Low Attomole Limit of Quantification on an Orbitrap Fusion Lumos Tribrid Mass Spectrometer

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Overview

Purpose: Demonstrate improved limit of quantification (LOQ) possible for the new Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid Mass Spectrometer utilizing several targeted and data independent (DIA) methods for peptide quantitation.

Methods: A set of stable isotope-labeled synthetic peptides were spiked in various concentrations into high complexity matrices. They were analyzed using high resolution accurate mass (HRAM) MS1/MS2 DIA methods and HRAM MS2 targeted methods.

Results: Limit of quantitation (LOQ) down to 1 attomole (CV<15%), while still maintaining linearity, was observed when using the targeted strategy which proved to be the most sensitive of all the tested techniques. Several orders of dynamic range were observed from the quantitation results of the DIA methods which exhibited limits of quantification down to 100 attomoles.

Introduction

One of the major challenges in Proteomics is the quantification of low abundance proteins and peptides of biological relevance such as transcription factors or low stoichiometry post translational modified proteins. The quantification of these analytes of interest is complicated by the very large dynamic range observed for cellular protein expression, especially in humans. Various strategies have emerged to overcome the high matrix/background effect and quantify low level targets such as Selected Reaction Monitoring (SRM) and Selected Ion Monitoring (SIM). With the development of new generations mass spectrometry platforms providing high resolution and multiple detector versatility, new strategies are available to push the limit of quantification. Common targeted and untargeted quantitation methods such as PRM (Parallel Reaction Monitoring, Peterson *et al*., 2011), WiSIM (Wide Isolation Selected Ion Monitoring, Kiyonami *et al.,* 2014), classic DIA (cDIA) and DDA (Data Dependent Acquisition) were performed with the Orbitrap Fusion Lumos MS that offers both the speed of the current generation of the Orbitrap Fusion and the enhanced sensitivity afforded by the brighter ion source and improved ion transmission.

Methods

Sample Preparation: Heavy Lysine and Arginine isotope-labeled synthetic peptides (Pierce Peptide Retention Time Calibration Mixture, PRTC) were spiked in various concentrations into a high complexity matrix of 200 ng Pierce HeLa Protein Digest Standard or 1000 ng into a non-small lung cancer cell protein digest spanning from 1 attomole to 100 femtomole per microliter.

Liquid Chromatography: Sample separation and introduction into the mass spectrometer was performed using a reversed phase high performance chromatography with a two component mobile phase system: 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A Thermo Scientific™ Easy nLC™ 1000 nano-uHPLC system and 75 uM x 50 cm Thermo Scientific™ PepMap™ Easy-Spray™ column were employed at a flow rate of 300 nL/min. Peptides were eluted using a gradient of 5% to 25% B over 120 minutes followed by 25% to 40% B over a subsequent 35 minutes (DIA experiments), or, using a gradient of 5% to 25% B over 20 minutes followed by 25% to 40% B over a subsequent 8 minutes (PRM experiments).

Mass Spectrometry: Data was acquired on the new Orbitrap Fusion Lumos Tribrid mass spectrometer which features a brighter ion source and improved ion transmission (Figure 1); DDA, PRM, WiSIM and cDIA experiments were performed using CID and HCD fragmentations.

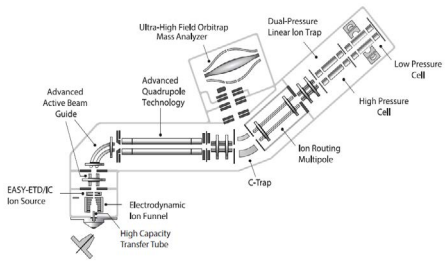

Figure 1: The new Thermo Scientific Orbitrap Fusion Lumos™ Tribrid Mass Spectrometer offers a High Capacity Ion Transfer Tube and Electrodynamic Ion Funnel which increase ion flux, while the segmented quadrupole powered by Advanced Quadrupole Technology improve parent ion transmission.

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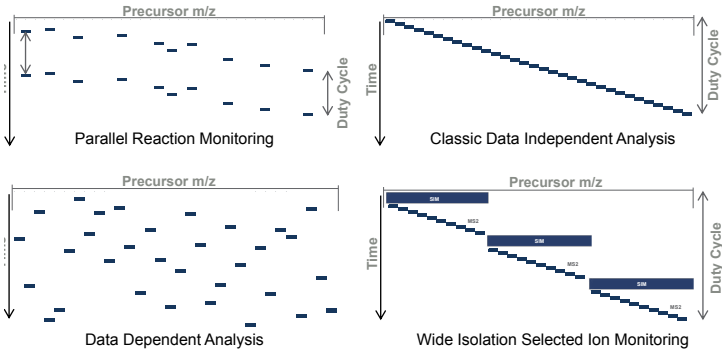

Figure 2: DDA, PRM and DIA strategies

A DDA scheme was used with an MS1 full scan resolution of 120,000 FWHM (at *m/z* 200) followed by as many subsequent MS2 scans on selected precursors as possible within a 3 second maximum cycle time. MS/MS was performed in the Orbitrap using HCD at a resolution of 30,000 FWHM. PRM analysis was performed using 0.7 u isolation window and MS2 scans on targeted precursors were analyzed by into the Orbitrap at a resolution of 60,000 FWHM. WiSIM experiment covered three 200 u wide SIM windows at 240,000 resolution with a range from 400 to 1000, in parallel to each SIM scan experiment, 17 sequential CID MS2 scans covering 12 u were performed in the ion trap. cDIA acquisition method followed using small (15 u) sequential MS2 windows at 30,000 resolution and an 120,000 resolution MS1 full scan. The range surveyed by these experiments was between *m/z* 400 and 1000 with a maximum injection time of 60 msec.

Data Analysis: Spectral .raw files from data dependent acquisition were analyzed using Thermo Scientific™ Proteome Discoverer™ 2.0 software with SEQUEST®HT search engines in order to generate the library for the DIA data analysis. Data was searched against human Uni-Prot® at a 1% spectrum level FDR criteria using Percolator (University of Washington). MS1 mass tolerance was constrained to 10 ppm. For Ion Trap MS2 data mass tolerance was limited to 0.6 Da, for Orbitrap MS2 data mass tolerance was limited to 0.02 Da. Heavy Lysine (13C(6) 15N(2), +8.014) and heaving Arginine ((13C(6) 15N(4), +10.008) were considered as variable modifications to retrieve the PRTC peptides. Additional variable modifications included Carbamidomethylation of Cysteine (+57.021) and/or oxidation of Methionine (+15.995). Label free quantification of the results was undertaken using Skyline 2.6 (University of Washington) and Spectronaut™ (Biognosys Proteomics Solutions).

Results

The acquisition of full MS scans at 240,000 FWHM resolution on the Orbitrap Fusion Lumos Tribrid MS using a WiSIM DIA approach enabled confident identification and quantification of PRTC peptides spiked into 1 µg of matrix down to 100 attomoles; as shown below in Figure 3 for the peptide ELASGLSFPVGFK. These results highlight the ability of the Orbitrap Fusion Lumos Tribrid to reach unprecedented LOQs in highly complex matrices. Lower LOQs will be further investigated to determine the minimal amount possible to accurately quantify using this strategy.

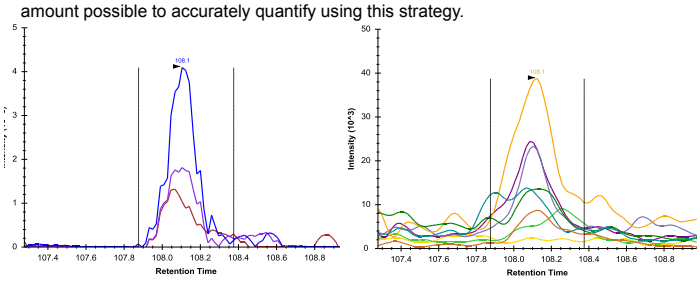

Figure 3: Detection of the synthetic peptide ELASGLSFPVGFK at 100 amol loaded on column in 1000 ng of human lung cancer cell digest by WiSIM. Left, reconstructed XICs of the monoisotopic, A+1 and A+2 peaks measured at 240,000 resolution with the Orbitrap. Right, reconstructed XICs of the measured transitions by rapid MS²-CID analyzed with the Linear Ion Trap for confident peptide validation.

Spectral libraries were created from DDA experiments in which 6501 protein groups were identified (1% FDR), subsequent cDIA analysis allowed for the quantification of 5964 proteins with a 6.9% median CV from only 500 ng of a HeLa digest (Pierce) loaded on column (Figure 4). Thus, the Orbitrap Fusion Lumos Tribrid MS was able to retrieve near 92% of the available proteins from the DDA library. A direct comparison with the Orbitrap Fusion Tribrid MS (Senko *et al*., 2013) showed the Orbitrap Fusion Lumos Tribrid MS was able to identify up to 30% more protein groups and unique peptides with more confidence (Figures 5 and 6) with only 100 ng or 500 ng of HeLa digest loaded on column.

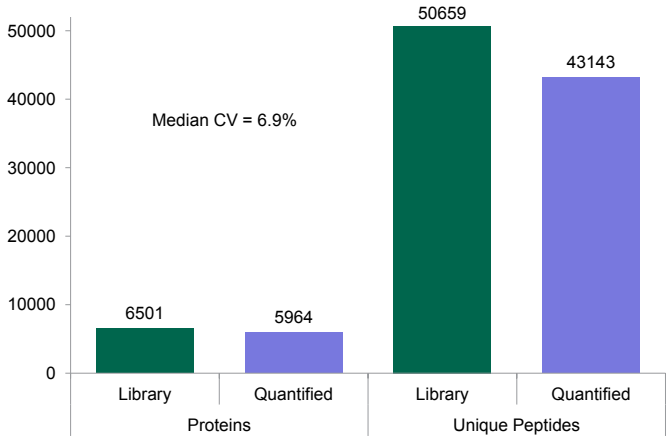

Figure 4: Number of identified HeLa protein groups and unique peptides quantified using the cDIA strategy with the Orbitrap Fusion Lumos Tribrid MS

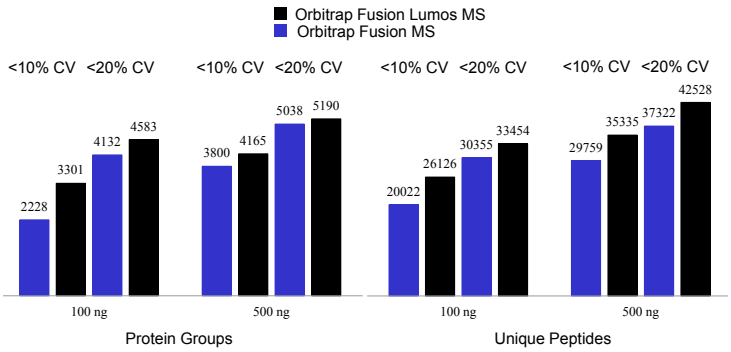

Figure 5: Number of confident identified protein groups (right) and unique peptides (left) with the Orbitrap Fusion Tribrid and the Orbitrap Fusion Lumos tribrid MS using the cDIA strategy. The CV<10% and CV<20% for both 100 ng and 500 ng of HeLa digest loads on column are shown here.

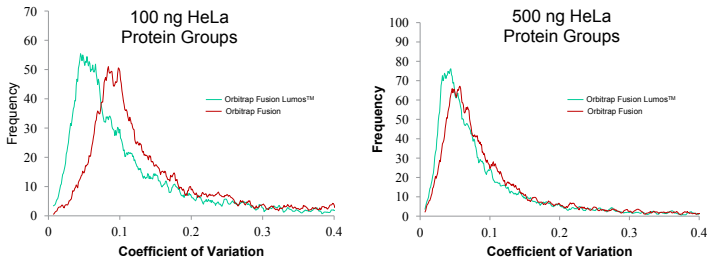

Figure 6: Coefficient of variation distribution (1=100%) comparisons between the Orbitrap Fusion Lumos tribrid MS and the Orbitrap Fusion Tribrid MS for both loads of HeLa digest on column 100 ng (Left) and 500 ng (right).

The Orbitrap Fusion Lumos Tribrid MS provided accurate LOQ down to 1 attomole for 5 PTRC peptide spiked into 200 ng of HeLa matrix and down to 5 attomoles for 5 other PRTC peptides with average CVs lower than 11% (Figure 7) while keeping excellent quantification linearity over 5 orders of magnitude of concentration (1 attomole to 100 femtomoles per microliter, Figure 8). Direct comparison using the PRM approach showed that Orbitrap Fusion Lumos Tribrid MS outperforms Orbitrap Fusion for confident quantification of the spiked PRTC peptides by detecting more fragment ions with better S/N as illustrated in Figures 9 and 10.

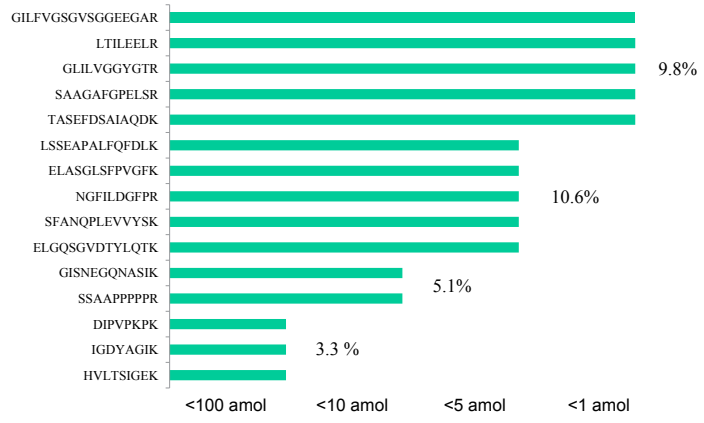

Figure 7: The Orbitrap Fusion Lumos Tribrid MS provides accurate quantitation of 15 PRTC peptides spiked into 200 ng of HeLa digest matrix, down to 1 attomole levels. Average CV% for each LOQ level is shown. Peptide quantitation is based on multiple fragment ions, as shown in Figures 9 and 10 for the peptide LSSEAPALFQFDLK.

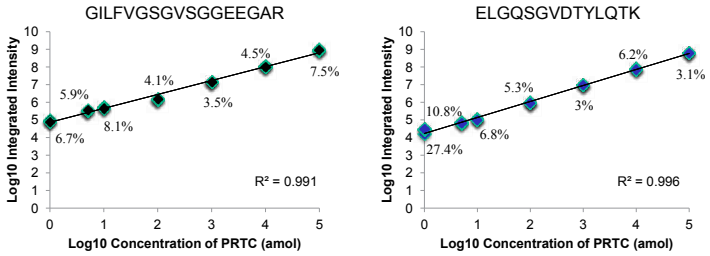

Figure 8: The Orbitrap Fusion Lumos Tribrid MS achieves excellent quantification linearity down to 1 attomol of spiked PRTC into the matrix as illustrated for the peptide GILFVGSGVSGGEEGAR and down to 5 amol for the peptide ELGQSGVDTYLO\QTK Coefficient variations (CV) are indicated for each spiked PRTC peptide concentration and measure the dispersion between four individual runs.

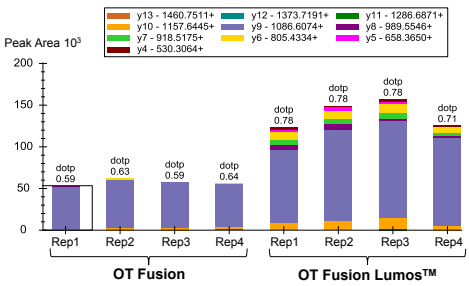

Figure 9: The Orbitrap Fusion Lumos Tribrid MS outperforms the Orbitrap Fusion MS for confident quantification of 5 attomoles of the peptide LSSEAPALFQFDLK spiked into 200 ng of HeLa matrix, detecting more fragment ions with better S/N.

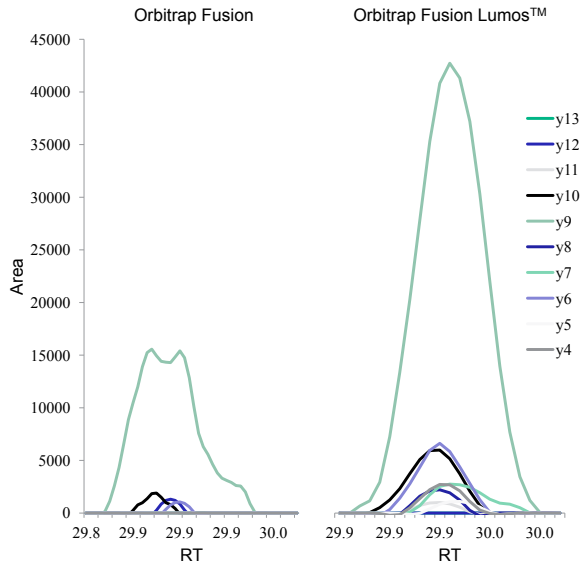

Figure 10: The Orbitrap Fusion Lumos Tribrid MS outperforms the Orbitrap Fusion MS for confident quantification of 5 attomoles of the peptide LSSEAPALFQFDLK spiked into 200 ng of HeLa matrix, detecting more fragment ions with better S/N. Here are shown the XICs of individual fragments for one replicate run per MS instrument.

Summary

- A single DDA analysis with 1 ug HeLa digest loaded on column resulted in the identification of over 5,300 protein group and 32,000 Unique Peptides (FDR<1%) with either the Linear Ion Trap or Orbitrap MS2 detection.
- The Orbitrap Fusion Lumos Tribrid MS using the WiSIM approach enables confident identification and quantification of 100 attomole PRTC peptides spiked into 1 ug of matrix with a 155 min gradient.
- cDIA analysis allowed for the confident identification and quantification of over 3,330 proteins (CV<10%) or 4500 proteins (CV<20%) with only 100 ng of HeLa digest loaded on column with a 130 min gradient, retrieving over 70% of the available protein groups from the DDA library.
- The Orbitrap Fusion Lumos Tribrid MS provided accurate LOQ lower than 5 attomoles for 10 PTRC peptide spiked into 200 ng of HeLa matrix with a gradient of only 28 min highlighting that the PRM method widely benefits from the brighter source and Advanced Quadrupole Technology of the new Tribrid system.

References

-Novel Parallelized Quadrupole/Linear Ion Trap/Orbitrap Tribrid Mass Spectrometer Improving Proteome Coverage and Peptide Identification Rates. Senko *et al.,* 2013, *Anal. Chem.*, *85* (24), pp 11710–11714

-Large Scale Targeted Protein Quantification Using WiSIM-DIA Workflow on an Orbitrap Fusion Tribrid Mass Spectrometer. Kiyonami *et al.,* 2014, AN64085

-Parallel Reaction Monitoring for High Resolution and High Mass Accuracy Quantitative, Targeted Proteomic. Peterson *et al.*, 2011, Molecular & Cellular Proteomics, 11, 1475-1488.

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