High-Precision Quantitative Proteomics Using Tandem Mass Tags on an LTQ Orbitrap Velos Hybrid MS

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Overview

Purpose: Evaluate the performance of a new hybrid linear ion trap - Orbitrap mass spectrometer featuring a unique dual-pressure ion trap configuration and improved HCD collision cell for relative quantification of TMT[6]-labeled peptides.

Methods: An enzymatic digest of reduced and alkylated *E. coli* and MCF-7 cells proteins were labeled with *m/z* 126, 127, 128, 129, 130, and 131 TMT tags, and analyzed by LC-MS/MS.

Results: The changes to the ion trap and HCD collision cell translated to significant improvements in the sensitivity of the HCD analysis. From the identified peptides, over 80% were quantifiable at all investigated sample amounts, including 40 ng injections. More than 80% of the quantified peptides had less than 15% RSD.

Introduction

Isobaric mass tagging techniques such as Tandem Mass Tags® (TMT®) [1] and iTRAQ® [2,3] are well established as effective approaches for analysis of protein expression[4]. Isobaric tags allow for accurate and precise relative quantitation of peptides in multiple samples simultaneously[1,4]. Higher-energy collisional dissociation (HCD) has previously proved to be very effective for mass analysis of isobarically-labeled peptides[5]. In this report we evaluated the performance of a new hybrid ion trap/Orbitrap mass spectrometer featuring a unique dual-pressure ion trap configuration and improved HCD collision cell[6]. Together, they provided robust and sensitive relative quantification of TMT- and iTRAQ-labeled peptides.

Methods

Sample Preparation

An enzymatic digest of reduced and alkylated *E. coli* proteins was divided into six aliquots, labeled with *m/z* 126 - 131 TMT tags according to the manufacturer's protocol and mixed in 1:1 ratios. Human breast cancer MCF-7 cells were treated with 17β-estradiol (E2) for 30 min or 60 min before they were harvested and lysed. The lysates were then precipitated with acetone, digested, and individually labeled with TMT reagents as follows: control (126, 129), 30 min (127, 130), and 1 hr (128, 131).

LC/MS

Experiments were performed on a Thermo Scientific LTQ Orbitrap Velos mass spectrometer with a nano-ESI source that was coupled to a Thermo Scientific Surveyor MS Pump with a flow splitter. For LC-MS/MS analysis, protein digests were separated on a Magic C_{18}, 15 cm or 20 cm x 75 µm i.d. column (New Objective, Woburn, MA). Gradient elution was performed from 5%-40% acetonitrile in 0.1% formic acid over 180 min at a flow rate of ~300 nL/min.

Data Processing

Thermo Scientific Proteome Discoverer software (version 1.1) with a Mascot™ search engine and Swiss-Prot database was used for data analysis. Figure 1 shows the TMT quantification data analysis workflow set up in Proteome Discoverer™ 1.1 software.

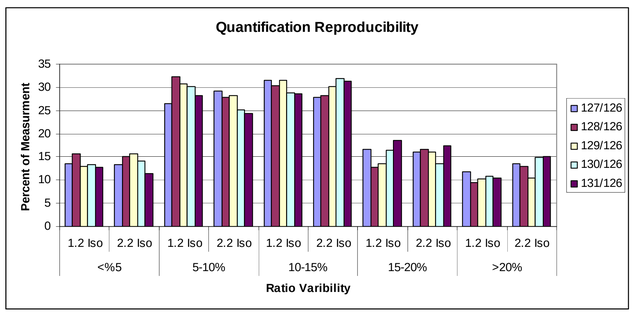
Results

Systematic evaluation of HCD instrumental parameters such as normalized collision energy settings, isolation width windows, and number of microscans was performed to optimize reporter-based relative quantification workflow on the LTQ Orbitrap Velos™ mass spectrometer. TMT[6]-labeled *E. coli* cell lysate digest was used as a sample.

HCD Parameters Optimization

Introduction of an integrated C-trap/HCD collision cell combination[6] with the LTQ Velos Orbitrap mass spectrometer enables rapid extraction of all ions from the HCD cell and allows increased speed and sensitivity of the instrument in HCD mode. As a result, the recommended target values for peptide fragmentation in HCD mode are reduced from 50,000-200,000 in the Thermo Scientific LTQ Orbitrap XL hybrid MS to only 20,000-50,000 for detection in the LTQ Orbitrap Velos instrument.

FIGURE 1. Effect of precursor isolation width on quantification reproducibility and protein identification.

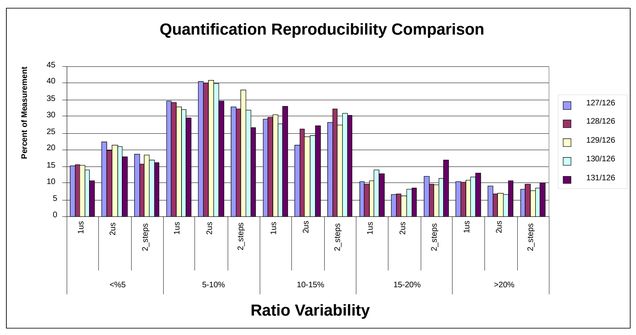


Isolation Window	Proteins	Peptides	% Quan Pep	Unique Pep
2.2	680	7267	80	4769
1.2	691	7229	77	4819

All experiments presented in this study were conducted at a 50,000 AGC target. For reporter-based quantification, precursor co-isolation will have a major effect on quantification accuracy and precision. As shown in Figure 1, a 1.2 amu (the narrowest isolation width to cover a complete isotope envelope for charge 2+ precursors) isolation window provides better quantification precision. Approximately 90% of the quantified peptides have less than 20% ratio variability at this isolation width. Alternatively, a wider isolation window (2.2 amu) yielded slightly more quantifiable peptides but fewer protein and unique peptide identifications. Overall, more consistently accurate results were obtained with a 1.2 amu isolation width. Collision energy in the HCD experiments is automatically adjusted (normalized) based on the parent ion *m/z* and charge state. To determine the optimal HCD fragmentation conditions, three different normalized collision energies (NCE), 40%, 45%, and two-step NCE values (40%-50%), were evaluated (Figure 2).

FIGURE 2. Comparison of identification and quantification results using different numbers of microscans and CE settings.

HCD settings	Proteins	Peptides	% Quan Peptides	Unique Peptides
1 µs, 40% CE	691	7229	77	4819
2 µs, 40% CE	643	5735	82	4350
1 µs, 45% CE	685	9398	87	4500



As shown in the table in Figure 2, HCD scans acquired at 45% NCE and 1 µscan provided the best overall results for both protein identification and quantification. Two-step NCE methods yielded the highest percentage of quantifiable peptides, as expected, at 90%(results are not shown) versus 87%, but the lowest rate of peptide identifications with a comparable rate of quantification reproducibility across the all three conditions.

FIGURE 3. Base peak chromatograms for LC-MS/MS runs of 40 ng (A) , 60 ng (B), and 80 ng (C) of *E. coli* digest.

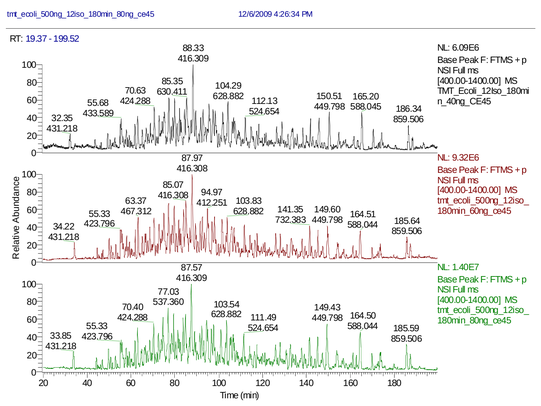
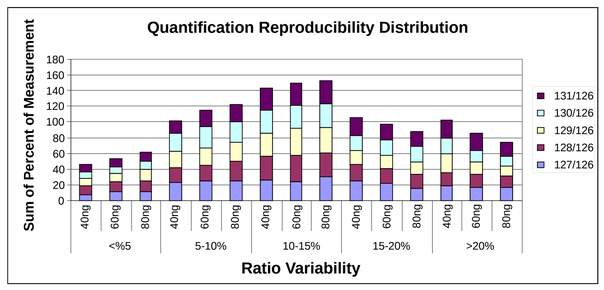


FIGURE 4. Protein identification and quantitative precision at the protein level for 40, 60, and 80 ng of *E. coli* digest.

Protein Amount	Proteins	Peptides	% Quan Peptides	Unique Peptides
40 ng	507	5096	74	3795
60 ng	573	6594	78	4263
80 ng	619	7321	81	4658
500ng	685	9398	87	4500



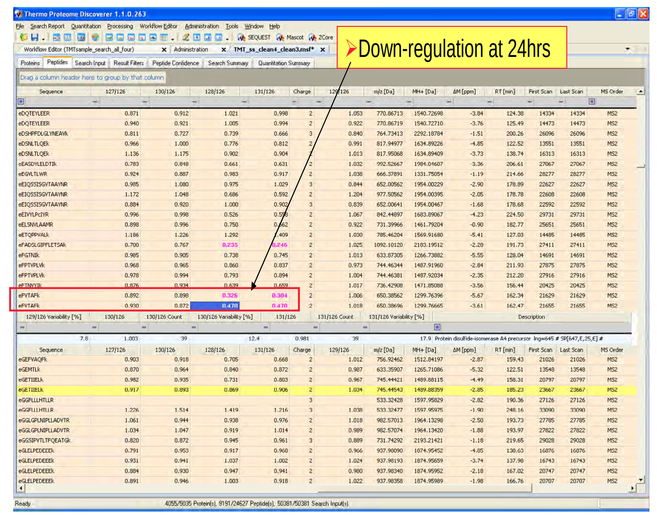
Because 45% NCE, 1 µscan, and an isolation width of 1.2 amu provided the highest protein identification rate and also produced the most reproducible TMT reporter ion intensities, these settings were used for all subsequent experiments.

Sensitivity

The higher transmission ion source, the dual pressure ion trap, and the improved HCD cell in the LTQ Orbitrap Velos mass spectrometer results in significantly increased sensitivity for shotgun proteomics experiments[8]. In the next series of experiments we examined the sensitivity of the LTQ Velos Orbitrap system for TMT quantification experiments using the HCD optimal settings as described above. Figure 3 shows the base peak chromatograms from analysis of low sample loads, and Figure 4 shows identification and quantification results for low sample amount versus a standard sample amount of 500 ng. Remarkably, more than 500 proteins were identified and 74% peptides quantified for 40 ng of sample load. These results can be explained by excellent extraction and fragmentation efficiency of the new HCD device. Even with a 40 ng sample load most proteins were quantified with less than 20% error (Figure 4).

The majority of quantitative experiments are designed to investigate changes in the abundance of individual peptides rather than their corresponding proteins. Figure 5 shows quantification reproducibility on the peptide level in an experiment in which a human cancer cell line was treated with drug for 2 or 24 hrs. In this experiment, the control sample was labeled with TMT 126 and 129, the 2 hr sample was labeled with TMT 127 and TMT 130 tag and the 24 hr sample was labeled with TMT 128 and TMT 131. HCD scans were acquired using optimal settings (see above). Results shown correspond to four independent LC-MS/MS runs and demonstrate relative variability (measure of precision) of TMT ratios averaging 10% RSD at the peptide level. As a result, we concluded that peptide EFVTAFK is indeed down-regulated after 24 hrs of drug treatment.

FIGURE 5. Quantification reproducibility at the peptide level for a digest of MCF-7 cells treated with 17β-estradiol.



Conclusions

- Optimal HCD settings for isobaric tag-based quantification using an LTQ Orbitrap Velos mass spectrometer are an FT AGC MSn target at 50000, an isolation width of 1.2 amu, 1 µscan, and 45% NCE.
- Using optimal HCD settings, more than 500 proteins were identified and quantified using HCD fragmentation in 40 ng of *E. coli* digest.
- On average, 90% of all identified peptides were quantified and ~83% of these peptides had less than 15% error.

References

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