

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

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## 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<sup>1,2</sup> 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<sup>3</sup>.

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/H<sub>2</sub>O; 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: 1 μL

A schematic diagram of the EASY-MS instrument layout. The diagram shows the path of ions from the ion source through various stages of mass analysis. Key components labeled include: EASY ETD Ion Source, S-Lens Ion Source, Active Beam Guide, Quadrupole Mass Filter, C-Trap, Ion Routing Multipole, Dual-Pressure Linear Ion Trap, Ultra-High Field Orbitrap Mass Analyzer, High Pressure Cell, and Low Pressure Cell. The layout is a complex, interconnected system of tubes and traps.

**Orbitrap analyzer**  
R:240,000  
AGC: 2E4

**SIM 400-600 Da**

**SIM 600-800 Da**

**SIM 800-1000 Da**

**Linear Trap**  
AGC: 1E4

406 412  $m/z$   $m/z$  574 586 598  $m/z$   $m/z$   $m/z$  606 618  $m/z$   $m/z$  774 786 798  $m/z$   $m/z$   $m/z$  806 818  $m/z$   $m/z$  974 986 998  $m/z$   $m/z$   $m/z$

17 sequential CID MS/MS scans with 12 Da isolation windows

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**3.6 second cycle time**

**Simultaneous Qual/Quan**

RT: 25.64  
AA: 846425942

**Quantification using HR/AM SIM (R: 240,000)**

**Sum of extracted peak areas of precursor ions of 613.3167 + 613.8182 using 10 ppm mass window**

**Peptide sequence confirmation using cid ms/ms (12 amu isolation window)**

**Peptide sequence is confirmed by querying existence of specific fragment ions per targeted peptide relying on spectral library**

Relative Abundance

Time (min)

Relative Abundance

$m/z$

**y4** 355.31 **b5** 426.35 **y5** 484.24 **y6** 501.34 **y7** 540.32 **y8** 587.38 **y9** 604.66 **y10** 651.44 **b11** 668.42 **y7** 725.43 **y8** 854.53 **y9** 871.48 **y10** 901.55 **b11** 958.45 **y10** 1055.56 **b11** 1071.62

## MS

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

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

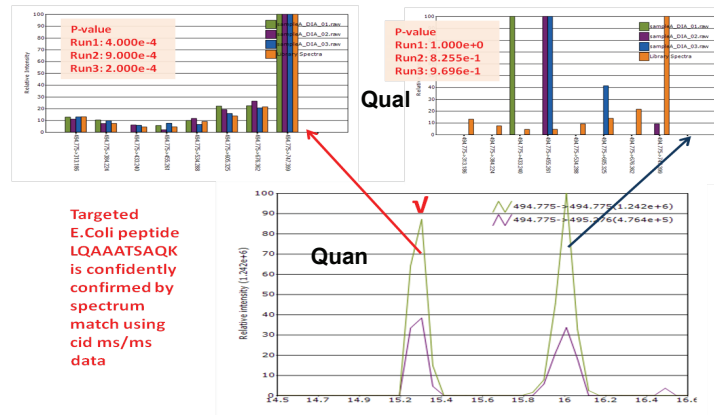
CID MS/MS: Rapid CID MS/MS, AGC target : 1+E04; Isolation width: 12 amu using Q for isolation.

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<sup>12</sup> and C<sup>13</sup> precursor ions per targeted peptide are used for quantification with  $\pm 5$  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.**



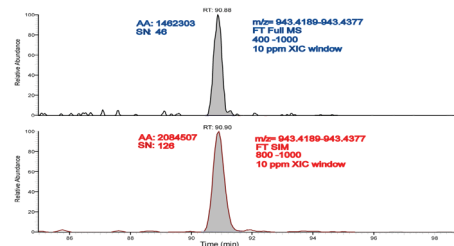
## Results

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

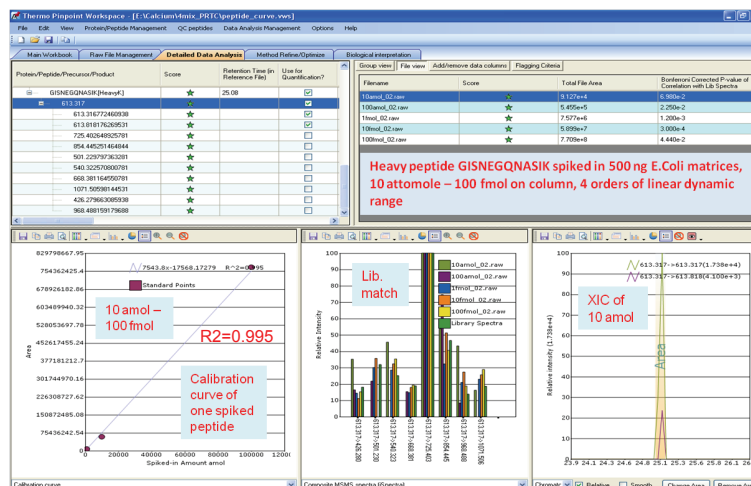
The detection limits and the quantitative dynamic range of this DIA workflow were evaluated using a mixture of seven isotopically labeled yeast peptides spiked into *E. coli* digests (500 ng/μL) at five different concentrations (0.01 fmol/μL, 0.1 fmol/μL, 1 fmol/μL, 10 fmol/μL and 100 fmol/μL).

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%.

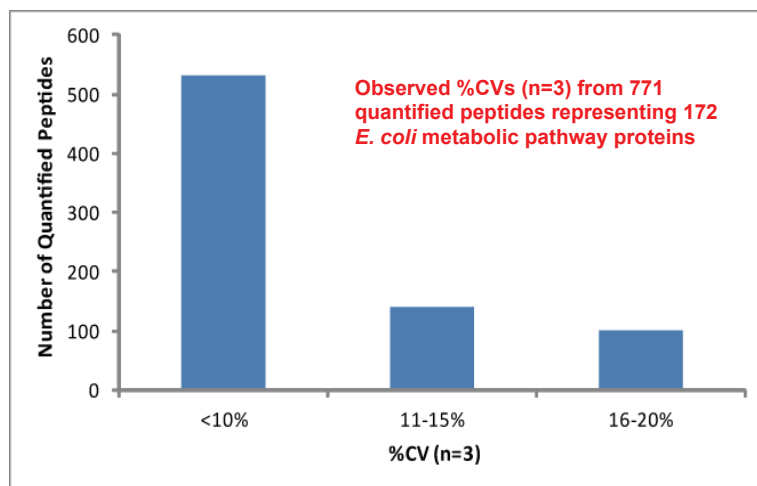
**Table 1. Quantifying six bovine proteins spiked in 500 ng *E. coli* matrix at five orders of dynamic concentrations in a single experiment.**

	Retention Time	CV% (n=3)	Run1	Run2	Run3
<b>&gt;spi02769IALBU_BOVIN Serum albumin (10 attomole on column)</b>					
HLVDEPGLNIK	51.06	22	8.40E-05	1.32E+06	1.47E+06
<b>&gt;spi02662ICASA1_BOVIN Alpha-S1-casein (100 attomole on column)</b>					
FFVAPFPEVFGK	110.66	8	2.33E+06	2.39E+06	2.00E+06
<b>&gt;spi00366IDHE3_BOVIN Glutamate dehydrogenase (1 fmol on column)</b>					
LQHGTLGFFPK	56.78	21	5.47E+06	5.55E+06	3.37E+06
QASVEDK	21.11	6	1.02E-07	9.30E+06	9.19E+06
MVEGFDFR	68.04	7	3.46E+06	3.07E+06	2.94E+06
RDDGSWEIEGYR	67.64	28	1.91E+06	3.46E+06	2.03E+06
HGGTIPIVPTAEFGDR	68.8	1	3.38E+06	3.43E+06	3.43E+06
C[Carboxymethyl]AVVDVFFGG	73.73	14	1.63E+07	1.60E+07	1.17E+07
YSTDVSDEK	46.35	10	2.18E+07	1.70E+07	1.77E+07
<b>&gt;spi00921ICAH2_BOVIN Carbonic anhydrase (10 fmol on column)</b>					
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
EPISVSSQMLK	55.15	9	1.07E+07	8.92E+06	8.69E+06
AVVDQFALKPLALYYGEATSR	91.67	1	3.46E+07	3.54E+07	3.58E+07
VGDGTAAGQFDGLAVGVFI	113.84	10	3.50E+06	4.54E+06	4.26E+06
<b>&gt;spi00025IPERL_BOVIN Lactoperoxidase (100 fmol on column)</b>					
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
IHGFDLAINLQK	73.44	3	1.14E+09	1.13E+09	1.06E+09
GFCC[Carboxymethyl]GLSQPK	49.46	9	1.57E+09	1.32E+09	1.23E+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]DNTHIK	33.78	3	3.56E+08	3.78E+08	3.56E+08
DYLPIVLGSEMGK	94.67	7	1.11E+08	1.24E+08	1.32E+08
FVVENPVGVFTEK	102.01	5	4.59E+08	4.84E+08	4.24E+08
AGFVC[Carboxymethyl]PTFPY	77.27	3	2.22E+09	2.24E+09	2.08E+09
<b>&gt;spi02754ILACB_BOVIN Beta-lactoglobulin (1 pmol on column)</b>					
IDALNENK	25.32	11	9.15E+09	8.75E+09	6.98E+09
TPFVDDEALEKFDK	53.86	5	2.17E+10	2.13E+10	1.95E+10
VLVLDTOYKK	46.5	4	1.29E+09	1.20E+09	1.16E+09
TPFVDDEALEK	41.33	6	4.28E+10	3.72E+10	3.76E+10
TPFVDDEALEKFDK	59.77	5	2.17E+10	2.13E+10	1.95E+10
VENGECC[Carboxymethyl]AQK	22.78	5	3.64E+08	3.30E+08	3.30E+08
LSFNPTGLEEQCC[Carboxymethyl]	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  $\pm 5$  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

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