Improved Throughput and Reproducibility for Targeted Protein Quantification Using a New High-Performance Triple Quadrupole Mass Spectrometer

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Overview

Purpose: Demonstrate increased sensitivity, throughput, and reproducibility for targeted protein quantification in biologically complex samples using a new triple stage quadrupole instrument and Thermo Scientific™ Pinpoint™ 1.3 software.

Methods: Thermo Scientific™ TSQ Quantiva™ triple stage quadrupole mass spectrometer equipped with a Thermo Scientific™ EASY-Spray™ source were used for all experiments. A dilution series containing enzymatic digest mixtures of BSA and Enolase spiked in *E. coli* digest matrices were used to evaluate the improved quantitative performances and reproducibility of the new instrument. A separate sample set was used to evaluate the quantitative accuracy while applying the new triple stage quadrupole instrument to more complex samples. Pinpoint 1.3 software was used for rapid selected reaction monitoring (SRM) assay development based on discovery data and data processing.

Results: The new instrument design improved SRM assay sensitivity, reproducibility, and linear dynamic range significantly. Both BSA and Enolase were detected at 1 attomol on column with excellent reproducibility. Five orders of linear dynamic range was observed.

Pinpoint software allowed rapid SRM assay development and refinement for targeted proteins. Pinpoint software also allowed automatic data processing for quantifying protein expression ratios between two different sample groups with good precision.

Introduction

The triple stage quadrupole instrument-based SRM method has emerged as the method of choice for targeted protein quantification. In order to accurately quantify proteins of interest from complex biological samples in which the concentration of targeted proteins could change over six-orders of magnitude, high sensitivity and wide dynamic range are required. In most cases, sample-specific peptide standards that can be used for SRM assay development may not be available. It is critical to have software that enables rapid SRM assay development utilizing previous discovery data.

Here we report that a new triple stage quadrupole instrument was able to push detection limits of targeted proteins down to the 1-attomol level with five orders of linear dynamic range. We also demonstrate that an optimized SRM assay to quantify 12 proteins from *E. coli* complex matrices could be developed in a few hours using Pinpoint software relying on data previously acquired in the discovery phase of the project.

Methods

Sample Preparation

Sample Set 1: A sample dilution series that spiked BSA and Enolase digest mixture into 10 ng *E. coli* digest matrix at six different concentration levels (0.0005 fmol/µl, 0.005 fmol/µl, 0.05 fmol/µl, 0.5 fmol/µl, 5 fmol/µl, 50 fmol/µl) were prepared.

Sample Set 2: Two *E. coli* digest complex samples (A and D) that contain 12 protein standards with different concentration ratios were prepared. Sample A was prepared by spiking 12 protein standard digests into 500 ng *E. coli* matrices at a static concentration level of 5 fmol/µl. Sample D was prepared by spiking 12 protein standard digests into 500 ng *E. coli* matrix at three different concentration levels of 0.5 fmol/µl, 5 fmol/µl and 50 fmol/µl.

Nano-LC:

System:	Thermo Scientific™ EASY-nLC™ 1000 liquid chromatograph
Separation Column:	EASY-Spray column (50 µm ID × 15 cm)
MA:	0.1% FA-H2O
MB:	0.1%FA-MeCN
Gradient:	5%B – 45%B in 40 min
Flow rate:	300 nL/min
Injection amount:	2 µl

Mass Spectrometry:

Instrument:	TSQ Quantiva MS with an EASY-Spray nano source
Spray voltage:	1800 V
Sheath gas:	0
Auxiliary gas:	0
Capillary T:	275 °C
Collision gas:	Ar, 1.5 mTorr
Q1 and Q3 resolution:	0.7 Da
Cycle time:	1.5 sec; CE=0.03 × m/z of precursor ion + 6.0
SRM:	Non-scheduled for initial SRM assay development Scheduled SRM with 2 min scheduled time window for the refined SRM assay

Data Analysis:

Thermo Scientific™ Xcalibur™ 2.2 and Pinpoint 1.3 software were used for all data processing.

Results

Quantitative Performances Acquired From the BSA-Enolase Dilution Series

By implementing a novel ion source and optics with active ion management (AIM) technology with a 10% imbalance in the RF amplitude applied to the hyperbolic quadrupoles, the new instrument improved detection sensitivity significantly. Both BSA and Enolase peptides were clearly detected at 10 attomol levels on column in 10 ng of complex *E. coli* matrix (Figure 1). In combination with the innovative EASY-Spray nano source, the signal intensity variations from run-to-run were very small and the observed percentage of coefficient of variation was less than 7%, even at low attomol concentration levels (Figure 2). With a completely redesigned ion detection system, the dynamic range of the new instrument has also been improved significantly. Figure 3 shows that the calibration curve generated from the BSA peptide of AEFVEVTK for which five orders of linear dynamic range was observed.

FIGURE 1. Extracted chromatograms of BSA peptide of LVNELTEFAK and Enolase peptide of NVNDVIAPAFVK from the dilution series (0.0005 fmol/µl)

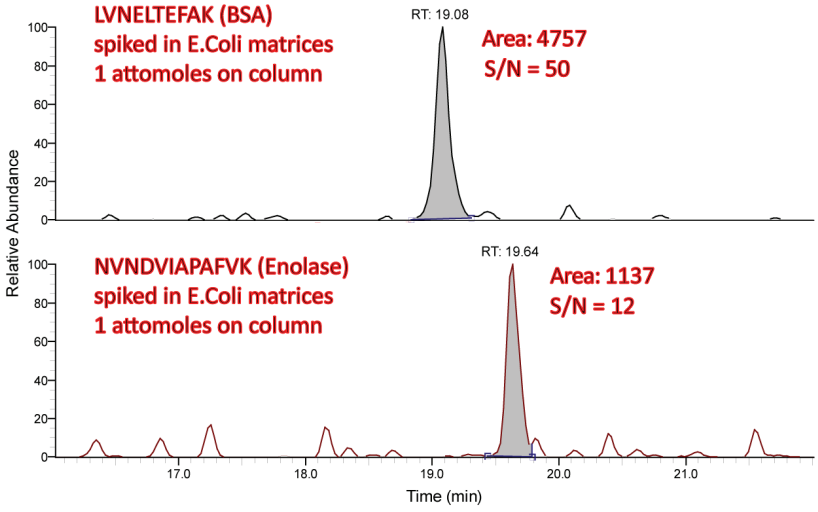


FIGURE 2. The %CV for peptide LVNELTEFAK from BSA and for peptide NVNDVIAPAFVK from Enolase for the low attomol injections of the dilution series

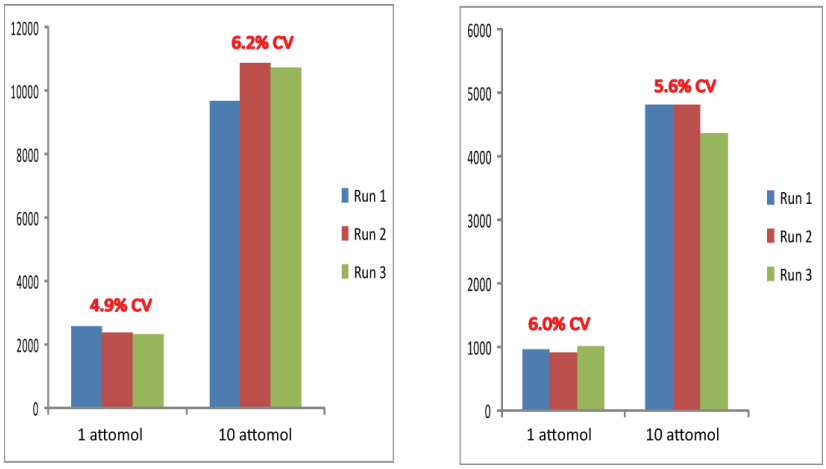
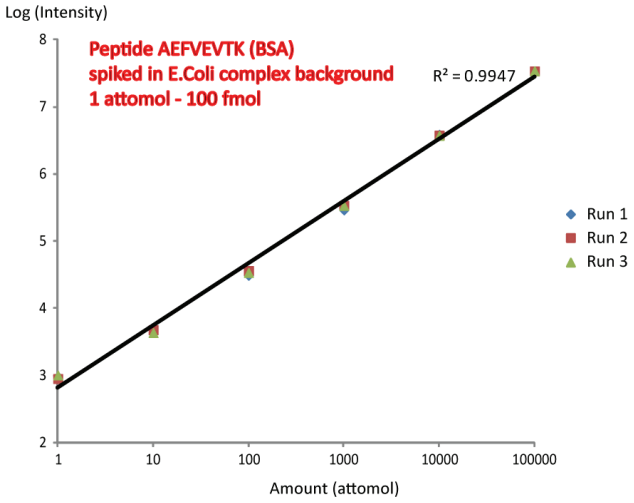


FIGURE 3. Calibration curve of spiked BSA peptide LVNELTEFAK. Over 5-orders of linear dynamic range was observed.



Rapid SRM Assay Development and Refinement Using Pinpoint Software

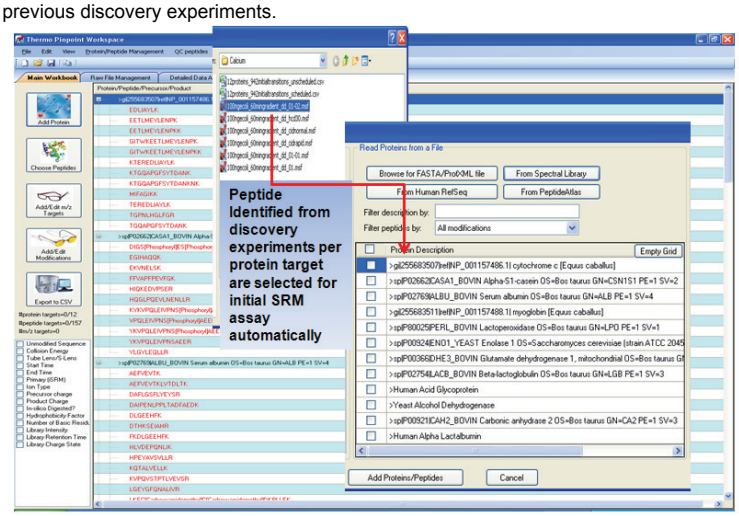
The new triple stage quadrupole instrument was applied to determine the differential ratios of 12 proteins spiked in 500 ng of complex *E. coli* matrices for sample A and sample D. The SRM assay development and refinement were carried on using Pinpoint 1.3 software and previous 12 protein identification results. Figure 4 shows the steps to develop the initial SRM assay relying on the discovery data and refine the final SRM assay using the data from the initial SRM assay. Only two HPLC MS/MS runs were required to generate the final SRM assay for quantifying the 12 protein targets from both samples.

The optimized final SRM assay was used for both sample A and sample D. Each sample was run in triplicate. Pinpoint software was used to process all the data and calculate the group ratios between sample A and sample D and the %CV of each targeted peptides (Figure 5).

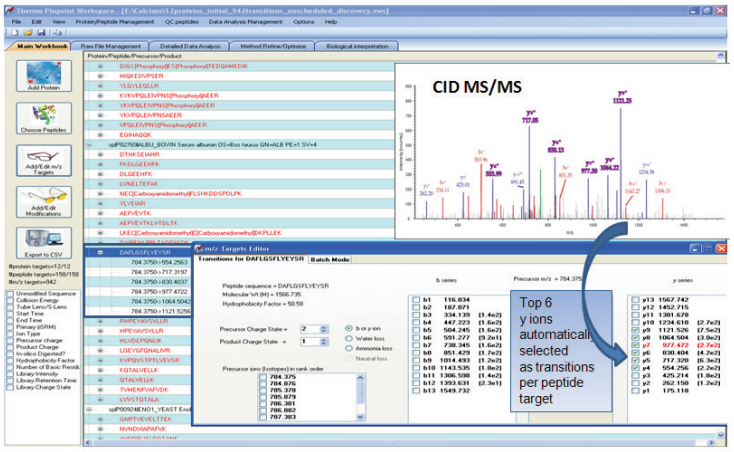
The calculated differential group ratios of each targeted protein between sample A and sample D are shown in Figure 6. The observed differential ratios are consistent with the expected ones. The calculated %CVs were all less than 15% (Figure 6).

FIGURE 4. SRM development workflow using Pinpoint 1.3 software

Step 1: Develop initial SRM assay by selecting all identified peptides from the previous discovery experiments.



Step 2: Generating the initial SRM assay by selecting top six detected y-type fragment ions for each peptide from the discovery data. The new instrument allows 500 SRM transitions per second, providing rapid initial SRM assay development by running 1000 unscheduled transitions in a single HPLC run.



Step 3: Refinement of SRM assay by automatically selecting up to top 5 detected peptides per protein from the initial run. All transitions are scheduled automatically for the refined SRM assay.

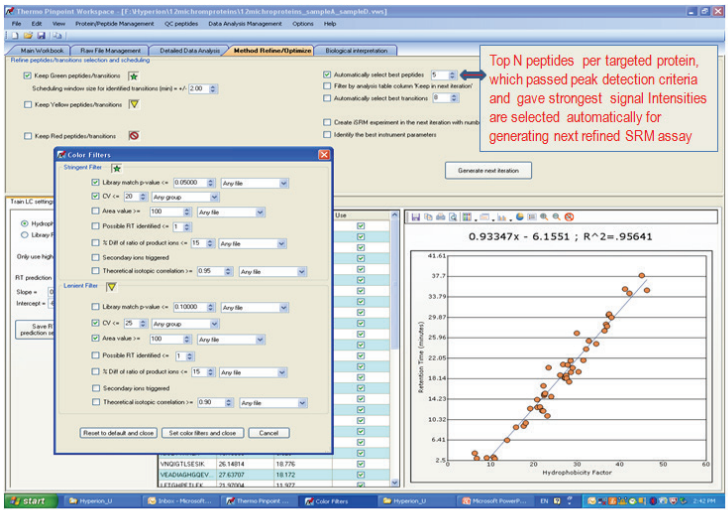


FIGURE 5. Automatic data processing using Pinpoint 1.3 software

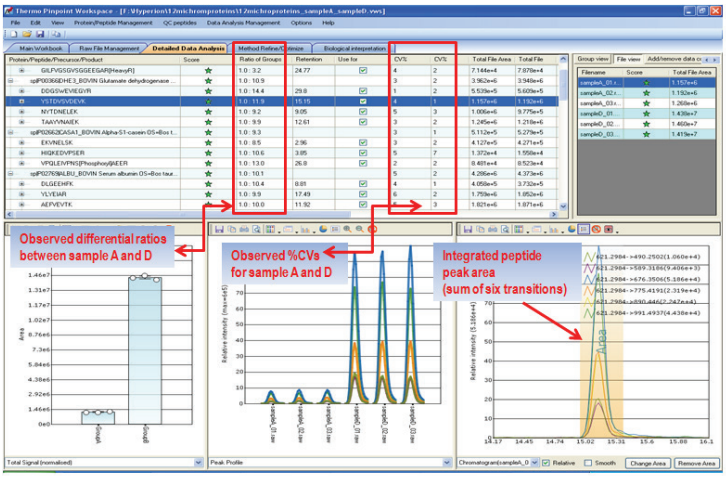
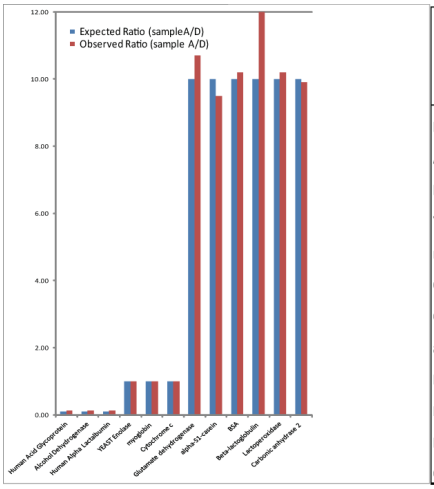


FIGURE 6. Observed differential ratio of each targeted protein between samples A and D. The observed differential ratios are in good match with the expected ratios.



Protein Target	Expected Ratio (sampleA/D)	Observed Ratio (sample A/D)
Human Acid Glycoprotein	0.10	0.12
Alcohol Dehydrogenase	0.10	0.12
Human Alpha Lactalbumin	0.10	0.12
YEAST Enolase	1.0	1.0
myoglobin	1.0	1.0
Cytochrome c	1.0	1.0
Glutamate dehydrogenase	10.0	10.7
alpha-S1-casein	10.0	9.5
BSA	10.0	10.2
Beta-lactoglobulin	10.0	12.2
Lactoperoxidase	10.0	10.2
Carbonic anhydrase 2	10.0	9.9

Conclusion

- Low attomol LOD/LOQ of targeted proteins in complex biological samples was achieved with a new triple stage quadrupole instrument demonstrating ultimate sensitivity for peptide quantification.
- Excellent reproducibility with %CV better than 6.5 was observed even at low attomol concentration levels.
- In excess of five-orders of linear dynamic range was observed for a peptide in the presence of a complex matrix.
- In combination of Pinpoint 1.3 software and fast SRM scan speed of the new triple stage quadrupole instrument, an optimized SRM assay to quantify 12 proteins in complex *E. coli* matrix was developed in a few hours requiring only two iterative experiments.
- Precise relative quantification results were observed using a rapidly developed SRM assay in four iterations of optimization using Pinpoint software.

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