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Improving high-throughput top-down proteomics using a modified hybrid quadrupole-ultra-high-field-Orbitrap mass spectrometer

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ABSTRACT

Purpose: Demonstrate improved Top-Down performance using a Thermo Scientific™ Q Exactive™ HF-X mass spectrometer and advanced Data-Dependent Acquisition (DDA) algorithms

Methods: Improved 'Medium-High' and 'High-High' Top-Down DDA methods

Results: Redundant fragmentation is significantly reduced and more proteoforms are identified using the Q Exactive HF-X mass spectrometer and improved Top-Down DDA methods

INTRODUCTION

Over the last few years, Top-Down mass spectrometry has benefited from enormous improvements in FTMS instrumentation in terms of resolution, speed, sensitivity and fragmentation techniques. In spite of all these improvements, there is still a need for higher sensitivity and more intelligent data dependent acquisition methods in order to successfully conduct high-throughput large scale Top-Down proteomics studies. Furthermore, improvement to the current generation of charge state assignment and deconvolution algorithms to handle complex Top-Down data will lead to more efficient, complete and accurate protein identification. Here we demonstrate the improved performance of a modified Thermo Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap mass spectrometer in a series of high-throughput Top-Down proteomics experiments.

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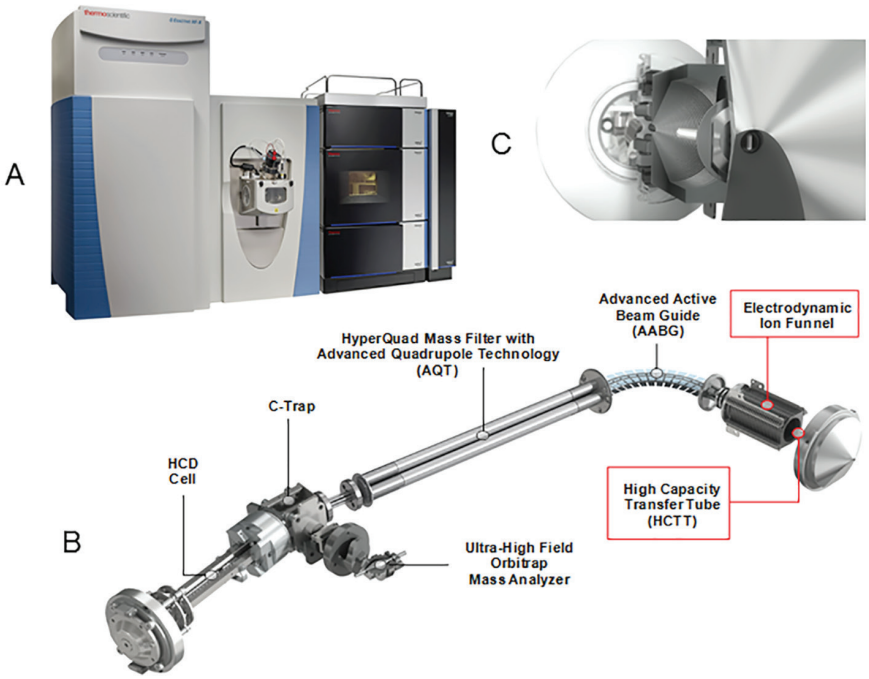
MATERIALS AND METHODS

Several hardware and software improvements have been implemented into an existing Q Exactive HF instrument: (a) a brighter ion source interface using a high-capacity transfer tube (HCTT) and an electrodynamic ion funnel (EDIF) for improved ion transmission, (b) on-the-fly charge state assignment and deconvolution for both, medium and high resolution Orbitrap spectra using advanced algorithms, and (c) improved data-dependent decisions for 'High-High' and 'Medium-High' Top-Down analysis by selecting only one charge state per protein and applying optimum collision energy for HCD fragmentation.

Reversed-Phase chromatographic separation of intact protein mixtures prior to top-down analysis was performed using a Thermo Scientific™ MabPac-RP 2.1x100mm, 4µm column and a Thermo Scientific™ Vanquish™ Horizon UHPLC system. Data processing was performed using the Thermo Scientific™ BioPharma Finder™ 2.0 and Thermo Scientific™ ProSight™ PD node in Thermo Scientific™ Proteome Discoverer™ 2.1 software.

Figure 1. A) Photograph of the Q Exactive HF-X mass spectrometer and Vanquish Horizon UHPLC system. B) Ion path schematic of the Q Exactive HF-X mass spectrometer. C) Photograph showing the High Capacity Transfer Tube and the Electrodynamic Ion Funnel.



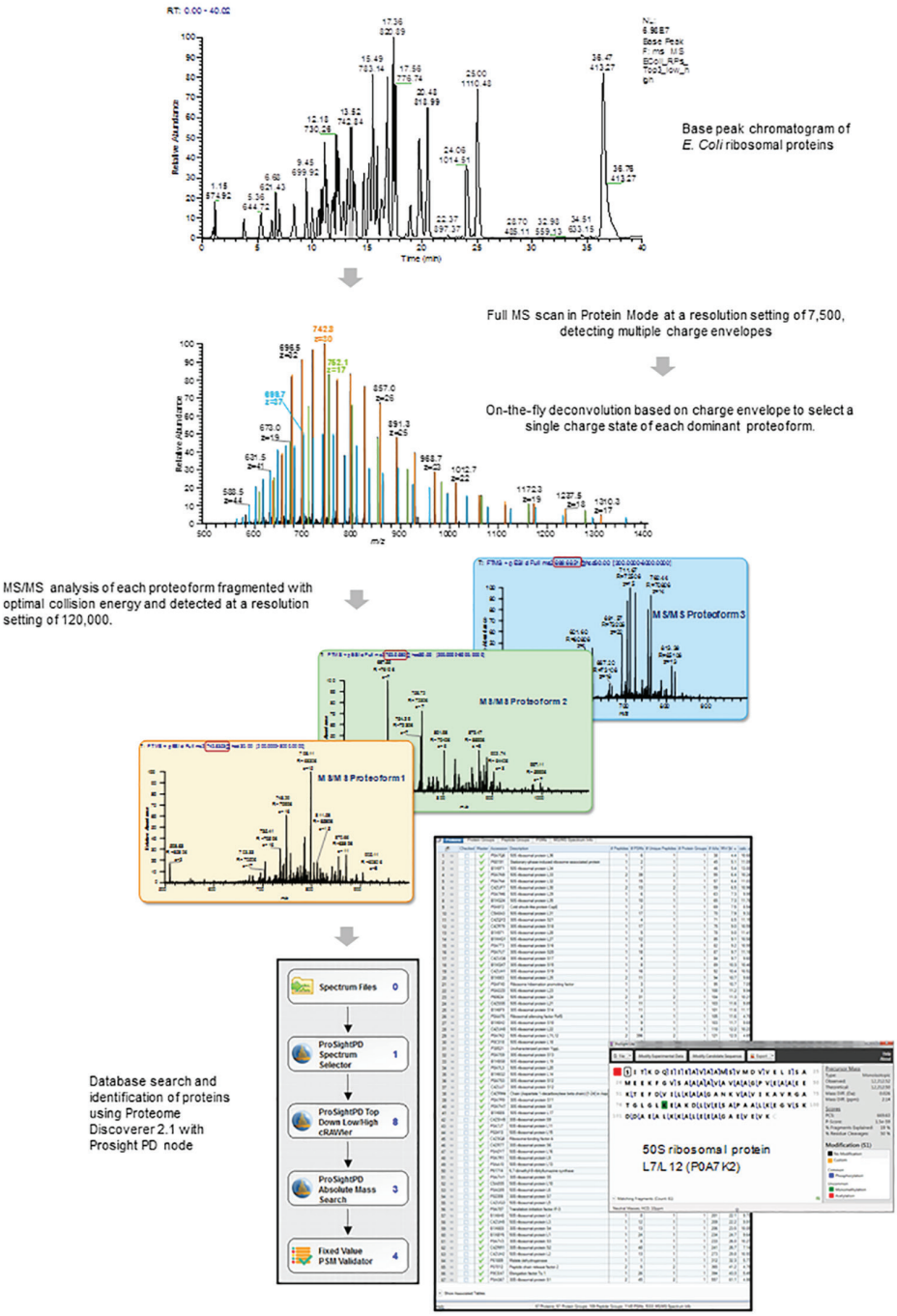
RESULTS

Improved Top-Down DDA Workflow

One of the challenges in Top-Down DDA approaches is the limited dynamic range of proteoform characterization. Using standard TopN methods, the abundant proteoforms are fragmented multiple times, and their multiple detection can hinder new, lower abundant species from identification. Here we present an improved, more intelligent Top-Down DDA workflow (see Figure 2) which eliminates redundant fragmentation and could reveal deeper and more interesting insights from Top-Down proteomics data.

During the MS1 scan, advanced algorithms are used to perform on-the-fly charge state assignment and deconvolution for both, medium and high resolution Orbitrap spectra. This step is followed by selection of only one, most abundant charge state per proteoform while redundants are placed on an exclusion list. For the following MS2 scans, smart algorithms automatically determine the optimal collision energy for the fragmentation of each selected precursor to generate high-resolution accurate mass spectra for confident protein identification.

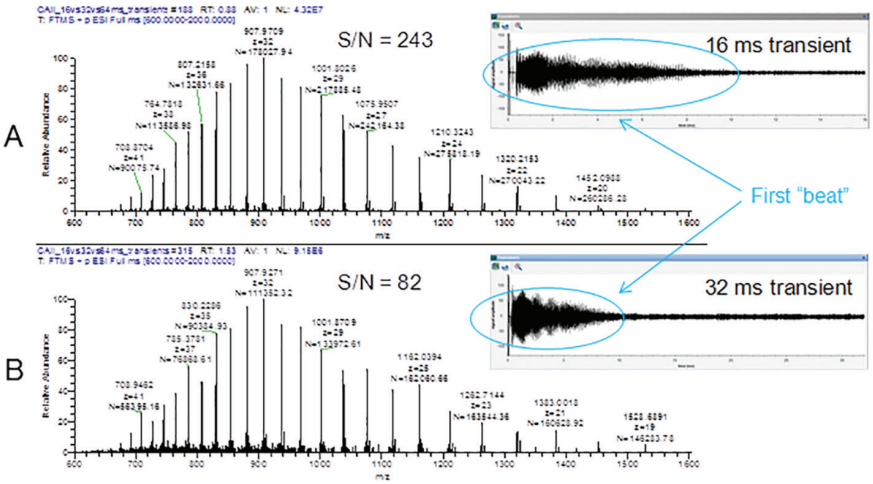
Figure 2. Improved Top-Down DDA workflow applied for the analysis of intact *E. Coli* ribosomal proteins



Evaluation of the Top-Down DDA workflow in direct infusion experiments

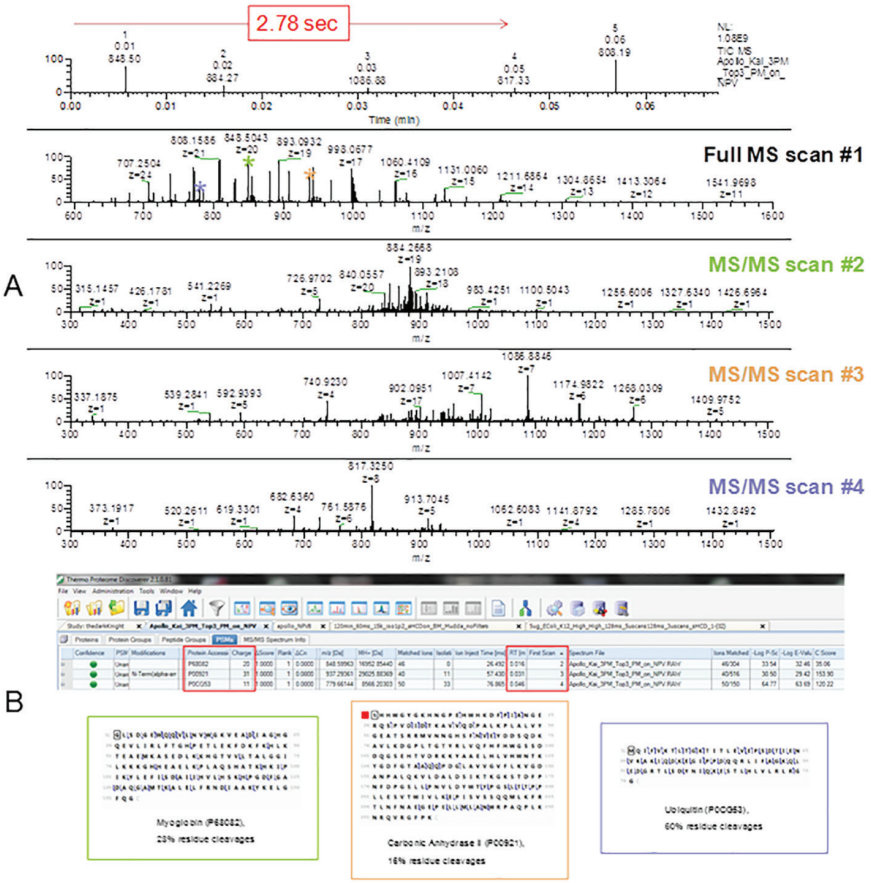
Time domain signals from multiply-charged protein ions show characteristic beat patterns due to the closely spaced frequencies of the various isotopic constituents. Useful frequency information is extracted only from these isotopic "beats" while no signal is observed for the time span between the "beats". The new resolution setting of 7,500 which corresponds to a transient length of 16 ms aims to improve signal-to-noise ratio of intact proteins by collecting only useful frequency information from the first "beat". Figure 3 shows a three-fold increase in S/N for intact Carbonic Anhydrase II (29 kDa protein) using 16 ms transients compared to 32 ms transients.

Figure 3. Full MS spectra of intact Carbonic Anhydrase II protein using A) 16 ms and B) 32ms transients



The new improved Top-Down DDA workflow was tested in a direct infusion experiment using an intact protein mix containing Ubiquitin, Apo-Myoglobin and Carbonic Anhydrase II. Figure 4 shows the unambiguous identification of all three proteins in less than 3 seconds using the improved workflow.

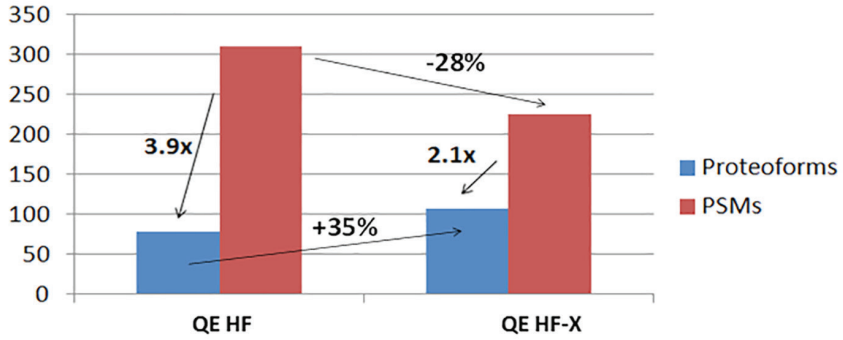
Figure 4. Top-Down DDA analysis of a three-protein mix using the improved workflow. A) Full MS scan (5 uscans) at 7.5k resolution followed by three MS/MS scans (3 uscans for each scan) at 120k resolution. B) ProSight PD identification results



Evaluation of the Top-Down DDA workflow in LC-MS/MS experiments

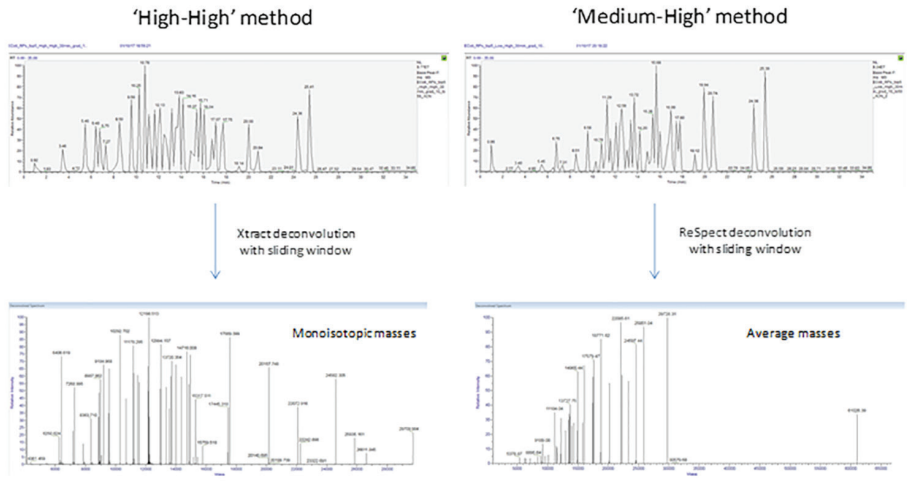
Top-Down LC-MS/MS analyses of several intact protein mixtures were carried out to evaluate the improved ability of the modified instrument and improved DDA workflow to perform high-throughput Top-Down analysis. We started with an *E. Coli* protein extract, which was first purified by solid phase extraction and then analyzed by Top-Down LC-MS/MS using 15 minutes LC gradient and the TopN 'High-High' method. With the modified Q Exactive HF instrument and using the improved workflow we were able to eliminate redundant fragmentation (the ratio between the number of PSMs and number of proteoforms was ~ 2x lower) and identify 35% more proteoforms than with the standard Q Exactive HF instrument (see Figure 5). The 'High-High' method was employed here to deliver high resolution (120,000@m/z200) and high mass accuracy in both MS and MS/MS modes for the identification of proteins with MWs below 40kDa.

Figure 5. Average number of Proteoforms and PSMs identified from duplicate runs on the Q Exactive HF MS and Q Exactive HF-X MS. Results are from a SPE purified *E. Coli* protein extract separated using a 15 min LC gradient.



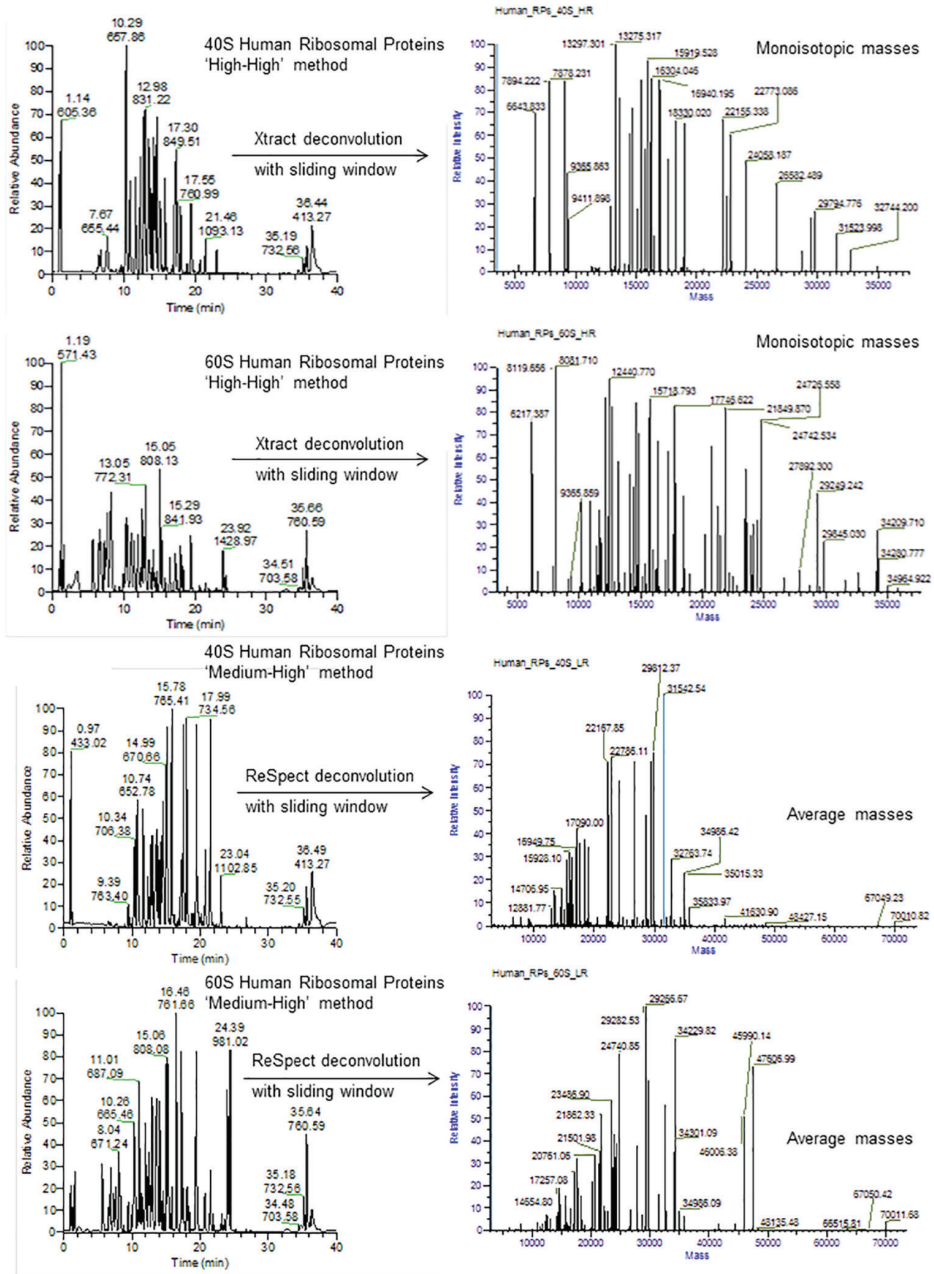
Furthermore, purified *E. Coli*, Human 40S and 60S ribosome samples were analyzed using a combination of 'High-High' and 'Medium-High' methods. For the *E. Coli* ribosome sample (Figure 6), a total of 67 proteins were unambiguously identified. All expected *E. Coli* ribosomal proteins as well as other ribosome-associated proteins with MWs ranging from 4.4 to 61 kDa, were identified. The 'Medium-High' method employed 16 ms transients (R=7,500@*m/z* 200) for Full MS scans and 256 ms transients (R=120,000@*m/z* 200) for MS/MS scans. The main advantages of the 'Medium-High' method is that it provides higher S/N at higher Full MS scanning speed allowing sequencing of more proteins, including large ones (> 40kDa).

Figure 6. Base peak chromatograms and deconvoluted spectra from the 'High-High' and 'Medium-High' runs of the *E. Coli* ribosomal proteins sample.



Top-Down analysis of Human 40S ribosome sample resulted in the identification of 32 from a total of 33 expected subunits with MWs ranging from 6.5 to 35kDa. For the Human 60S ribosome sample 45 out of 47 subunits were identified with MWs ranging from 3.4 to 47.6 kDa. Figure 7 shows base peak chromatograms and deconvoluted spectra from the 'High-High' and 'Medium-High' runs of the Human 40S and 60S ribosome samples.

Figure 7. Base peak chromatograms and deconvoluted spectra from the 'High-High' and 'Medium-High' runs of the 40S and 60S Human ribosome samples



CONCLUSIONS

- High-throughput Top-Down analysis is significantly improved by using Q Exactive HF-X mass spectrometer and a more intelligent DDA workflow.
- Redundant fragmentation is dramatically reduced and 35% more proteoforms are identified in a SPE purified *E. Coli* protein extract.
- The improved Top-Down workflow was successfully applied for the analysis of *E. Coli* and Human 40S and 60S ribosomal proteins.

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