Quantifying Peptides in Complex Mixtures with High Sensitivity and Precision Using a Targeted Approach with a Hybrid Linear Ion Trap Orbitrap Mass Spectrometer

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Key Words

- LTQ Orbitrap Velos
- Pinpoint Software
- Targeted Peptide Quantitation
- High Resolution
- Protein Identification

Targeted HR/AM Peptide Quantitation Workflow at a Glance:

- Four orders of quantitative dynamic range
- Low-attomole detection limits
- Ultra-high selectivity with >100K resolution
- High quantitative precision with >9 points across the peak and average CV <4.7%
- Automated method development and data analysis with Pinpoint software

Introduction

High-resolution, accurate-mass (HR/AM) mass spectrometry (MS) is routinely used in discovery-based proteomics experiments for both identification and relative quantitation of peptides present in complex mixtures. In these experiments, relative quantitation is most commonly performed using stable-isotope labeling[1], isobaric tags[2], or label-free techniques using precursor extracted ion chromatograms (XICs)[3]. Quantitation using a discovery-based approach is routinely used to identify putative protein targets whose abundances are changing between conditions or following treatment. After protein candidates have been identified, targeted peptide quantitation can be used to more accurately quantify a wide range of peptides with high sensitivity and precision across a larger number of samples. Although considered essential for discovery-based experiments, the application of HR/AM MS to targeted peptide analyses, including verifying the identity and dynamics of putative biomarkers found in the discovery phase and their absolute quantitation, has been less explored until recently[4].

For the targeted HR/AM quantitation approach, Thermo Scientific LTQ Orbitrap instruments share the same detection advantages as the data-dependent peptide discovery approach. These include the high mass accuracy, high intrascan dynamic range, low detection limits and ultra-high resolution of the Orbitrap™ mass analyzer combined with the high sensitivity, high mass range, and fast cycle time of a linear ion trap mass analyzer. These instrument capabilities allow targeting peptides across a wide mass range with high selectivity, sensitivity, specificity, and throughput in complex biological matrices. Additionally, the high mass accuracy, resolution, and MS/MS mass range up to 4000 amu enable targeting larger (non-tryptic), heavily modified peptides using the same instrument acquisition methods applied to smaller, unmodified tryptic peptides.

Targeted peptide quantification can be performed on peptides identified previously in discovery-based experiments or directly using *a priori* knowledge/hypotheses of protein/peptide presence. The development of a targeted assay begins with the verification of the identity and quantitative dynamics of the targeted peptides. Frequently, changes occur in the chromatographic separation or sample source (i.e. from tissue to blood) when transitioning from discovery to targeted assays. Due to these changes and the complexity of biological samples, confirmation of precursor mass and retention time alone are not sufficient for verifying the identity of a targeted peptide, even when performing HR/AM MS analysis[5]. Figure 1 highlights this issue, showing two isobars belonging to doubly charged peptides eluting within a one-minute window. These two peaks have a mass difference of less than 5 ppm. The identity of the peak of interest was confirmed at the MS/MS level because the two peaks produce two distinctly different MS/MS spectra. This exemplifies the need for sequence confirmation via MS/MS during the early verification stage of targeted quantitative assay development. After an assay has been optimized and standardized, it is possible to perform targeted quantitation by relying on accurate mass and retention time alone, especially if retention time standards are employed[6].

Here we describe a targeted HR/AM peptide quantitation workflow on a Thermo Scientific LTQ Orbitrap Velos hybrid mass spectrometer[7,8]. Identities of targeted peptides present in a complex mixture are verified using selected ion monitoring (SIM) and scheduled MS/MS. In addition, we detail the utility of Thermo Scientific Pinpoint software for generating the targeted HR/AM instrument methods in an automated fashion from previously acquired raw data and/or known protein targets.
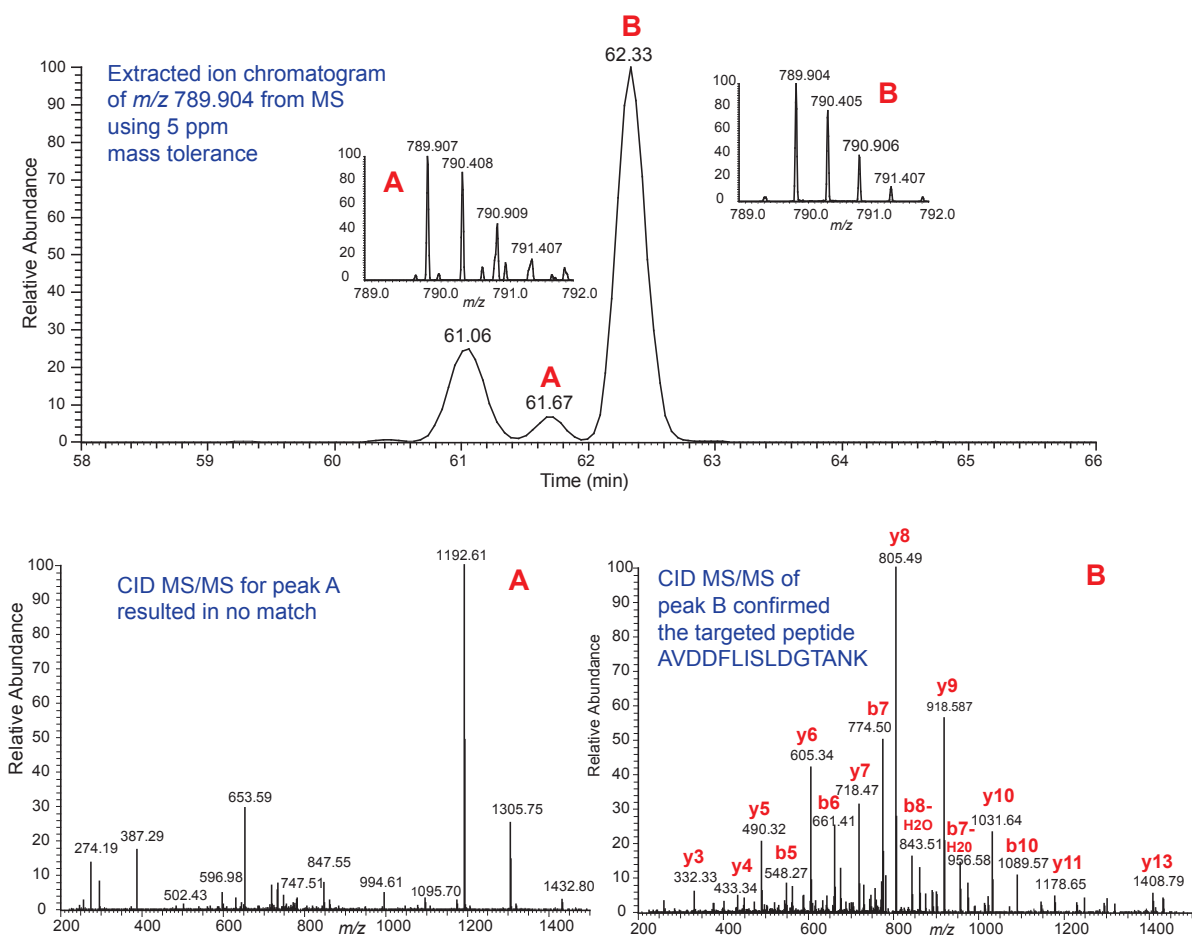
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Figure 1. The targeted peptide cannot be confidently confirmed using accurate mass (5 ppm extraction window) and retention time alone.

Experimental

Sample 1: To evaluate workflow detection limits, a peptide mixture containing seven isotopically labeled yeast peptides (GAIAAAHYIR*, GILFVGSGVSGGEEGAR*, LGNDDEVILFR*, LVEDPQVIAPFLGK*, ELASGLSF-PVGFK*, GISNEGQNASIK*, LTILEELR*) were spiked into a yeast digest (1 µg/µL) to varying final concentrations (0.01 fmol/µL, 0.1 fmol/µL, 1 fmol/µL, 10 fmol/µL, 100 fmol/µL).

Sample 2: To evaluate workflow throughput and robustness, a triptically digested yeast cell lysate at 1 µg/µL was used.

LC/MS Analysis, Data Processing, and Method Optimization

One-microgram samples were loaded onto a PicoFrit™ C18 column (75 µm x 100 mm, 10 µm tip) run at a flow rate of 300 nL/min. The gradient was 5% to 45% of 0.1% formic acid/ACN over 40 or 60 minutes. The LTQ Orbitrap Velos hybrid mass spectrometer was used with capillary temperature, 250 °C; spray voltage, 1800 V; and S-lens voltage, 50%. The automatic gain control (AGC) target was 5e4 for SIM scans in the Orbitrap mass analyzer and 1e4 for MS/MS scans in the ion trap mass analyzer. Precursor ions were detected at 30,000 or 100,000 FWHM resolution, as indicated. The wide SIM windows were defined between 100 and 200 amu over the mass range *m/z* 450 -1250. Targeted MS/MS was performed in the linear ion trap using global scheduled inclusion lists developed using Pinpoint™ 1.1 software. Pinpoint 1.1 software was also used to further optimize the targeted quantitation method to target a maximum of 4 peptides per protein of interest. Quantitation analysis was performed in an automated fashion using the software with a 5 ppm window for XICs.

Results and Discussion

Selected Ion Monitoring Improves Detection Limits of the Targeted Assay

SIM is used to selectively accumulate precursor ions of a desired mass range in the ion trap before transferring them to the Orbitrap analyzer for detection. Using a narrow mass range (100-200 amu) permits more ions of a particular species, significantly improving both dynamic range and detection limits of the assay while retaining high selectivity afforded by HR/AM. As shown in Figure 2, using a 100 amu SIM window and accurate mass XICs significantly improves both signal-to-noise (S/N) and peak area, even with only half of the peptide amount loaded on the column.
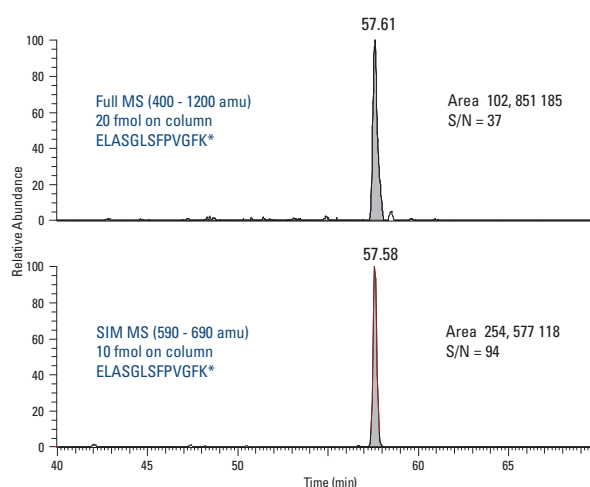
Figure 2. Comparison of detection limits between full-scan and a SIM MS. When comparing 5 ppm XICs, using a 100-amu wide SIM significantly improves both S/N and peak area, even with only half of the peptide amount loaded on the column.

Ultra-High Resolution of the Orbitrap Uniquely Enables the Detection of Isobaric Quantitation Interferences

Quantitation of precursor ions using the full scan or SIM modes typically requires high mass resolution to distinguish between precursor ions of interest and isobaric co-eluters, which are common in complex mixtures, especially for targets of low S/N. As shown in the top panel of Figure 3, >100,000 FWHM resolution is used to distinguish two isobaric 2+ ions at m/z 620.3. When the instrument is operated with lower resolution, for example 26,000 FWHM as shown in the bottom panel of Figure 3, peptide detection and subsequent verification/quantitation are compromised. Improved mass accuracy measurement of the underlying isobaric species and lower detection limits are additional advantages of high resolution.

Targeted Protein Quantitation Workflow on the LTQ Orbitrap Combines High Sensitivity, Selectivity, Precision, and Throughput

We have developed a workflow that uses wide mass range HR/AM SIM for quantitation and scheduled MS/MS for confirming targeted peptide sequences. The instrument method is developed in a fast and automated fashion using Pinpoint software based on discovery data previously acquired on the same instrument[9]. Alternatively, *a priori* knowledge about the studied system can be used to design the targeted assay within Pinpoint software with or without previously acquired MS/MS data. As described in the experimental section, the method consists of four sequential SIM windows followed by scheduled MS/MS of the targeted peptides. The SIM scans decrease the peptide detection limit while improving the accuracy and precision of the quantitation results. The scheduled MS/MS for the targeted peptides enables peptide identity confirmation while keeping cycle times low. This allows more MS data points across the elution profile of the peptides, which further improves the precision and accuracy of the quantitation results. As the ion trap is able

to accumulate ions, high quality MS/MS spectra can be obtained, even for ions of low intensity. This permits a large quantitative dynamic range (four orders of magnitude in this study). Additionally, the workflow allows reprocessing for unforeseen peptide targets within the wide SIM window.

Excellent Detection Limits and Quantitation Over a Large Dynamic Range in a Complex Mixture

The detection limits and the quantitative dynamic range of the workflow were evaluated using seven isotopically labeled peptides spiked into a yeast digest over a large concentration range (0.01-100 fmol/µL). The LTQ Orbitrap Velos mass spectrometer provided excellent sensitivity and quantitative results for all seven peptides. At each concentration level, the peptides were successfully verified using MS/MS spectra acquired in the linear ion trap mass analyzer and quantified using HR/AM MS XICs with a 5-ppm mass tolerance.

Figure 4A shows the 5-ppm XICs of the targeted peptide ELASGLSFPVGFK* from 0.01 fmol to 100 fmol. With the sensitivity of the ion trap mass analyzer, the peptide was confirmed by MS/MS at all concentrations including 10 amol (Figure 4B). Figure 5 shows similar results for an additional peptide GISNEGQNASIK*. Remarkably, the results show a quantitative dynamic range up to four orders of magnitude without the use of an internal standard. This is made possible by the AGC
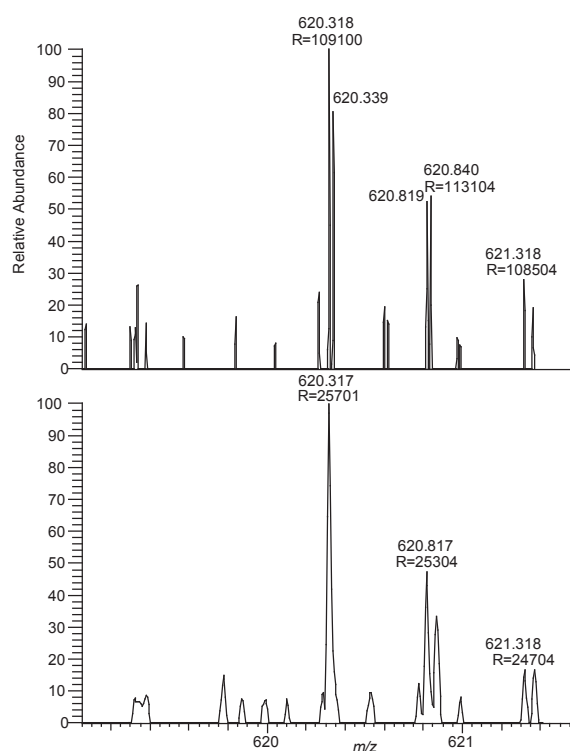

Figure 3. HR/AM is required for accurate peptide detection and quantitation. Peptides at m/z 620.3 detected with >100,000 (top) and 26 000 (bottom) FWHM show the necessity of high resolution to distinguish between isobaric precursor ions different by less than m/z 0.02.
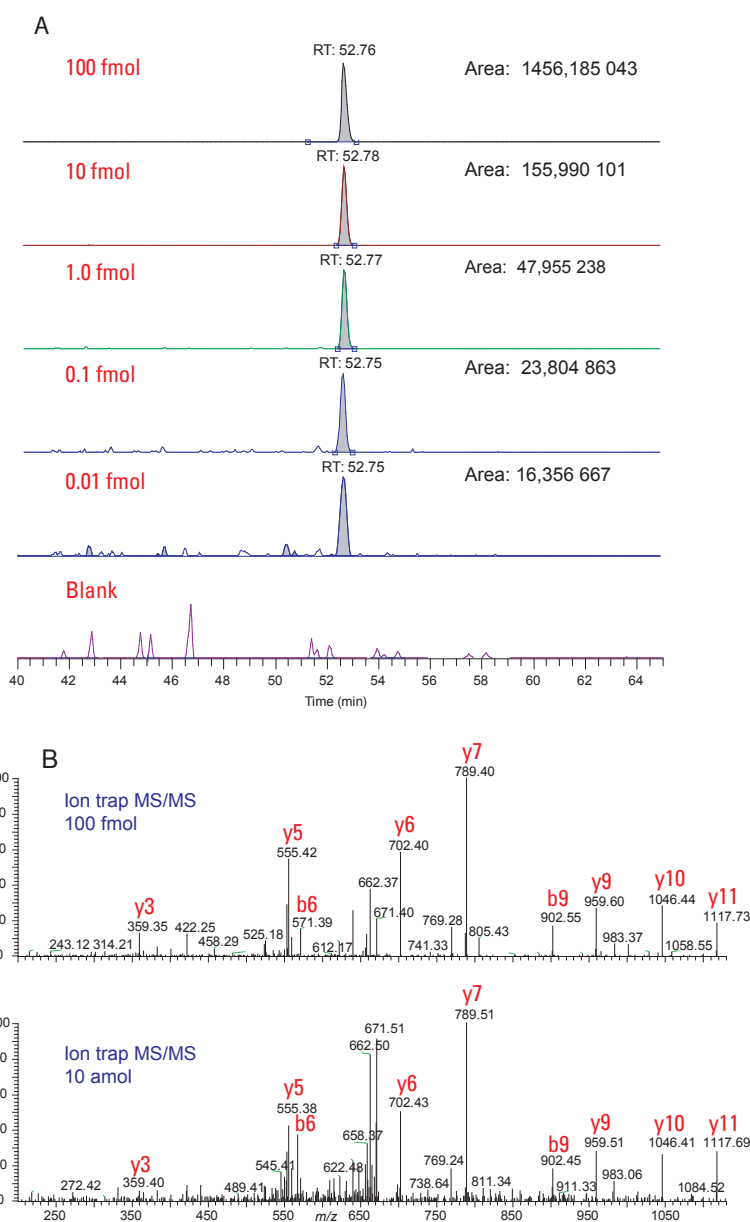
Figure 4. The targeted HR/AM SIM workflow on the LTQ Orbitrap Velos mass spectrometer enables detection, identification, and quantitation at low peptide levels. (A) HR/AM XICs of the targeted peptide ELASGLSFPVGFK* spiked into yeast matrix at varying levels; (B) MS/MS data acquired from 0.01 fmol and 100 fmol samples. When searched against the spectral library[9], both spectra matched to the targeted peptide spectrum with a P-value <0.01.
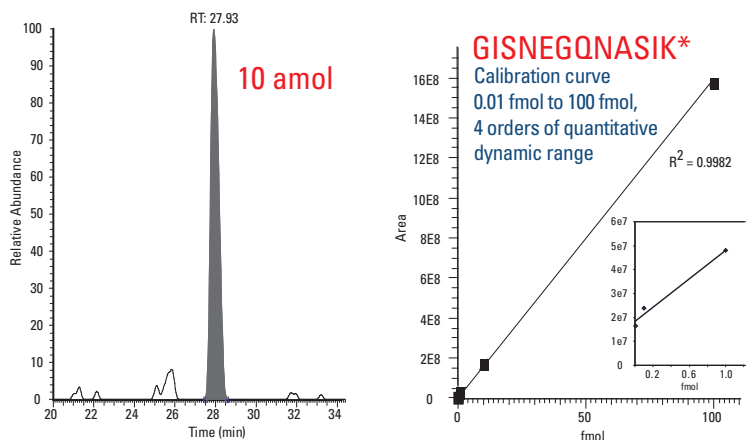
function of the linear ion trap, which maintains a consistent charge density for each scan event, minimizing detector saturation effects common to time-of-flight instruments. AGC enables robust peptide quantitation in the presence of high sample complexity. Using the method described, all peptides were detected and verified by MS/MS at the 10-amol level with the exception of GILFVGSGVS-GGEEGAR*.

Workflow Reproducibility and Throughput: Targeting 25 Yeast Proteins in a Single LC-MS/MS Experiment Using Automated Method Development

The workflow described uses multiple wide SIM windows, making it possible to detect a wide range of targeted peptides with high quantitative accuracy while maintaining an LC-compatible cycle time. Here, we performed a targeted analysis of 25 yeast proteins in a single LC-MS/MS run to evaluate its reproducibility and throughput. Pinpoint software was used to automate the development and refinement of the targeted peptide quantitation instrument method and to perform subsequent data analysis. The instrument method was developed based on discovery data previously acquired on the same instrument. The raw file was imported into the software, which analyzed the data and determined the optimal peptide targets for the proteins of interest. The software determined the retention time window and accurate *m/z* ratio for each targeted peptide and produced a mass list used directly in the acquisition method for scheduled MS/MS. One-hundred and ninety-two previously detected peptides were selected by the software and then exported as a retention time-dependent inclusion list to build

Figure 5. The targeted HR/AM SIM workflow on the LTQ Orbitrap Velos mass spectrometer enables quantitation with 4 orders of dynamic range. Quantitative results for the spiked heavy peptide GISNEGQNASIK*: (left) Low detection limit with excellent S/N; (right) Quantitative dynamic range spanning four orders of magnitude without the use of an internal standard.

an initial instrument acquisition method. Because of the complexity of the yeast samples, we developed this initial high-resolution method to determine if there are iso-baric interferences that would affect quantitation. In this method, a single MS scan from 300 to 1500 Da at a mass resolution of 100,000 FHWM was performed, followed by up to ten ion trap MS/MS scans (from the scheduled inclusion list). The software was used to verify the peptide sequences and to quantify peptide signals. For all targeted peptides, there were no co-eluting isobars detected that would interfere with quantitation.

Further optimizations of the acquisition method can be performed within Pinpoint software to improve cycle time, sensitivity, and accuracy. This is particularly useful when targeting a large number of proteins within a complex sample. For example, the software can refine the scheduled MS/MS inclusion list based on the most intense peptides per protein. To illustrate this, we chose up to four verified peptides per protein according to their precursor signal intensity rank, which yielded a total of 77 peptide targets representing the 25 yeast proteins. The 77 peptides were exported as a global MS/MS inclusion list with a time-scheduled window for use in the refined instrument method. In this method, four SIM scans (m/z 450–650, m/z 650–850, m/z 850–1050, and m/z 1050–1250) at a mass resolution of 30,000 FWHM were followed by MS/MS scans from the global time-scheduled MS/MS list. Figure 6 shows a screen shot of the acquisition method detailing the mass spectrometer parameters used.

The sample was run six times using the refined instrument method. All raw data files were processed using Pinpoint software (Figure 7). The resulting cycle time allowed for 9 to 11 data points across the chromatographic peak for all targeted peptides. Ninety-seven percent of the targeted peptides had CVs below 10%, and 71% of the peptides had CVs less than 5% (Figure 8).
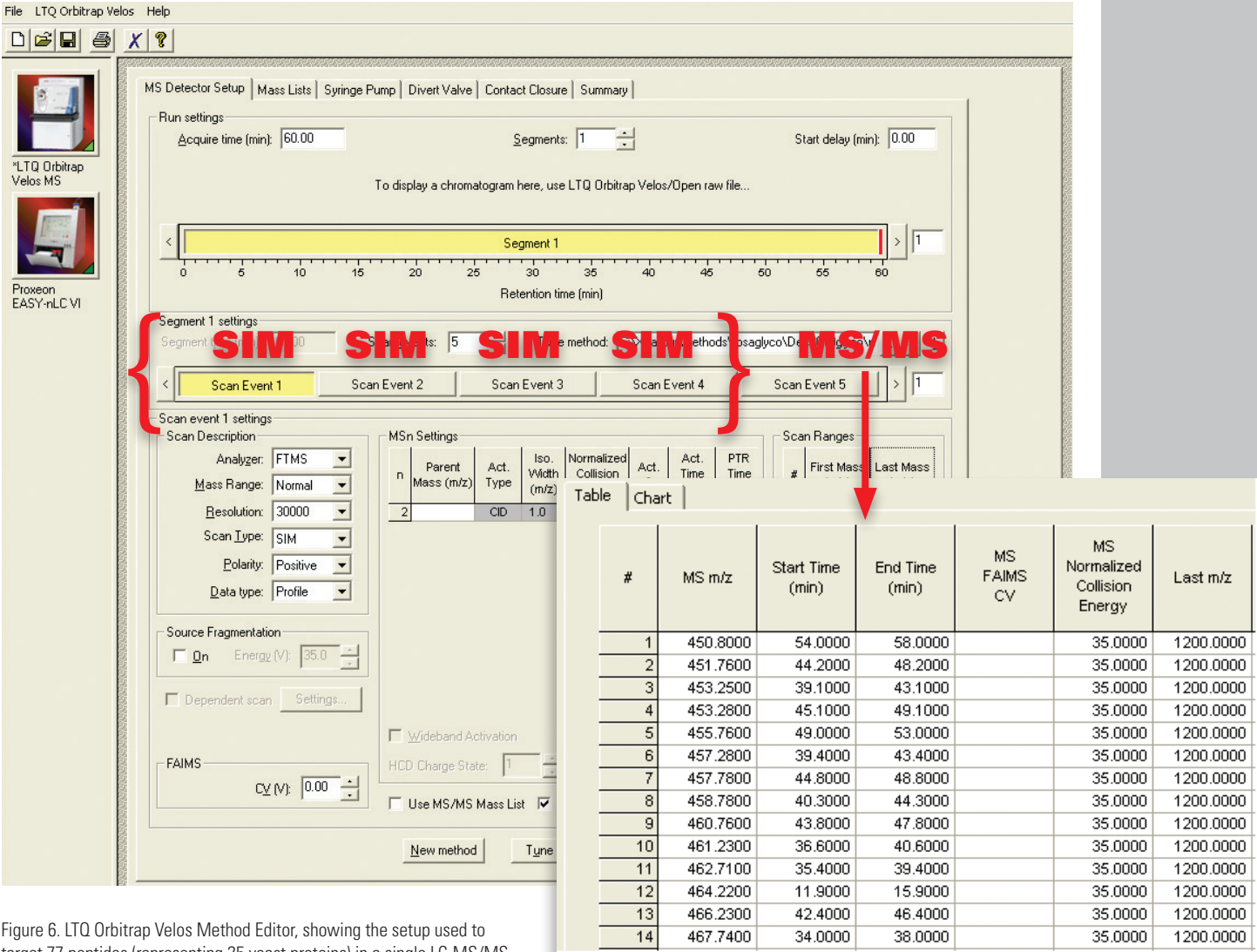

Figure 6. LTQ Orbitrap Velos Method Editor, showing the setup used to target 77 peptides (representing 25 yeast proteins) in a single LC-MS/MS analysis. Scan events 1-4 were SIM scans in the Orbitrap analyzer and scan event 5 was time scheduled MS/MS in the ion trap. The inset shows the global scheduled inclusion MS/MS list.

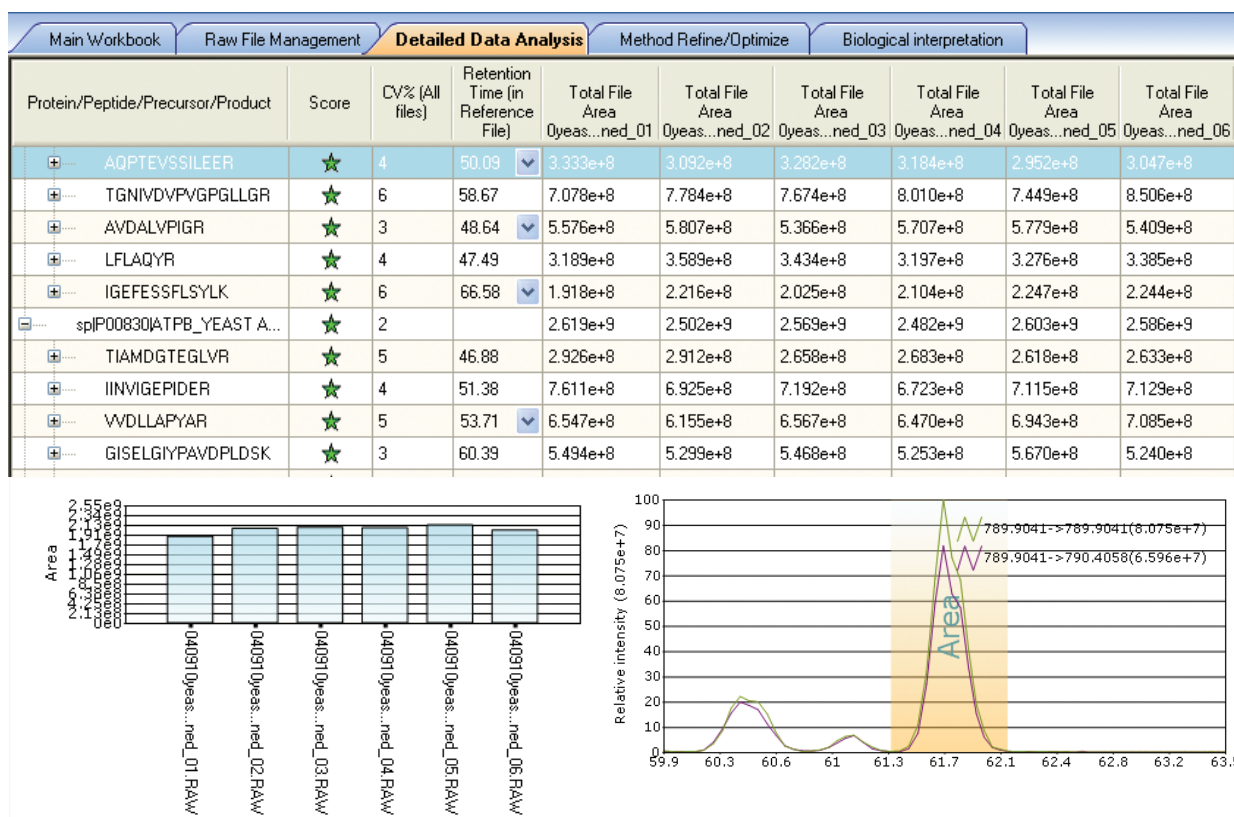| Protein/Peptide/Precursor/Product	Score	CV% (All files)	Retention Time (in Reference File)	Total File Area 0yeas...ned_01	Total File Area 0yeas...ned_02	Total File Area 0yeas...ned_03	Total File Area 0yeas...ned_04	Total File Area 0yeas...ned_05	Total File Area 0yeas...ned_06
⊞ AQPTEVSSILEER	★	4	50.09 ⌄	3.333e+8	3.092e+8	3.282e+8	3.184e+8	2.952e+8	3.047e+8
⊞ TGNIVDVPVGPGLLGR	★	6	58.67	7.078e+8	7.784e+8	7.674e+8	8.010e+8	7.449e+8	8.506e+8
⊞ AVDALVPIGR	★	3	48.64 ⌄	5.576e+8	5.807e+8	5.366e+8	5.707e+8	5.779e+8	5.409e+8
⊞ LFLAQYR	★	4	47.49	3.189e+8	3.589e+8	3.434e+8	3.197e+8	3.276e+8	3.385e+8
⊞ IGEFESSFLSYLK	★	6	66.58 ⌄	1.918e+8	2.216e+8	2.025e+8	2.104e+8	2.247e+8	2.244e+8
⊟ sp|P00830|ATPB_YEAST A...	★	2		2.619e+9	2.502e+9	2.569e+9	2.482e+9	2.603e+9	2.586e+9
⊞ TIAMDGTEGLVR	★	5	46.88	2.926e+8	2.912e+8	2.658e+8	2.683e+8	2.618e+8	2.633e+8
⊞ IINVIGEPIDER	★	4	51.38	7.611e+8	6.925e+8	7.192e+8	6.723e+8	7.115e+8	7.129e+8
⊞ VVDLLAPYAR	★	5	53.71 ⌄	6.547e+8	6.155e+8	6.567e+8	6.470e+8	6.943e+8	7.085e+8
⊞ GISELGIYPAVDPLDSK	★	3	60.39	5.494e+8	5.299e+8	5.468e+8	5.253e+8	5.670e+8	5.240e+8

Figure 7. Pinpoint software displays simultaneous identification and quantitation results for the 77 targeted peptides from a single LC/MS run. The top panel shows the summary of quantitative results using the integrated areas of primary ions. The bottom left panel shows a histogram view of relative peak area differences for each targeted peptide between replicates (n=6). The right panel shows the XIC and integrated peak area of primary ions per targeted peptide.
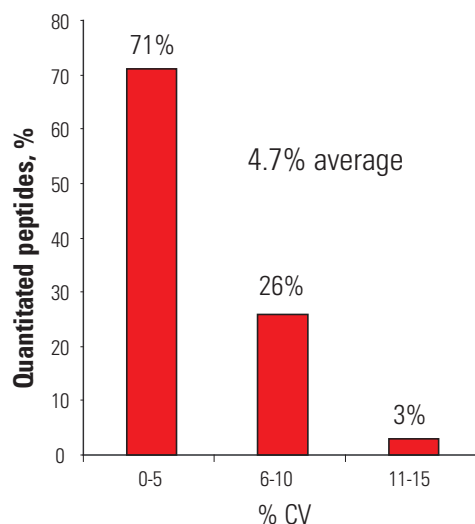

Figure 8. Analytical precision of the quantitative workflow targeting 25 yeast proteins.

Conclusion

A workflow for simultaneous quantification and identity verification of targeted peptides using the LTQ Orbitrap Velos hybrid mass spectrometer and Pinpoint software was developed and evaluated. The workflow employs wide mass range SIM scanning to improve the sensitivity and scheduled MS/MS for peptide verification. The high-resolution MS analysis (100,000 resolution FWHM) enables detection of co-eluting isobars that would interfere with quantitation. The detection limits were investigated by spiking isotopically labeled yeast peptides into a complex yeast digest matrix at different concentrations. The peptides were detected as low as 10 amol for all but one spiked heavy peptide and the quantitative dynamic range was 4 orders of magnitude.

The 77 peptides selected to represent the 25 targeted yeast proteins in the optimized instrument method were successfully quantified and verified with high analytical precision. Ninety-seven percent of the targeted peptides gave a CV of less than 10%, and the average CV was 4.7%. Workflow detection limits, precision and specificity were enhanced using wide SIM isolations and a time-scheduled global MS/MS list.

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