

# Comparison and Optimization of First and Second Generation Quadrupole Dual Cell Linear Ion Trap Orbitrap MS for Glycopeptide Analysis

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## Overview

**Purpose:** To optimize instrument parameters for the Thermo Scientific™ Orbitrap Fusion™ Lumos MS and compare performance against the Thermo Scientific™ Orbitrap Fusion™ MS for intact glycopeptide analysis.

**Methods:** Glycopeptides enriched from various sources were analyzed on the Orbitrap Fusion Lumos MS and Orbitrap Fusion mass spectrometers. Multiple instrumental parameters were tested to maximize intact glycopeptide identifications. Data analysis were performed using Byonic™ software.

**Results:** Improvement in performance for intact glycopeptide analysis was observed on the Orbitrap Fusion Lumos MS relative to the Orbitrap Fusion MS.

## Introduction

Large scale intact glycopeptide analysis remains challenging due to complexities associated with the glycopeptide structure. Not only must one sequence the peptide backbone, but glycosylation site localization and glycan composition are also required for intact glycopeptide analysis. The challenge is further compounded by the fact that traditional fragmentations are not ideal for glycopeptide sequencing. The emergence of electron-transfer dissociation (ETD) and by extension electron-transfer and higher-energy collision dissociation (EThcD) have alleviated a lot of these issues. Here we present a performance evaluation comparison of first and second generation quadrupole dual cell linear ion trap Orbitrap hybrid mass spectrometer (Orbitrap Fusion Lumos MS and Orbitrap Fusion MS) for glycopeptide analysis. Parameters and workflows will be presented that highlight large scale glycoproteomics.

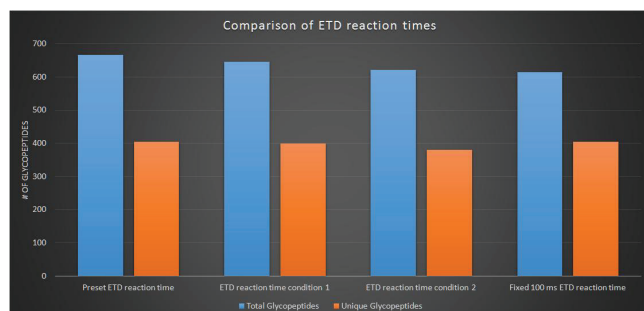
## Methods

Glycopeptides were enriched from human serum and HeLa cell lysates digests using strong anion exchange (SAX) columns. The enriched glycopeptides were analyzed using a Thermo Scientific™ EASY-nLC™ 1000 with a Thermo Scientific™ C18 PepMap™ column (2µm, 100Å, 75µm×50 cm) coupled to an Orbitrap Fusion Lumos MS and Orbitrap Fusion MS. Various ETD reaction times, AGC target values, isolation windows, supplemental activation collision energy and RF were tested to maximize glycopeptides identification. Data analysis were performed using Byonic software (Protein Metrics Inc.).

## Results

ETD is ideal for intact glycopeptide analysis due to the fact that it is a 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. Our initial experiments focused on optimizing ETD parameters to improve glycopeptides data on Orbitrap Fusion Lumos MS. Typically, longer ETD reaction times are needed for glycopeptides relative to conventional peptides. Various ETD reaction times, fixed or varied, dependent upon charge states were tested to maximize spectral quality, a crucial aspect of intact glycopeptide analysis.

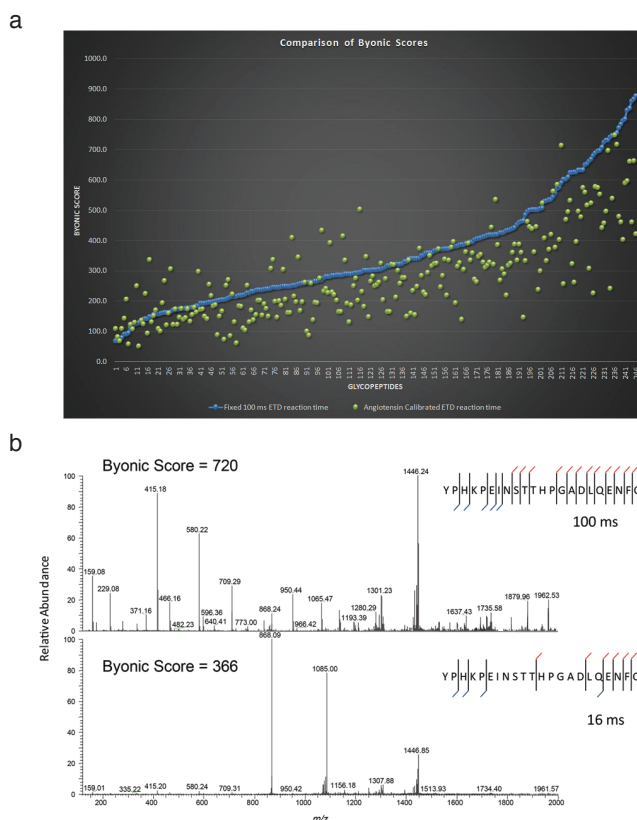
Figure 1. Comparison of ETD reaction times



Charge State	3	4	5	6-8
ETD reaction time condition 1 (ms)	70	50	40	20
ETD reaction time condition 2 (ms)	154	98	63	40
Fixed 100 ms ETD reaction time (ms)	100	100	100	100

In general preset calibrated ETD reaction times were suitable for intact *N*-linked glycopeptide analysis. These are values that can be optimized infusing angiotensin into the mass spectrometer. However, longer reaction times for glycopeptides is ideal as it can significantly improve spectral quality (Figure 2). Which can increase confidence for glycosylation site localization.

Figure 2. Comparison of the quality of spectrum between Angiotensin calibrated ETD reaction time and Fixed ETD reaction time of 100ms. (a) Comparison of Byonic score for 246 glycopeptides common between the two runs. (b) Example of spectral quality



In total, 11 parameters were tested with 21 individual runs to maximize performance on the Orbitrap Fusion Lumos MS. After optimization, experiments were conducted on both the Orbitrap Fusion MS and Orbitrap Fusion Lumos MS to examine performance of the platforms relative to each other. Human serum glycopeptides were used in the comparison. All data were acquired using the product ion triggered approach (HCD-pd-ETD, HCD-pd-EThcD). In our ETD comparison, Orbitrap Fusion Lumos MS identified 9% more unique glycopeptides relative to Orbitrap Fusion MS (Figure 3a). In EThcD comparison Orbitrap Fusion Lumos MS identified 43% more unique glycopeptides than Orbitrap Fusion MS (Figure 3b). Comparison of EThcD to ETD within Orbitrap Fusion Lumos MS resulted in 49% more unique glycopeptides identified by EThcD over ETD (Figure 4). Closer examination of the data showed that the increase in identification by Orbitrap Fusion Lumos MS and EThcD came from large glycopeptides which are challenging in mass spectrometry experiments (Figure 5a and b). We also observed spectrum quality was better in EThcD compared to ETD (Figure 6). Due to the observed increase in glycopeptide identifications by EThcD over ETD, for all our subsequent experiments EThcD was used for sequencing. An important parameter for EThcD is the amount of supplemental activation collision energy used in EThcD fragmentation. We observed supplemental activation collision energy between 20-25 was optimal for maximizing glycopeptide identification and spectrum quality (Figure 7). Comprehensive sequence coverage is very crucial for glycopeptide analysis. Especially dealing with *O*-linked glycopeptides. These can occur on both serine (Ser) and threonine (Thr), in clusters and on multiple sites on a single peptide. In general we observed that EThcD relative ETD improved glycopeptide sequence coverage. Figure 8 shows the importance of having good sequence coverage and the advantage of EThcD. This particular glycopeptide has two potential *O*-glycosylation site. Since Ser and Thr are adjacent to each other. Mis-assignment can occur without good sequence coverage.

Figure 3. Comparison of *N*-linked glycopeptides identified by (a) ETD (b) ETHcD on Orbitrap Fusion Lumos MS and Orbitrap Fusion MS

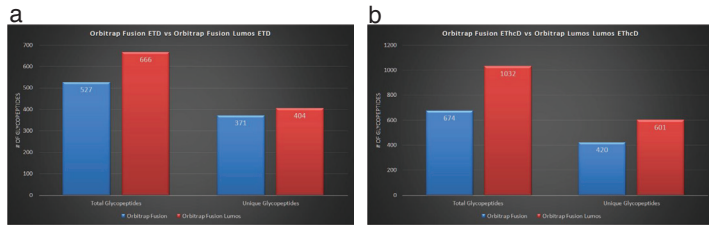


Figure 4. Comparison ETHcD to ETD in Orbitrap Fusion MS and Orbitrap Fusion MS Lumos

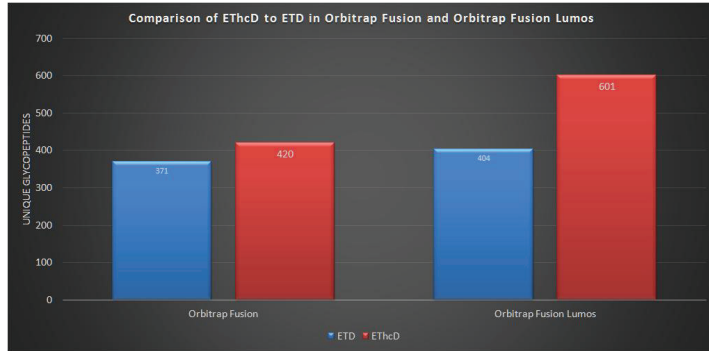


Figure 5. Distribution of identification by peptide mass: (a) ETHcD identifications in Orbitrap Fusion Lumos vs. Orbitrap Fusion (b) ETHcD vs ETD identifications on Orbitrap Fusion Lumos

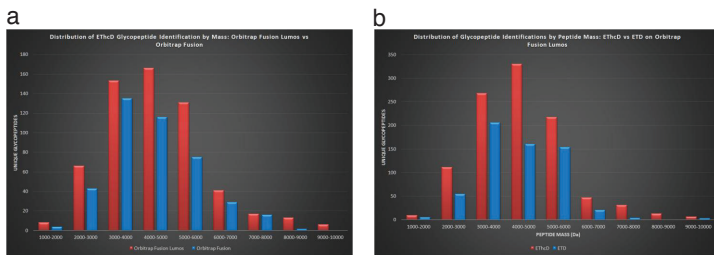


Figure 6. Comparison of the quality of spectra: ETD vs ETHcD on Orbitrap Fusion Lumos

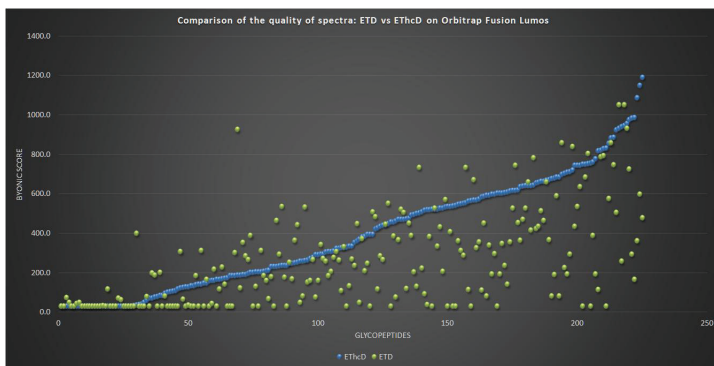


Figure 7. Maximizing glycopeptide identifications: Effect of supplemental activation collision energy on ETHcD identifications

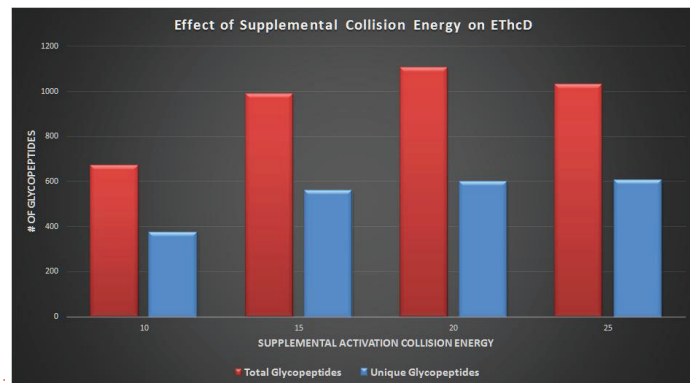
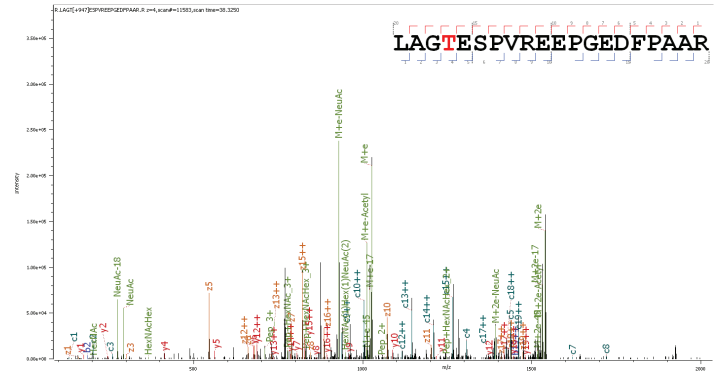
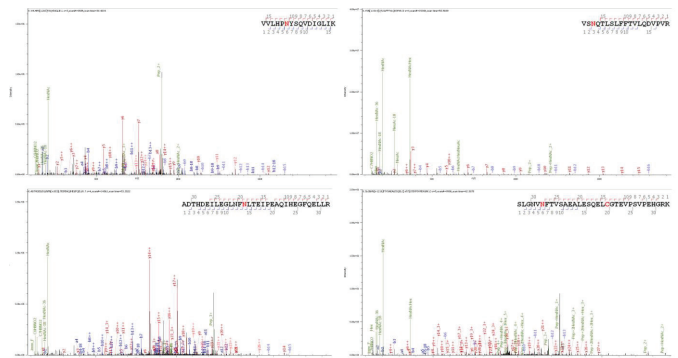


Figure 8. ETHcD FT-MS/MS spectrum of *O*-linked glycopeptide



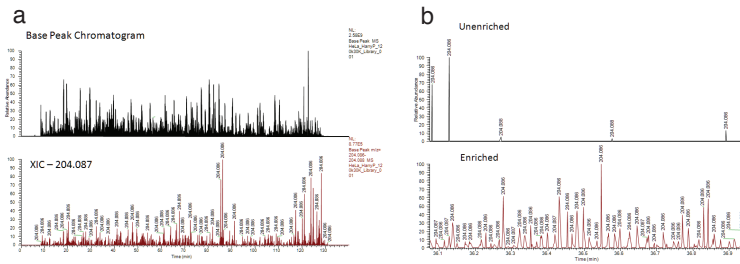
The primary focus of our experiments were on ETD and ETHcD, however, we observed that the quality of HCD spectra were superior to spectra acquired on other platforms for intact glycopeptides. Typically, b and y ions generated from peptide backbone of a glycopeptides are low abundant and are difficult to detect on commercial mass spectrometers. But in the Tribrid™ mass spectrometers, we could easily detect and use them for sequencing (Figure 9).

Figure 9. HCD MS/MS spectra from intact *N*-linked glycopeptides from Human serum

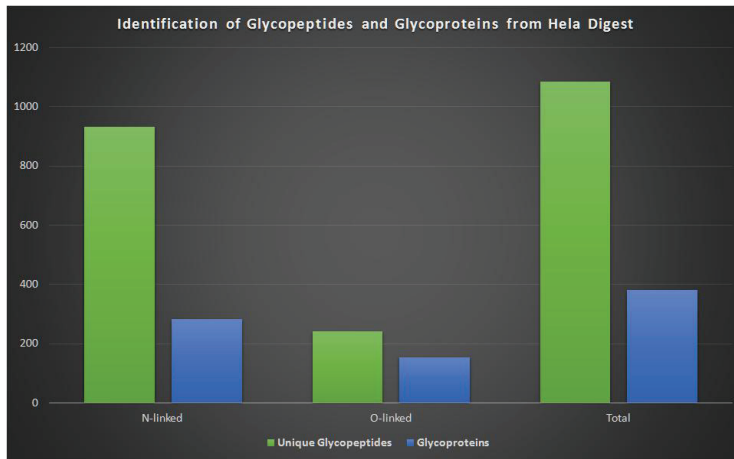


Specific changes in glycosylation is a hallmark of cancer. Unfortunately, proteomics studies tend to ignore this particular post-translational modification in cancer cell line analysis. For example in an unenriched HeLa digest 15-20% of spectra are glycopeptides (Figure 10a). However, in an unenriched sample we are still limited by the dynamic range of a mass spectrometer and only detect a fraction of all possible glycopeptides (Figure 10b). With improved capability of Orbitrap Fusion Lumos MS it is possible to sequence these modifications and discover a number of intact glycoproteins (Figure 11).

**Figure 10. (a) Unenriched LC-MS of tryptically digested HeLa. Top chromatogram shows the base peak chromatogram while the bottom chromatogram is the XIC of 204.087 which is indicative of HexNAc (b) Top chromatogram shows a 1 minute window of the unenriched run while bottom show an SAX enriched run**



**Figure 11. Identification of N- and O-linked glycopeptides from tryptically digested HeLa.**



## Conclusions

- > 40% increase in identifications using EThcD on Orbitrap Fusion Lumos MS relative to EThcD on Orbitrap Fusion MS for human serum N-linked glycopeptides
- Superiority of EThcD relative to ETD is better exemplified on Orbitrap Fusion Lumos MS than on Orbitrap Fusion MS
- Supplemental activation collision energy used in EThcD has effect on the quality of spectrum. Optimal supplemental activation collision energy is 20-25 %
- EThcD does a far better suited for sequencing and localizing site of glycosylation compared to ETD or HCD on Orbitrap Fusion Lumos MS
- ETD reaction time has effect on quality of ETD spectrum. Longer reaction times will result in better spectra quality.
- Tribrid's hidden secret: HCD for sequencing glycopeptides is very good. However, ETD or EThcD is still recommended over HCD

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