Application Note: 557

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

- Orbitrap Elite
- Pinpoint software
- EASY-nLC II
- High resolution, accurate mass
- Targeted peptide quantitation

Introduction

Hybrid ion trap-Orbitrap™ mass spectrometers are routinely used in discovery-based proteomics experiments for both identification and relative quantitation of peptides present in complex mixtures[1-4]. However, application of high-resolution, accurate-mass (HR/AM) mass spectrometry to targeted quantitative proteomics tasks, such as verifying and validating the putative protein biomarkers identified in the discovery phase, has been less explored until recently[5]. For targeted HR/AM quantitation, hybrid ion trap-Orbitrap mass spectrometers offer the same detection advantages as they do for the data-dependent peptide discovery. The Orbitrap mass analyzer provides high mass accuracy, high intra-scan dynamic range, low detection limits as ions of a specific *m/z* can be accumulated/enriched in the mass spectrometer prior to detection, and ultra-high resolution. Simultaneously, the linear ion trap provides high sensitivity and fast cycle time.

The development of a targeted HR/AM assay begins with selecting peptides to serve as quantitative surrogates of the targeted proteins. The peptides must be unique and allow highly sensitive analyses. They can be selected based on previous discovery data or directly using *a priori* knowledge/hypotheses of protein/peptide presence. The identity and quantitative dynamics of the selected peptides need to be verified in the developed HR/AM assay. Due to the complexity of biological samples, in most cases, the 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[6]. Figure 1 highlights this situation, showing two isobaric species 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 only 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.

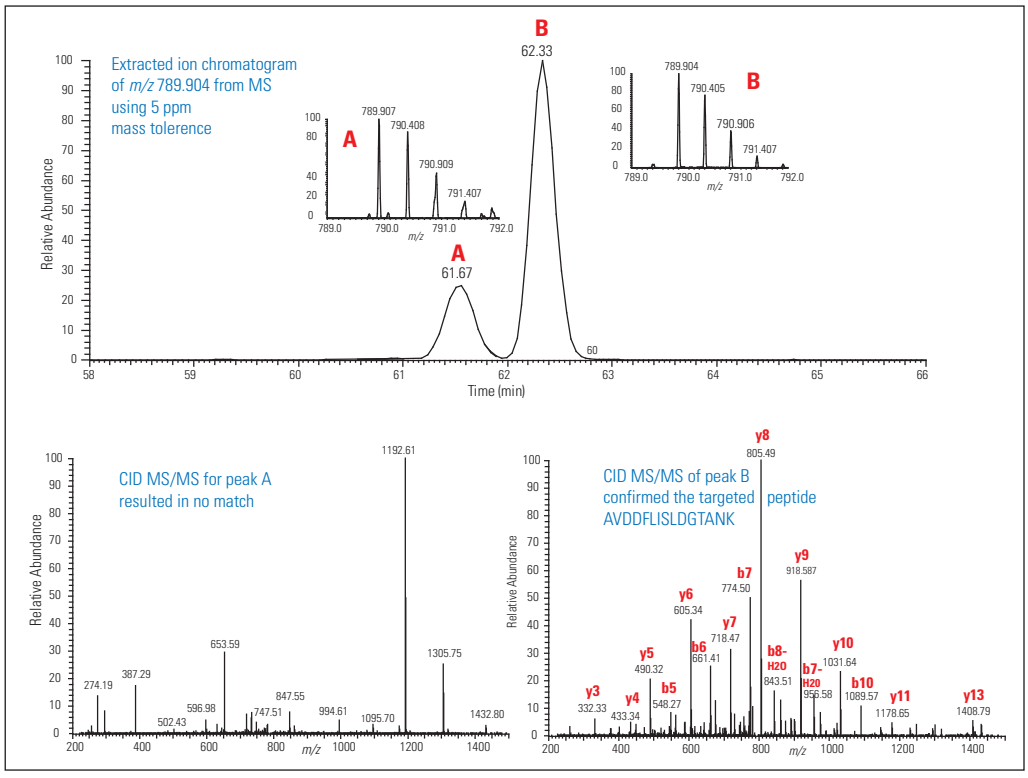

Figure 1. The targeted peptide cannot be confidently confirmed using accurate mass (5 ppm extraction window) and retention time alone.

To address this issue, we developed a targeted HR/AM peptide quantitation workflow[5] that uses HR/AM selected-ion monitoring (SIM) for quantitation and time-scheduled ion trap MS/MS for simultaneous peptide verification. Figure 2 shows the workflow employed on a new next-generation hybrid ion trap-Orbitrap mass spectrometer. For targeting a large number of peptides in one HPLC-MS run, multiple SIM scans with 200 amu isolation windows followed by targeted data-independent CID MS/MS with scheduled time windows were used. The relatively large SIM isolation windows resulted in collection and quantitation of all the ions within that window, effectively "enriching" those ions while excluding ions outside the mass range of interest. This enrichment resulted in a lower limit of quantification in much the same way that selectively collecting peptides on a trapping column would. Because Orbitrap analyzer detection is the largest contributor to the cycle time in this workflow, the new hybrid Orbitrap instrument with a 4-times faster acquisition rate (at 60,000 FWHM), compared to most current hybrid Orbitrap instruments,[7] is expected to offer better analytical precision and throughput.

Here we report the results of the targeted quantification workflow combining HR/AM SIM with simultaneous CID MS/MS on the new Thermo Scientific Orbitrap Elite hybrid mass spectrometer. Attention is focused on the detection limits, dynamic range, and the analytical precision of the assay.

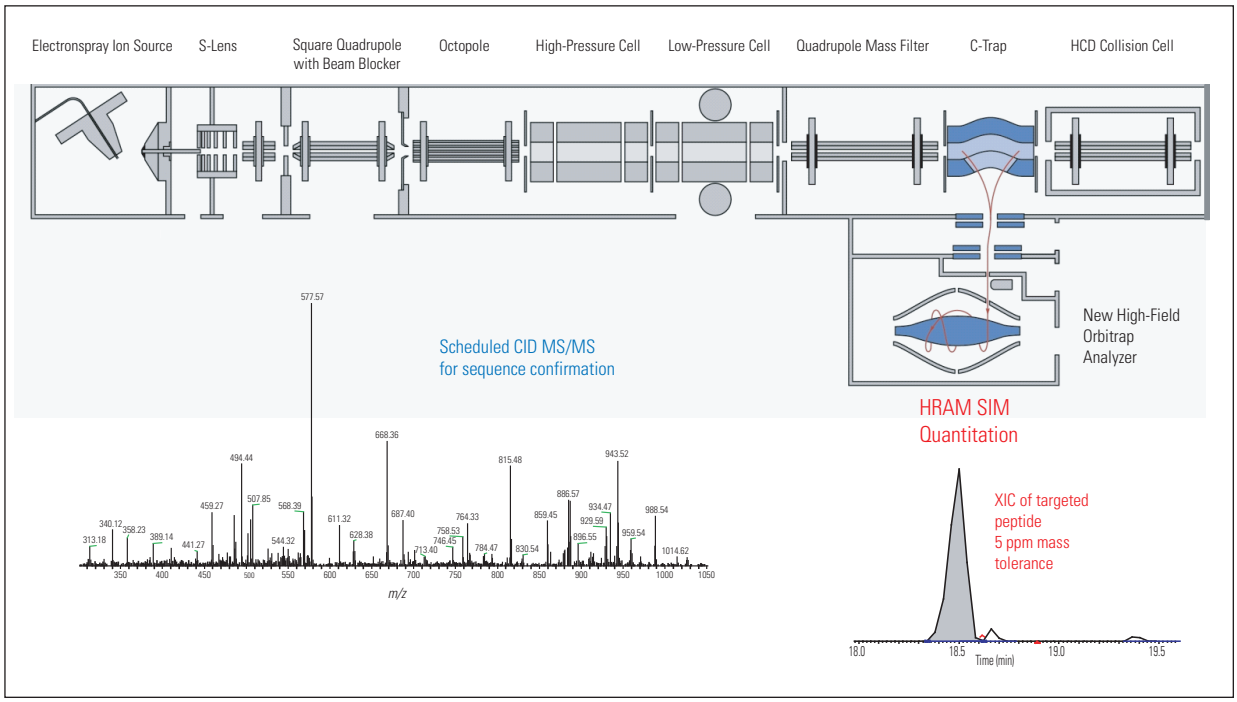

Figure 2. Targeted peptide quantitation workflow using an HR/AM approach on the Orbitrap Elite hybrid mass spectrometer. Targeted peptides were quantified using accurate mass SIM and confirmed simultaneously using time-scheduled CID MS/MS within a single HPLC-MS run.

Goal

To evaluate the sensitivity, analytical precision, dynamic range and throughput of the targeted quantification workflow for simultaneously verifying and quantifying multiple targeted proteins/peptides on a new hybrid linear ion trap-Orbitrap mass spectrometer, the Orbitrap Elite™. Accurate masses of targeted peptides in high-resolution selected ion monitoring (SIM) mode are collected for quantitation and the MS/MS spectrum of each targeted peptide is used for sequence confirmation.

Experimental

Sample Preparation

Sample 1:	A mixture of six isotopically labeled yeast peptides was spiked into a 1-µg yeast digest 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 yeast digest mixture (1 µg/µL)

Liquid Chromatography

A Thermo Scientific EASY n-LC II was used for all experiments. The LC conditions were as follows:

Column:	PicoFrit® Magic C18 column (75 µm x 150 mm, 3 µm particle size) (New Objective, Woburn, MA)
Flow rate:	300 nL/min; Buffer A: 0.1% formic acid/H_2O; Buffer B: 0.1% formic acid/acetonitrile
Gradient:	5% B to 45% B in 60 min
Sample loading:	Directly loaded on column
Injection amount:	1 µL

Mass Spectrometry

An Orbitrap Elite™ hybrid ion trap-Orbitrap mass spectrometer equipped with a Thermo Scientific Nanospray Flex ion source was used for all experiments. The MS conditions were as follows:

Capillary temperature:	220 °C
Spray voltage:	1800 V
S-lens RF level:	50%
FT Resolution:	FHWM at *m/z* 400
AGC target for FT SIM:	1E5
Isolation width of FT SIM:	50 amu for sample 1; 200 amu for sample 2

Targeted data—independent MS/MS lists with scheduled time windows were used for targeted peptide verification. For sample 1, three SIM scans were used to cover all precursor ion mass ranges of the spiked peptides and the targeted MS/MS list included all of the six precursor ions without scheduled time windows. For sample 2, four SIM scans were used to cover 86 yeast peptides representing 26 yeast proteins. The targeted MS/MS list included 86 precursor ions with scheduled time windows. Each sample was run in triplicate.

Workflow to Generate a Targeted MS/MS List for Sample 2

1. A total of 26 yeast proteins were selected from the literature[8,9]. Proteins were selected with estimated cellular abundances ranging from less than 50 copies per cell to more than 1,000,000 copies per cell to evaluate the dynamic range of the workflow. The majority of the selected proteins were of low abundance (<128 copies/cell).
2. Based on either previous discovery data or *in-silico* digestion, peptides with sequences unique to the targeted proteins were selected as putative candidates for quantification using Thermo Scientific Pinpoint software version 1.1. The 2+ and 3+ precursor *m/z* list of each candidate was exported as global inclusion list. The elution time for each peptide was predicted using linear correlation based on a Thermo Scientific Pierce Peptide Retention Time Calibration (PRTC) kit. 4-minute windows were used per peptide.
3. The yeast digest was analyzed on the Orbitrap Elite instrument using full scan at 60,000 resolution in the Orbitrap mass analyzer and rapid CID MS/MS in a targeted, data-dependent fashion in the linear ion trap. Only the precursor ions in the global inclusion list were triggered. All unidentified candidate peptides in the first run were exported as an additional global MS/MS list with a larger 10-minute time window using Pinpoint™ software for the second run.
4. The two raw files were searched against the yeast database with Thermo Scientific Proteome Discoverer software version 1.3. The first and the second search results were compiled together to establish a MS/MS spectral library using Pinpoint software. In the final HR/AM SIM method, 86 identified peptides representing the 26 targeted proteins were targeted using four SIM scans (400–600 amu, 600–800 amu, 800–1000 amu, 1000–1200 amu), followed by a data-independent CID MS/MS scan with a global MS/MS list. The four SIM scans were acquired constantly over the total run, which facilitated method set up by eliminating the need for scheduling SIM scans. The time schedule of the global MS/MS list was automatically generated by Pinpoint software based on the detected retention time of each targeted peptide. A 4-minute time window per peptide was used.

Results and Discussion

Detection Limits, Analytical Precision and Linear Dynamic Range of the Workflow

The six heavy peptides spiked in the 1-µg yeast digest were targeted in five samples with different concentrations. Each sample was run in triplicate at each of the five different concentrations to evaluate the analytical precision. All six peptides were detected clearly from the 10-amol level to the 100-fmol level on column and simultaneously verified by CID MS/MS. Figure 3 shows one of the targeted peptide clearly detected and quantified from the complex yeast matrix at the 10-amol (lowest) concentration level. The peptide identity was confirmed by CID MS/MS in the same run. Four orders of linear dynamic range were observed with very good analytical precision (Figure 4). The %CV was less than 21% for the peptides at 10-amol level and less than 10% for the four other concentration levels. Table 1 shows the summary of the %CV for all five concentration samples.

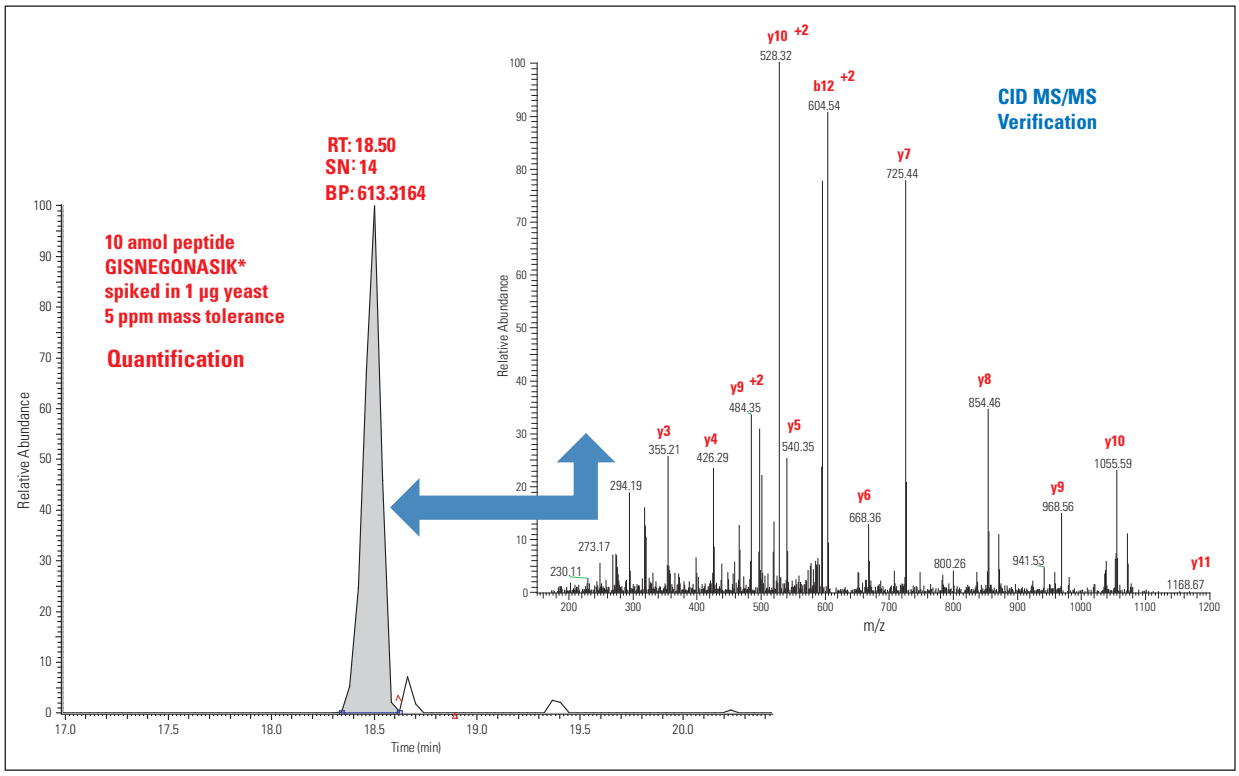

Figure 3. The spiked, isotopically labeled peptide GISNEGQNASIK* was simultaneously quantified using an extracted ion chromatograph from the SIM scan, and its sequence verified using CID MS/MS.

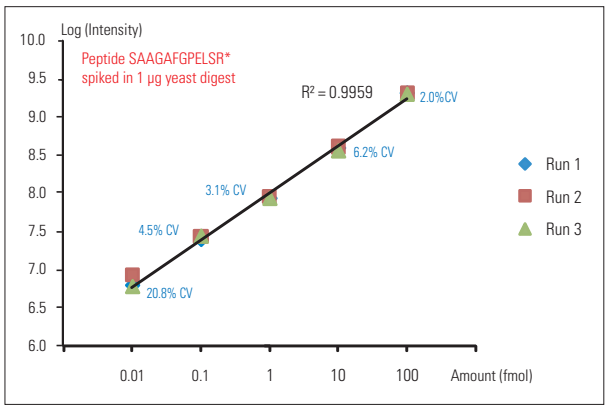

Figure 4. Four orders of linear dynamic range were observed for the spiked peptide SAAGAFGPELSR*.

Table 1. Analytical precision for the six targeted isotopically labeled peptides spiked into a 1-µg yeast digest at varying levels.

	0.01 fmol	0.1 fmol	1 fmol	10 fmol	100 fmol
	% CV (n=3)				
SSAAPPPPPR*	12.6	2.9	1.0	2.8	1.0
GISNEGQNASIK*	4.1	2.8	3.0	4.3	3.5
HVLTSIGEK*	15.1	1.3	2.2	2.9	1.0
IGDYAGIK*	8.2	4.2	8.2	4.1	5.5
TASEFDSAIAQDK*	12.6	1.3	7.7	8.5	4.0
SAAGAFGPELSR*	20.8	4.5	3.1	6.2	2.0

Detecting and Quantifying Low- and High-Abundance Yeast Proteins in a Single Run

To further evaluate the improved analytical precision of the workflow offered by the faster Orbitrap acquisition rates of the Orbitrap Elite instrument, a large HR/AM quantitative assay that targeted 86 peptides as quantitative surrogates for 26 targeted yeast proteins (Table 2) was developed and applied to the yeast digest sample (sample 2). Four SIM scans with a 200-amu isolation width and one additional targeted data-independent MS/MS scan using a global MS/MS list containing 86 precursor ions with scheduled 4-minute time windows were used. The analysis was performed in triplicate. All raw data files were processed using Pinpoint software (Figure 5). From the 5-ppm mass tolerance HR/AM SIM scan, the extracted ion chromatogram of the sum of the C^{12} and C^{13} intensities was used for quantitation. The CID MS/MS spectra were matched with the MS/MS spectral library entry for peptide sequence verification.

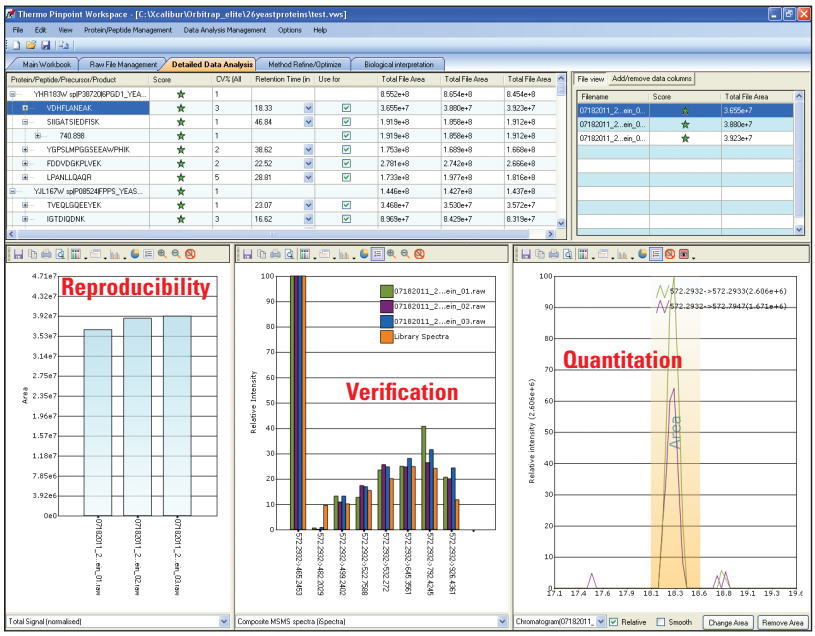

Figure 5. Automatic data processing using Pinpoint software

Because multiple co-eluting peptides can be isolated within the 2-amu precursor-ion isolation window, acquired CID MS/MS spectra can include fragment ions from different peptides. In those cases, there would likely be no good match with the spectra library while using all fragment ions from each MS/MS spectra. Pinpoint software features an innovative spectral match algorithm that uses multiple sequence-specific fragment ions [10] for spectral matching, with the number of fragment ions being defined by the user. It compares the relative intensities of the selected multiple fragment ions from the observed MS/MS spectra with the MS/MS spectral library. In this way, contaminant fragment ions from non-target sources are not involved in the spectral match process and do not influence the calculation of the spectra match score. As a result, Pinpoint software enables high-confidence peptide sequence conformation even with "contaminated CID MS/MS spectra". Details of the algorithm Pinpoint software uses for MS/MS spectral match can be found in the published paper by Prakash *et al.*[10]

Compared to earlier Orbitrap instruments, the faster Orbitrap acquisition scan speed offered by the Orbitrap Elite instrument allows shorter cycle times (1 second for four SIM scans plus 1-2 seconds of CID MS/MS time depending on how many precursor ions are in one specific time window), yielding more data points across the chromatographic peak for all targeted peptides (Figure 6). As a result, higher analytical precision was observed from the quantitative results acquired on the Orbitrap Elite instrument compared to that of the LTQ Orbitrap Velos™ instrument (Figure 6). Table 2 shows the partial summary of the %CVs for the targeted peptides representing proteins with different cellular abundances. All the targeted peptides had CVs below 15% and 96% of the targeted peptides had CVs below 10%.

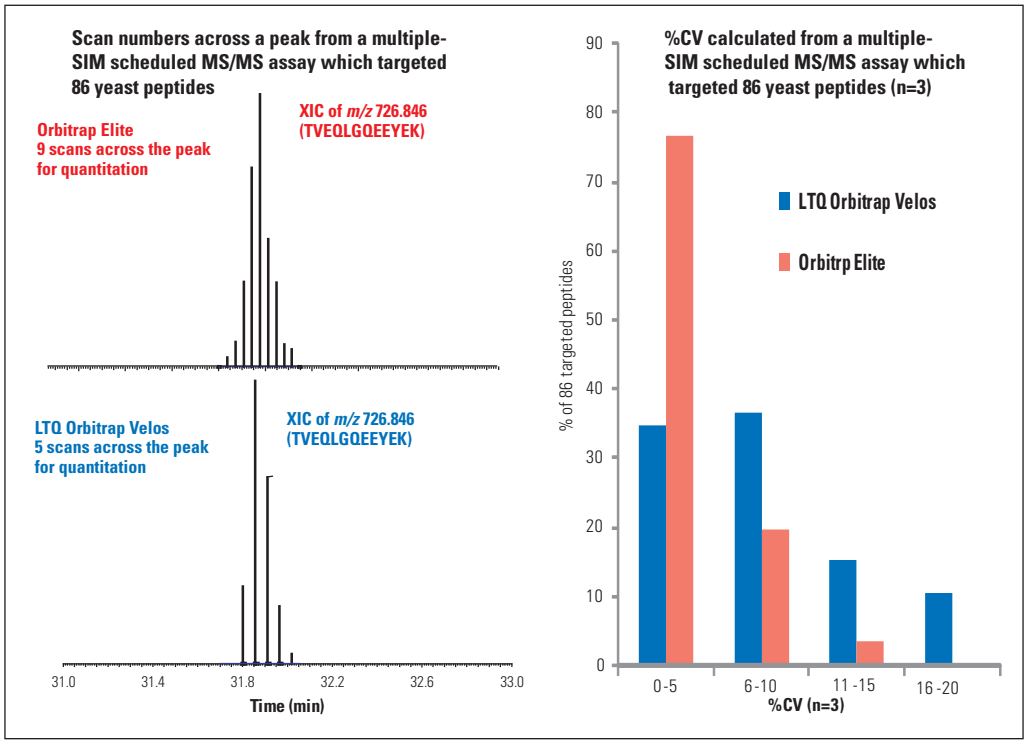

Figure 6. Analytical precision improvements due to faster Orbitrap mass analyzer scan speed. More scan points result in better precision.

Table 2. Partial summary results from the HR/AM assay in which 86 peptides representing 26 yeast proteins were detected and quantified across five orders of abundance range from over 1,000,000 copies/cell to less than 50 copies/cell in a single HPLC-MS run with excellent analytical precision.

Literature Abundance Range (Copies/Cell)*	Protein Names	%CV n=3	Retention Time	Total File Area run 1	Total File Area run 2	Total File Area run 3
1,018,216	YKL060C sp|P14540|ALF_YEAST Fructose-bisphosphate	2		3.85E+09	3.71E+09	3.78E+09
	TGVIVGEDVHNLFTYAK	1	54.15	2.67E+09	2.61E+09	2.66E+09
	GAIAAAHYIR	4	21.02	1.16E+09	1.08E+09	1.11E+09
	LLPWFDGMLEADEAYFK	5	63.48	1.61E+07	1.62E+07	1.76E+07
870,578	YLR249W sp|P16521|EF3A_YEAST Elongation factor	3		2.26E+09	2.37E+09	2.37E+09
	VLEELFQK	2	34.81	4.61E+08	4.75E+08	4.79E+08
	LSVATADNR	2	16.63	4.11E+08	4.23E+08	4.23E+08
	LVEDPQVIAPFLGK	6	44.81	6.38E+08	7.09E+08	7.03E+08
	SNFATIADPEAR	3	29.37	4.37E+08	4.60E+08	4.50E+08
	IAVIGPNGAGK	1	29.7	3.13E+08	3.05E+08	3.11E+08
< 128	YKL145W sp|P33299|PRS7_YEAST 26S protease regulatory subunit 7 homolog	1		3.16E+07	3.17E+07	3.23E+07
	SYGAAPYAAK	2	18.14	7.23E+06	7.53E+06	7.41E+06
	LGEEHPLQVAR	1	36.08	1.79E+07	1.82E+07	1.84E+07
	EVVELPLLSPER	4	43.53	6.48E+06	6.01E+06	6.53E+06
< 50	YJL167W sp|P08524|FPPS_YEAST Farnesyl pyrophosphate synthase	1		1.45E+08	1.43E+08	1.44E+08
	TVEQLGQEEYEK	1	23.92	3.47E+07	3.53E+07	3.57E+07
	IGTDIQDNK	4	17.24	8.97E+07	8.43E+07	8.32E+07
	IEQLYHEYEESIAK	10	35.16	2.02E+07	2.31E+07	2.48E+07
< 50	YDR150W sp|Q00402|NUM1_YEAST Nuclear migration protein	3		1.83E+07	1.75E+07	1.86E+07
	LGHTVVSNEAYSELEK	3	34.27	1.78E+07	1.70E+07	1.81E+07
	ILNILGDPSIDFLK	1	54.13	4.29E+05	4.36E+05	4.42E+05
< 50	YOR120W sp|P14065|GCY_YEAST Protein GCY	3		1.27E+07	1.33E+07	1.34E+07
	TWELMQELPK	3	43.09	1.27E+07	1.33E+07	1.34E+07
No expression detected	YJL008C sp|P47079|TCPQ_YEAST T-complex protein 1	5		7.56E+07	8.05E+07	8.36E+07
	QGYNSYSNADGQIIK	8	31.63	1.58E+07	1.62E+07	1.82E+07
	GTVLLHNAQEMLDFSK	8	46.42	1.11E+07	9.60E+06	9.78E+06
	LGAPTPEELGLVETVK	8	41.46	2.97E+07	3.40E+07	3.43E+07
	GATQNNLDDIER	6	22.79	1.91E+07	2.08E+07	2.13E+07
No expression detected	YNL208W sp|P40159|YNU8_YEAST Uncharacterized protein	3		1.28E+06	1.31E+06	1.36E+06
	TGAPNNGQYGADNGNPNGER	1	16.21	2.15E+05	2.18E+05	2.20E+05
	QQEQYGNSNFGGAPQGGHNNHHR	6	19.9	3.96E+05	4.49E+05	4.34E+05
	QDNNNNNGGFGGPGGPGGQGFGR	5	27.03	6.68E+05	6.43E+05	7.08E+05
No expression detected	YFL037W sp|P02557|TBB_YEAST Tubulin beta chain	3		1.22E+08	1.19E+08	1.14E+08
	MMATFSVLPSPK	1	40.11	1.58E+07	1.61E+07	1.60E+07
	YPGQLNSDLR	7	27.66	4.81E+07	4.49E+07	4.19E+07
	LAVNLVPFPR	5	42.86	3.47E+07	3.34E+07	3.12E+07
	NMMAAADPR	3	19.84	2.39E+07	2.50E+07	2.53E+07
No expression detected	YDR381W sp|Q12159|YRA1_YEAST RNA annealing protein YRA1	2		5.38E+07	5.14E+07	5.29E+07
	EFFASQVGGVQR	6	30.96	2.23E+07	2.03E+07	2.01E+07
	LNLIVDPNQRPVK	3	31.2	3.14E+07	3.12E+07	3.28E+07
No expression detected	YDR321W sp|P38986|ASPG1_YEAST L-asparaginase 1	2		6.95E+07	7.27E+07	7.13E+07
	LGTGGTIASK	3	21.42	3.37E+07	3.59E+07	3.47E+07
	TNANSLDSFNVR	4	29.66	1.20E+07	1.09E+07	1.14E+07
	SMDGMVPIANVPK	4	35.47	2.38E+07	2.59E+07	2.52E+07
No expression detected	YCL018W sp|P04173|LEU3_YEAST 3-isopropylmalate dehydrogenase	6		3.95E+05	3.88E+05	3.54E+05
	VNPIATILSAAMMLK	6	61.53	3.95E+05	3.88E+05	3.54E+05

* Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., Weissman, J.S. (2004) Global analysis of protein expression in yeast. *Nature.* 425: 737-41.

Conclusion

- A workflow for simultaneous quantification and verification of targeted peptides in complex mixtures was developed and evaluated using an Orbitrap Elite hybrid mass spectrometer. Targeted peptides were quantified using SIM with wide windows and HR/AM. Identities of the targeted peptides were verified using scheduled ion trap MS/MS in the same LC run.
- The LOD was as low as 10 amol for six heavy peptides spiked into the samples. Linear dilution curves were established across 4 orders of dynamic range – from 10 amol to 100 fmol – with good analytical precision for all six peptides.
- With the established workflow, targeted yeast proteins covering 5 orders of cellular abundance were detected and quantified in a single LC-MS run. The faster scan rate of the Orbitrap mass analyzer in the Orbitrap Elite instrument resulted in improved/excellent analytical precision within this experiment. All 86 targeted peptides had CVs below 15% and ninety-six percent of the targeted peptides had CVs below 10%.
- Throughput (number of targets) was significantly increased by using a wide isolation width (200 amu) for SIM in the Orbitrap mass analyzer and a time-scheduled global MS/MS list for the ion trap.
- The developed workflow can be used for targeted quantitation of any protein and delivers highly sensitive and reproducible quantitative results with high throughput.

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