Large Scale Targeted Protein Quantification Using HR/AM Selected Ion Monitoring with MS/MS Confirmation on a Novel Hybrid, Q-OT-qIT Mass Spectrometer

Reiko Kiyonami,[1] Michael Senko,[1] Vlad Zabrouskov,[1] Jarrett Egertson,[2]
Sonia Ting,[2] Michael MacCoss,[2] Andreas FR Hühmer[1]
[1]Thermo Fisher Scientific, San Jose, CA, USA; [2]University of Washington, Seattle, WA, USA

Thermo SCIENTIFIC

Overview

Purpose: Develop a highly sensitive and highly selective data independent acquisition (DIA) workflow for large-scale targeted protein quantification on the new Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer.

Methods: For data independent acquisition set-up, three high-resolution, accurate-mass (HR/AM) selected ion monitoring (SIM) scans (240,000 FWHM) with wide isolation windows (200 amu) were used to cover all precursor ions of 400 – 1000 *m/z*. In parallel with each SIM scan, 17 sequential ion trap MS/MS with 12 amu isolation windows were acquired to cover the associated 200 amu SIM mass range. Quantitative information for all precursor ions detected in three sequential SIM scans is recorded in a single run. Plus, all MS/MS fragment information over the mass range of 400 – 1000 *m/z* is recorded for sequence confirmation of any peptide of interest by querying specific fragment ions in the spectral library. The quantitative performances and throughput of this new approach were evaluated using various samples.

Results: The data collected from SIM scans with high resolving power provided unambiguous detection of targeted peptide peaks even at low concentration levels by separating interferences from matrix from peptide associated signal. Ten (10) attomole limits of detection (LODs) were observed for the isotopically labeled standards spiked in 500 ng *E. coli* digest matrix. Four (4) orders of linear dynamic range was observed with good precision. Highly reproducible and complete quantitative results were achieved by applying a targeted data extraction strategy after the data independent acquisition.

Introduction

Proteomics studies are turning from qualitative to quantitative to understand the biological function and interactions of proteins in biological systems. The extreme complexity and dynamic range of proteins in typical samples challenges traditional data dependent workflows by requiring very fast MS/MS acquisition to reproducibly and deeply interrogate the sample. Recently, several data independent acquisition approaches[1,2] have been explored to increase reproducibility and comprehensiveness for better quantification. These approaches use targeted extracted fragment ions from HR/AM MS/MS data collected with a wide isolation window such as 25 Da for quantification. The quantitative performance could be potentially compromised by interfering fragments from co-eluted background compounds[3].

The Orbitrap Fusion Tribrid MS is based on a mass resolving quadrupole, Orbitrap analyzer, collision cell, linear ion trap (Q-OT-qIT) architecture (Figure 1). The Orbitrap detector can collect data with resolving power of 240,000 in less than 0.5 second. The linear ion trap can collect more than 20 CID MS/MS data in 1 second. The unique architecture enables parallel Orbitrap SIM and rapid targeted ion trap MS/MS detection for maximized duty cycle. We developed a new DIA workflow which collects HR/AM SIM and collision-induced dissociation (CID) MS/MS in parallel (Figure 2). The quantification of targeted proteins after data acquisition are carried out using HR/AM full MS SIM data and the simultaneous peptide sequence confirmations are carried out using CID MS/MS at the expected retention time relying on a spectral library (Figure 2). By using precursor ions from HR/AM SIM scans, which were collected with 240,000 resolving power, this new DIA approach can quantify most detected peptide peaks without any interferences from background, yielding a complete and reproducible quantitative data set with high sensitivity and selectivity. The detail of the DIA workflow, its quantitative performances and automatic data processing using Thermo Scientific™ Pinpoint™ software are reported.

Methods

Sample Preparation

Sample 1: A mixture of seven isotopically labeled yeast peptides were spiked into either BSA or *E. coli* digests (500 ng/µL) at five different concentrations (0.01 fmol/µL, 0.1 fmol/µL, 1 fmol/µL,10 fmol/µL and 100 fmol/µL).

Sample 2: A standard mixture of 6 protein digests which covers five orders of magnitude concentration (0.01 fmol/µL, 0.1 fmol/µL, 1 fmol/µL,10 fmol/µL, 100 fmol/µL and 1000 fmol/µL) was spiked into an *E. coli* digest (500 ng/µL).

Nano-LC

System:	Thermo Scientific™ EASY-nLC™ 1000
Column:	Thermo Scientific™ EASY-Spray™ PepMap C18 column (2 µm, 75 µm x 50 cm)
Flow rate:	300 nL/min; Buffer A: 0.1% FA/H2O; Buffer B: 0.1% FA/ACN;
Gradient:	5% B to 25% B in 100 min, 25% B to 35% B in 20min
Sample loading:	Directly loaded on column; Injection amount: 1µL

FIGURE 1. Instrument layout of Orbitrap Fusion MS

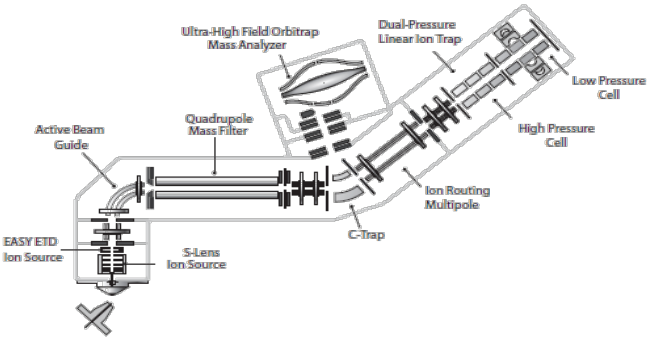
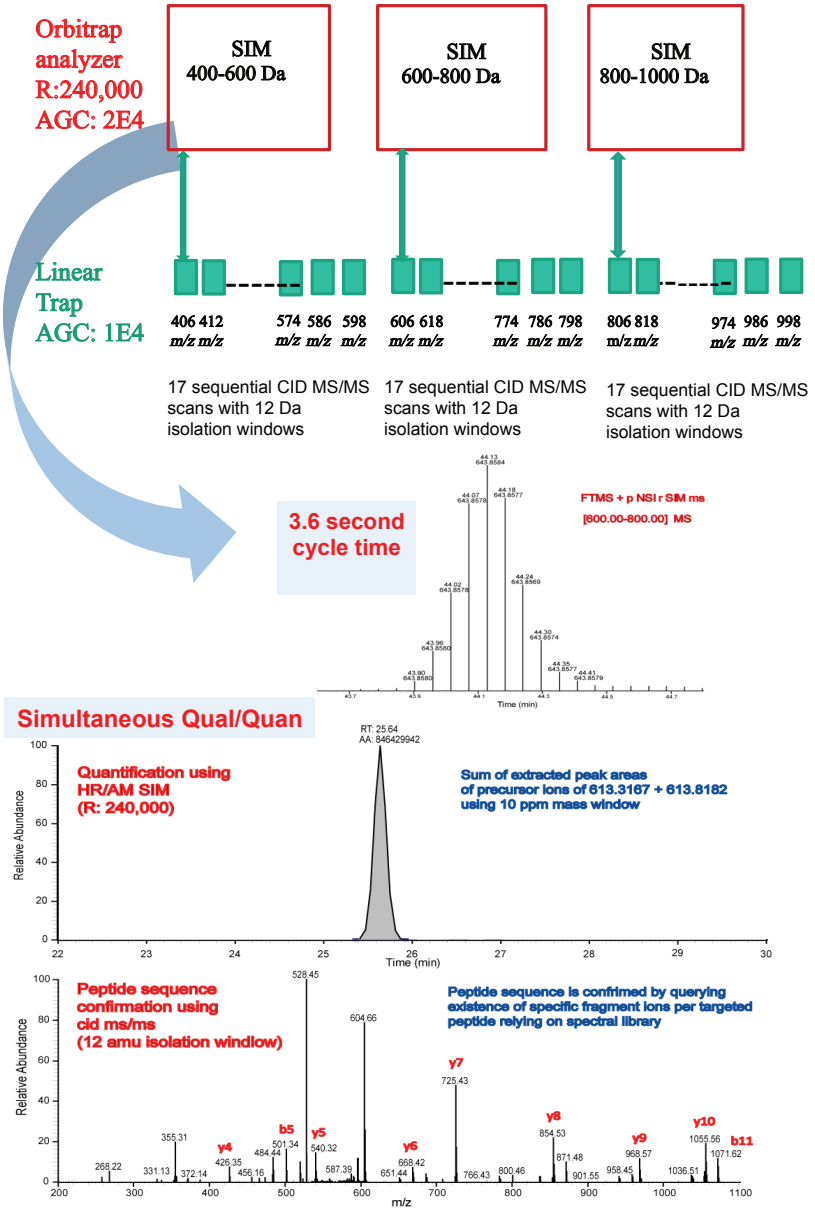


FIGURE 2. DIA workflow to collect HR/AM SIM at 240k resolution and 17 sequential CID MS/MS of 12 Da isolation width in parallel.



MS

Orbitrap Fusion MS equipped with a Thermo Scientific™ EASY-Spray™ source is used for all experiments. Capillary temperature: 275 ºC; Spray voltage: 1800 V.

FT SIM: Resolution: 240,000; AGC target : 2+E04; Isolation width: 200 amu. using trap for isolation.

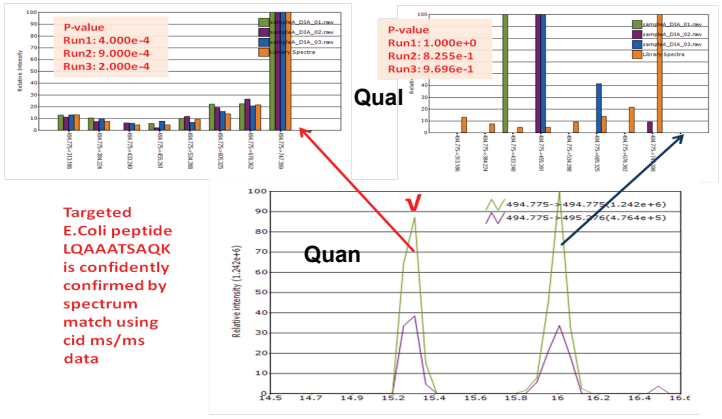
CID MS/MS: Rapid CID MS/MS, AGC target : 1+E04; Isolation width: 12 amu using Q for isolation.

Six scan events: Three SIM scan events (scan 1,3 and 5). Each SIM scan experiment is followed by one tMSn scan experiment which carries out 17 consecutive CID MS/MS events using predefined precursor ion inclusion list (Figure 2).

Data processing

Pinpoint 1.3 software is suitable for targeted qualitative and quantitative data extraction post-data acquisition. A spectral library is established using previous discovery data collected on Orbitrap Fusion MS. The XICs of isotope C^{12} and C^{13} precursor ions per targeted peptide are used for quantification with ± a 5 ppm window. Eight most intense fragment ions (b and y types) detected from discovery data are used for confirmation through spectral library match. A peptide with a P-value of less than 0.1 (as correlated with library spectra) is considered to be confirmed with high confidence by the spectral library match (Figure 3).

FIGURE 3. Simultaneous Qual/Quan using Pinpoint 1.3 software indicating low p-values and the correct target peptide at 15.3 min elution time in the presence of a similar contaminant at 16 min elution time.



Results

Evaluation of Detection Limits and Linear Dynamic Range of the DIA Workflow

The detection limits and the quantitative dynamic range of this DIA workflow were evaluated using a mixture of seven isotopically labeled yeast peptides spiked into *E. coli* digests (500 ng/µL) at five different concentrations (0.01 fmol/µL, 0.1 fmol/µL, 1 fmol/µL,10 fmol/µL and 100 fmol/µL).

Unlike full MS which isolates all precursor ions of the full mass range, the SIM scan with 200 amu isolation windows effectively "enriches" all precursor ions in that window while excluding all other ions outside the mass range of interest. This resulted in a lower limit of quantification in much the same way that selectively collecting peptides on a trapping column would. As a result, the SIM scan, even with a wide isolation window, can provide much higher sensitivity for low concentration peptides compared to full MS (Figure 4). Five spiked isotopically labeled peptides and BSA were detected at the lowest 10 attomol concentration level with four orders of linear dynamic range (Figure 5).

FIGURE 4. 10-fold increased sensitivity using SIM compared to using Full MS.

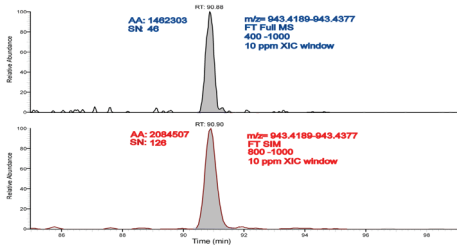
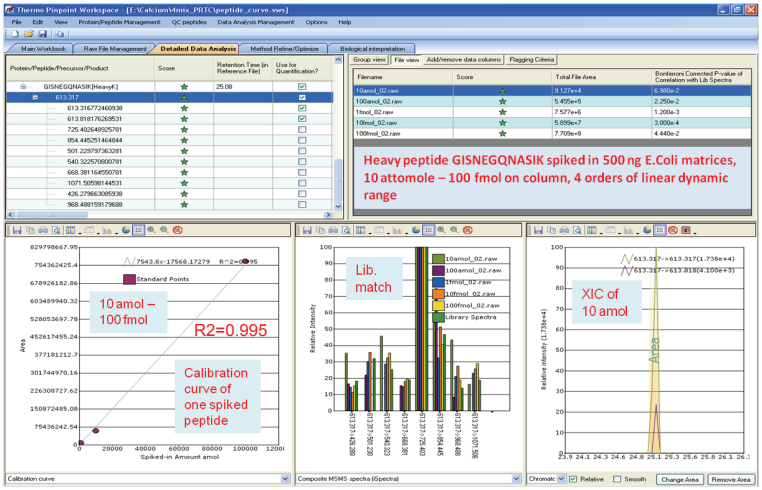


FIGURE 5. DIA workflow on the Orbitrap Fusion MS provided 10 attomole LOD with 4 orders of magnitude linear dynamic range.



Detecting and Quantifying Low-abundance and High-abundance Spiked Proteins with the DIA workflow in a Single Experiment

Six bovine protein digests were spiked into a 500 ng *E. coli* matrix at dynamic abundance levels. In the low-abundance sample, BSA was spiked at a level of 10 attomoles on column and in the high-abundance sample, beta-Lactoglobulin protein was added to achieve 1 pmol on column. The sample was run in triplicate using the developed DIA workflow. The HR/AM SIM provided high sensitivity, high selectivity and wide dynamic range to detect all six spiked proteins reproducibly over five orders of magnitude dynamic range. Table 1 summarizes the detected peptides per spiked protein and observed %CVs. Ninety percent of quantified peptides gave %CVs less than 10%.

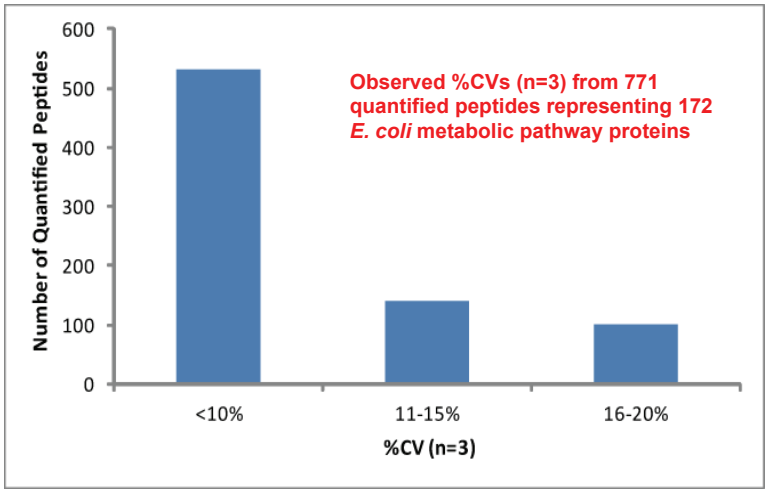
Table 1. Quantifying six bovine proteins spiked in 500 ng *E. coli* matrix at five orders of dynamic concentrations in a single experiment.

	Retention Time	CV% (n=3)	Run1	Run2	Run3
>sp|P02769|ALBU_BOVIN Serum albumin (10 attomole on column)					
HLVDEPQNLIK	51.06	22	8.40E+05	1.32E+06	1.47E+06
>sp|P02662|CASA1_BOVIN Alpha-S1-casein (100 attomole on column)					
FFVAPFPEVFGK	110.66	8	2.33E+06	2.39E+06	2.00E+06
>sp|P00366|DHE3_BOVIN Glutamate dehydrogenase (1 fmol on column)					
LQHGTILGFPK	56.78	21	5.47E+06	5.55E+06	3.37E+06
GASIVEDK	21.11	6	1.02E+07	8.98E+06	9.11E+06
MVEGFFDR	68.04	7	3.46E+06	3.07E+06	2.94E+06
RDDGSWEVIEGYR	67.64	28	1.91E+06	3.46E+06	2.03E+06
HGGTIPIVPTAEFQDR	68.8	1	3.38E+08	3.43E+08	3.43E+08
C[Carboxymethyl]AVVDVPFGG	73.73	14	1.63E+07	1.60E+07	1.17E+07
YSTDVSVDEVK	46.35	10	2.11E+07	1.70E+07	1.77E+07
>sp|P00921|CAH2_BOVIN Carbonic anhydrase (10 fmol on column)					
VLDALDSIK	65.52	4	2.39E+08	2.34E+08	2.15E+08
VGDANPALQK	23.66	7	1.89E+08	1.66E+08	1.64E+08
EPISVSSQQMLK	55.15	9	1.07E+07	8.92E+06	8.69E+06
AVVQDPALKPLALVYGEATSR	91.67	1	3.46E+07	3.54E+07	3.58E+07
YGDFGTAAQQPDGLAVVGVFI	113.84	10	3.60E+06	4.54E+06	4.26E+06
>sp|P80025|PERL_BOVIN Lactoperoxidase (100 fmol on column)					
SPALGAANR	20.17	3	1.05E+09	9.81E+08	9.94E+08
LFQPTHK	19.72	11	5.30E+08	4.64E+08	4.09E+08
DGGIDPLVR	58.98	5	1.18E+09	1.09E+09	1.05E+09
IHGFDLAAINLQR	79.44	3	1.14E+09	1.13E+09	1.06E+09
GFC[Carboxymethyl]GLSQPK	49.46	9	1.57E+09	1.32E+09	1.29E+09
RSPALGAANR	14.27	13	5.65E+08	5.66E+08	4.24E+08
IVGYLDEEGVLDQNR	76.67	1	3.49E+08	3.49E+08	3.39E+08
VPC[Carboxymethyl]FLAGDFR	88.27	6	1.73E+09	1.88E+09	1.63E+09
LIC[Carboxymethyl]DNTHITK	33.78	3	3.56E+08	3.78E+08	3.56E+08
DYLPIVLGSEMQK	94.67	7	1.11E+08	1.24E+08	1.32E+08
FWWENPGVFTEK	102.01	5	4.59E+08	4.84E+08	4.24E+08
AGFYC[Carboxymethyl]PTPPY	77.27	3	2.22E+09	2.24E+09	2.08E+09
>sp|P02754|LACB_BOVIN Beta-lactoglobulin (1 pmol on column)					
IDALNENK	25.32	11	9.15E+09	8.75E+09	6.98E+09
TPEVDDEALEKFDK	59.56	5	2.17E+10	2.13E+10	1.95E+10
VLVLDTDYKK	46.5	4	1.29E+09	1.20E+09	1.16E+09
TPEVDDEALEK	41.33	6	4.28E+10	3.72E+10	3.76E+10
TPEVDDEALEKFDK	59.77	5	2.17E+10	2.13E+10	1.95E+10
WENGEC[Carboxymethyl]AQK	22.78	5	3.64E+08	3.30E+08	3.30E+08
LSFNPTQLEEQC[Carboxymeth	89.46	2	4.13E+10	4.16E+10	4.00E+10

Detecting and Quantifying a Large Number of Proteins of Interest with the DIA Workflow in a Single DIA Experiment.

The same DIA data files acquired in triplicate to quantify six spiked bovine proteins were used to additionally quantify 172 *E. coli* proteins which are involved with metabolic pathway based on annotation results by Thermo Scientific™ Protein Center™ software. 771 peptides were quantified to represent these 172 proteins. Excellent analytical precision was observed (Figure 6). 69% of quantified peptides gave %CVs less than 10%. 88% of quantified peptides gave %CVs less than 15%. All quantified peptides gave CVs less than 20%.

FIGURE 6. Excellent analytical precision while quantifying large number of targeted proteins using the HR/AM SIM with the DIA workflow.



Conclusion

- A unique data independent acquisition workflow, which collects HR/AM SIM and rapid CID MS/MS in parallel on the new Orbitrap Fusion MS was developed. Any detected precursor ions in the HR/AM SIM can be quantified using XIC with ± 5 ppm window and simultaneously confirmed using CID MS/MS by applying a targeted data extraction approach post data acquisition.
- By using precursor ions collected on SIM with extremely high resolving power of 240,000 for quantification, high sensitivity and high selectivity are achieved through separation of most background interferences from analyte signal. Ten (10) attomole on column LOD and 4 orders of linear dynamic range are observed with the developed DIA workflow.
- Over 5 orders of magnitude protein abundance of six proteins were detected and quantified with good analytical precision in a single experiment.
- 771 peptides representing 172 *E. coli* metabolic pathway proteins were also detected and quantified in the same experiment with good precision and accuracy.

References

1. Gillet, L.C.; Navarro, P.; Tate, S.; Roest, H.; Selevsek, N.; Reiter, L.; Bonner, R.; Aebersold, R., Targeted dataextraction of the MS/MS spectra generated by data independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol Cell Proteomics, 2012, as Manuscript O111.016717.*
2. Egertson,J.D.; Kuehn, A.; Merrihew, G.; Bateman, N.; MacLean, B.; Ting, Y. S.; Canterbury, J. D.; Kellmann,M.; Zabrouskov, V.; Wu, C.; MacCoss, M. J., Multiplexed Data Independent Acquisition for Comparative Proteomics. *ASMS poster, 2012*.
3. Gallien. S.; Duriez. E.; Demeure. K.; Domon. B., Selectivity of LC-MS/MS analysis: Implication for proteomics experiments. *Journal of Proteomics, Volume 81, 9 April 2013*, *Pages 148–158.*

www.thermoscientific.com

Thermo Fisher Scientific, San Jose, CA USA is ISO 9001:2008 Certified.

Africa-Other +27 11 570 1840
Australia +61 3 9757 4300
Austria +43 1 333 50 34 0
Belgium +32 53 73 42 41
Canada +1 800 530 8447
China +86 10 8419 3588
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland/Norway/Sweden +46 8 556 468 00
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9434
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
Russia/CIS +43 1 333 50 34 0
South Africa +27 11 570 1840
Spain +34 914 845 965
Switzerland +41 61 716 77 00
UK +44 1442 233555
USA +1 800 532 4752

Thermo SCIENTIFIC

Part of Thermo Fisher Scientific

ASMS13_W490_RKiyonami_E 06/13S