparison of Orbitrap Elite MS to Orbitrap Fusion MS for the identification of human serum glycopeptides. Orbitrap Elite MS selects precursors based on intensity while Orbitrap Fusion MS can acquire data with intelligent precursor selection giving priority to highest charge precursors which are optimal for ETD fragmentation.



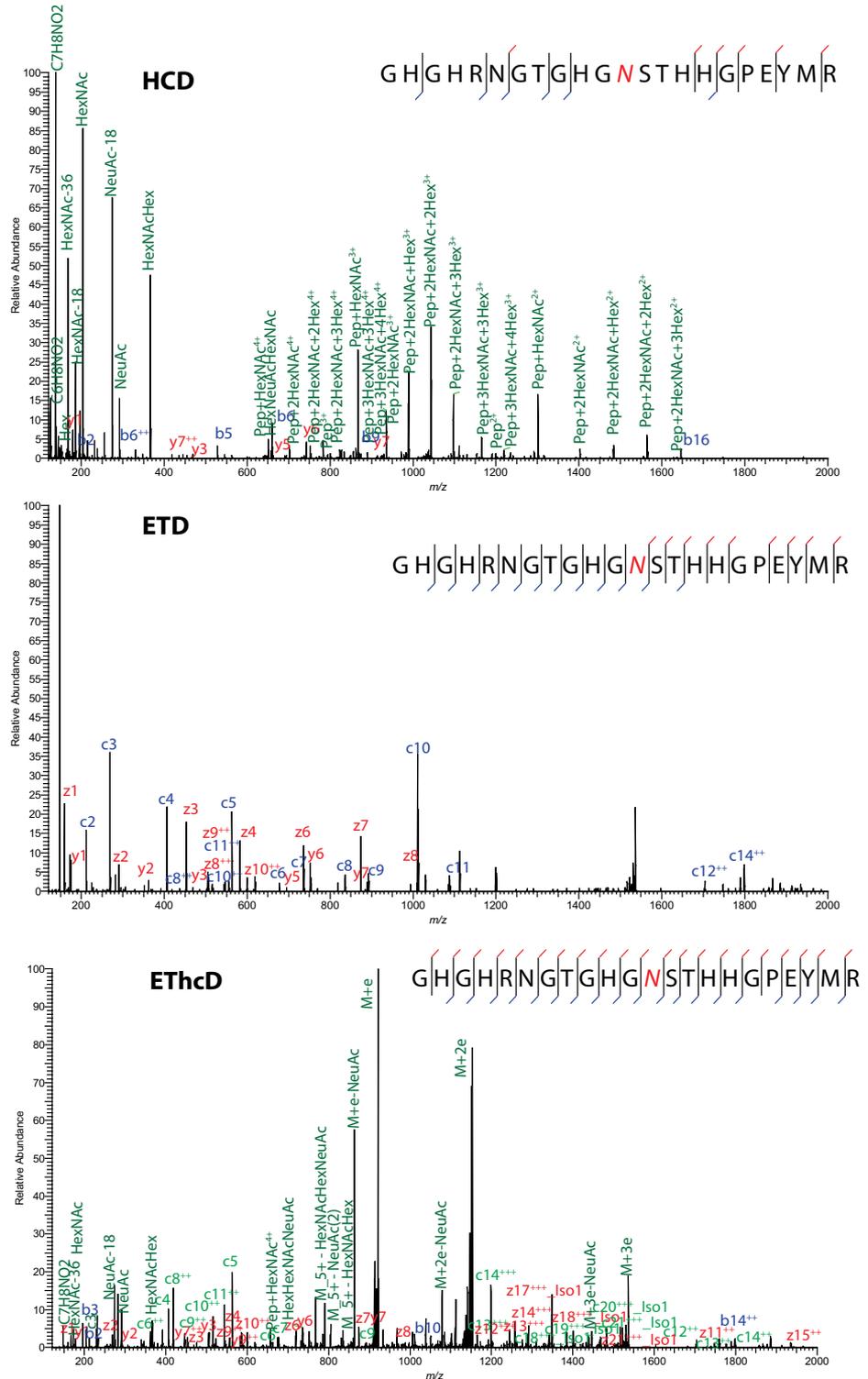
Several studies in the past have shown the importance of combining CAD and ETD fragmentation for intact glycopeptides analysis.¹³⁻¹⁶ However, all of these studies have used both types of fragmentation in a nonselective fashion. On the Orbitrap Fusion MS, we expand on this approach to implement an intelligent acquisition strategy termed HCD product-dependent ETD workflow (HCD-pd-ETD) that enables on-the-fly identification of glycopeptides and improves overall productivity of glycopeptide analyses. In this approach, the Orbitrap Fusion mass spectrometer acquires HR/AM HCD spectra in a data-dependent fashion. The instrument identifies glycan oxonium ions on the fly in the HCD spectra and triggers ETD spectra on the glycopeptide precursors only (Figure 7). This results in streamlined data analysis and improvement in dynamic range and duty cycle. The HCD-pd-ETD method is provided within the instrument control software for Orbitrap Fusion mass spectrometer. In addition to HCD-pd-ETD, Orbitrap Fusion MS can trigger any fragmentation based on oxonium ion presence including CID and HCD (HCD-pd-CID, HCD-pd-HCD). Triggering CID fragmentation based on the detection of oxonium ions is useful for elucidating glycan composition information as CID tends to produce more detailed glycan backbone fragmentation (Figure 7). This approach is useful as glycans are heterogeneous PTMs; multiple glycans can be present at a single amino acid site and requires complete characterization of all detected compositions.

Figure 7. Schematic representation of HCD-pd-ETD and HCD-pd-CID acquisition methods. The HCD spectrum shows diagnostic glycan oxonium ions in the low m/z region which are used to trigger ETD and/or CID spectrum. The ETD spectrum provides information about the peptide sequence and the site of glycosylation. The CID spectrum provides information about the glycan composition.



Orbitrap Fusion also introduces a novel fragmentation referred to as electron-transfer/higher-energy collision dissociation (ETHcD) that is unique to this platform. In this fragmentation ETD and HCD are combined in a single spectrum. In ETHcD, precursors are fragmented within the linear ion trap using ETD, the precursors, charge reduced precursors and ETD fragment ions are then transferred to the IRM for HCD fragmentation. The result is an ETHcD spectrum containing b-, c-, y- and z- ions, a spectrum that is combination of ETD and HCD fragments. Studies have shown that ETHcD data provides more complete fragmentation of unmodified and phosphorylated peptides than HCD or ETD alone, and it also increases confidence in localization of phosphorylation sites.^{17,18} ETHcD appears especially advantageous for *N*-linked glycopeptides, enabling better sequence coverage and glycosylation site localization (Figure 8). It should be noted that ETHcD can also be acquired in an HCD-pd- fashion for glycopeptides analysis.

Figure 8. Comparison of HCD, ETD and ETHcD spectra acquired on Orbitrap Fusion for a tryptically digested glycopeptide from Hemopexin protein. ETHcD shows near complete sequence coverage and confident glycosylation site localization relative to HCD or ETD.



In order to understand the functions of individual proteins and their place in complex biological systems, it is often necessary to measure changes in protein abundance relative to changes in the state of the system. These measurements have traditionally been performed using western blot analyses. More recently, modern proteomics has evolved to include a variety of technologies for the routine quantitative analyses of both known and unknown targets. Discovery-based relative quantification is an analytical approach that allows the scientist to determine relative protein abundance changes across a set of samples simultaneously and without the requirement for prior knowledge of the proteins involved. An isobaric chemical tagging approach is popular for relative quantitation. In a single analysis, they can be used to identify and quantify relative changes in complex protein samples across multiple experimental conditions. They can be used with a wide variety of samples including cells, tissues, and biological fluids. Thermo Scientific Tandem Mass Tags (TMT) reagents are isobaric chemical tags consisting of an MS/MS reporter group, a spacer arm, and an amine-reactive group. Amine-reactive groups covalently bind to peptide N-termini and to lysine residues. Each tag fragments during MS/MS, producing unique reporter ions. Protein quantitation is accomplished by comparing the intensities of the reporter ions. Unfortunately, this approach in the past could not be applied to glycoproteomics. Mainly because we could not use HCD or ETD for relative quantification of isobarically labeled peptides. In the case of ETD fragmentation, the reporter fragments are often uncharged and cannot be detected within the ETD spectra. In the case of HCD fragmentation, generation of reporter ions is limited as primary fragmentation occurs for glycosidic not peptide bonds for glycopeptides.

The implementation of synchronous precursor selection (SPS) exclusive to the Orbitrap Fusion MS overcomes these limitations.^{19,20} In this approach, the parent ion is selected in MS¹ scan, isolated in the quadrupole and fragment by ETD in the ion trap. Upon fragmentation, multiple MS² fragment ions are selected and isolated using single trap fill and waveform (synchronous precursor selection). Up to 20 fragments can be isolated simultaneously. Selected MS² fragment ions are transferred back into the IRM and HCD fragmentation is performed. MS³ fragments are detected in the Orbitrap for the most accurate MS³ based TMT quantitation. Using SPS dramatically increases the signal intensity and improves the ratio accuracy (due to counting statistics) and at the same time dramatically boosts sensitivity increasing the total number of peptides quantified significantly. Using this approach, multiplexed quantification of glycopeptides in a variety of biological samples is possible and this workflow is only possible on the Orbitrap Fusion mass spectrometer.

Conclusions

To summarize, the introduction of the Orbitrap Fusion MS provides a giant step forward for the glycomics and glycoproteomics community. The innovative instrument design contains new functionalities, including a mass selecting quadrupole coupled to both a linear ion trap and Orbitrap mass analyzer with highly optimized parallel operation. The instrument allows for increased scan rate, full flexibility of CID, HCD, ETD and EThcD dissociations at any stage of MSⁿ analysis, ultra high field Orbitrap detection with advanced signal processing, front-end compact and robust ETD source and dedicated internal mass calibration. These functionalities combine to produce a significant performance improvement for standard glycomics and glycoproteomics experiments. Additionally, the unique tribrid architecture makes a wide array of novel acquisition experiments possible facilitating workflows that were previously inaccessible with previous generation platforms.

References

1. M. Wührer, M. I. Catalina, A. M. Deelder et al., "Glycoproteomics based on tandem mass spectrometry of glycopeptides," *Journal of Chromatography B*, vol. 849, no. 1-2, pp. 115–128, **2007**.
2. J. J. Conboy and J. Henion, "High-performance anion exchange chromatography coupled with mass spectrometry for the determination of carbohydrates," *Biological Mass Spectrometry*, vol. 21, no. 8, pp. 397–407, **1992**.
3. M. J. Huddleston, M. F. Bean, and S. A. Carr, "Collisional fragmentation of glycopeptides by electrospray ionization LC/MS and LC/MS/MS: methods for selective detection of glycopeptides in protein digests," *Analytical Chemistry*, vol. 65, no. 7, pp. 877–884, **1993**.
4. J. Irungu, E. P. Go, Y. Zhang et al., "Comparison of HPLC/ESI/TOF MS versus MALDI-TOF/TOF MS for glycopeptide analysis of a highly glycosylated HIV envelope glycoprotein," *Journal of the American Society for Mass Spectrometry*, vol. 19, no. 8, pp. 1209–1220, **2008**.
5. E. Mirgorodskaya, P. Roepstorff, and R. A. Zubarev, "Localization of O-glycosylation sites in peptides by electron capture dissociation in a Fourier transform mass spectrometer," *Analytical Chemistry*, vol. 71, no. 20, pp. 4431–4436, **1999**.
6. N. V. Bykova, C. Rampitsch, O. Krokhin, K. G. Standing, and W. Ens, "Determination and characterization of site-specific N-glycosylation using MALDI-Qq-TOF tandem mass spectrometry: case study with a plant protease," *Analytical Chemistry*, vol. 78, no. 4, pp. 1093–1103, **2006**.
7. T. Geiger and S. Clarke, "Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation," *Journal of Biological Chemistry*, vol. 262, no. 2, pp. 785–794, **1987**.
8. N. E. Robinson and A. B. Robinson, "Prediction of protein deamidation rates from primary and three-dimensional structure," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 8, pp. 4367–4372, **2001**.
9. R. Tyler-Cross and V. Schirch, "Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides," *Journal of Biological Chemistry*, vol. 266, no. 33, pp. 22549–22556, **1991**.
10. H. T. Wright, "Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins," *Critical Reviews in Biochemistry and Molecular Biology*, vol. 26, no. 1, pp. 1–52, **1991**.
11. G. Palmisano, M. N. Melo-Braga, K. Engholm-Keller et al., "Chemical deamidation: a common pitfall in large-scale N-linked glycoproteomic mass spectrometry-based analyses," *Journal of Proteome Research*, vol. 11, no. 3, pp. 1949–1957, **2012**.
12. R. A. Zubarev, "Electron-capture dissociation tandem mass spectrometry," *Current Opinion in Biotechnology*, vol. 15, no. 1, pp. 12–16, **2004**.
13. L. M. Mikesch, B. Ueberheide, A. Chi et al., "The utility of ETD mass spectrometry in proteomic analysis," *Biochimica et Biophysica Acta*, vol. 1764, no. 12, pp. 1811–1822, **2006**.
14. M. I. Catalina, C. A. M. Koeleman, A. M. Deelder, and M. Wührer, "Electron transfer dissociation of N-glycopeptides: loss of the entire N-glycosylated asparagine side chain," *Rapid Communications in Mass Spectrometry*, vol. 21, no. 6, pp. 1053–1061, **2007**.
15. W. R. Alley Jr., Y. Mechref, and M. V. Novotny, "Characterization of glycopeptides by combining collision-induced dissociation and electron-transfer dissociation mass spectrometry data," *Rapid Communications in Mass Spectrometry*, vol. 23, no. 1, pp. 161–170, **2009**.

16. S. I. Snovida, E. D. Bodnar, R. Viner, J. Saba, and H. Perreault, "A simple cellulose column procedure for selective enrichment of glycopeptides and characterization by nano LC coupled with electron-transfer and high-energy collisional-dissociation tandem mass spectrometry," *Carbohydrate Research*, vol.345, no. 6, pp. 792–801, **2010**.
17. C.K. Frese, A.F. Altelaar, H. van den Toorn, D. Nolting, J. Griep-Raming, A.J. Heck, S. Mohammed, "Toward full peptide sequence coverage by dual fragmentation combining electron-transfer and higher-energy collision dissociation tandem mass spectrometry" *Analytical Chemistry*, vol. 84, no. 22, pp. 9668–9673, **2012**.
18. C.K. Frese, H. Zhou, T. Taus, A.F. Altelaar, K. Mechtler, A.J. Heck, S. Mohammed, "Unambiguous phosphosite localization using electron-transfer/higher-energy collision dissociation (ETHcD)," *Journal of Proteome Research*, vol. 12, no. 3, pp. 1520–1525, **2013**.
19. L. Ting, R. Rad, S.P. Gygi, W. and Haas, "MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics," *Nature Methods*, vol. 8, pp. 937-940, **2011**.
20. G. McAlister, E.L. Huttlin, W. Haas, L. Ting, M.P. Jedrychowski, J.C. Rogers, K. Kuhn, I. Pike, R.A. Grothe, J.D. Blethrow, S.P. Gygi, "Increasing the Multiplexing Capacity of TMTs Using Reporter Ion Isotopologues with Isobaric Masses," *Analytical Chemistry*, vol. 84, pp. 7469-7478, **2012**.

www.thermofisher.com

©2016 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is presented as an example of the capabilities of Thermo Fisher Scientific products. It is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details.

Africa +43 1 333 50 34 0	Denmark +45 70 23 62 60	Japan +81 45 453 9100	Russia/CIS +43 1 333 50 34 0
Australia +61 3 9757 4300	Europe-Other +43 1 333 50 34 0	Korea +82 2 3420 8600	Singapore +65 6289 1190
Austria +43 810 282 206	Finland +358 9 3291 0200	Latin America +1 561 688 8700	Spain +34 914 845 965
Belgium +32 53 73 42 41	France +33 1 60 92 48 00	Middle East +43 1 333 50 34 0	Sweden +46 8 556 468 00
Canada +1 800 530 8447	Germany +49 6103 408 1014	Netherlands +31 76 579 55 55	Switzerland +41 61 716 77 00
China 800 810 5118 (free call domestic) 400 650 5118	India +91 22 6742 9494	New Zealand +64 9 980 6700	UK +44 1442 233555
	Italy +39 02 950 591	Norway +46 8 556 468 00	USA +1 800 532 4752

Thermo
SCIENTIFIC

A Thermo Fisher Scientific Brand