Relative Quantitation of TMT-Labeled Proteomes – Focus on Sensitivity and Precision

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Key Words

Orbitrap Elite, LTQ Orbitrap Velos, Isobaric tagging, Quantitative precision, Quantitative accuracy

Introduction

The Thermo Scientific Orbitrap Elite mass spectrometer is the latest addition to the family of hybrid ion trap-Orbitrap™ mass spectrometers (Figure 1). Major design improvements include a compact high-field Orbitrap mass analyzer, advanced signal processing, and improved ion optics in the linear ion trap part that effectively prevent neutrals from entering the analyzer region (Table 1). The new features contribute to increased acquisition speed and sensitivity, enabling routine analysis at mass resolution 240,000 (FWHM at *m/z* 400) and improving the duty cycle of the instrument[1].

In quantitative proteomics, many approaches are available to measure relative abundances of proteins across two or more different samples. Isobaric tagging methods involving differential isotope labeling by chemical tagging are one of the most popular and universally applicable approaches. Two amine-reactive versions of isobaric tags are commercially available: Thermo Scientific Tandem Mass Tag (TMT)[2] reagents and isobaric tags for relative and absolute quantitation (iTRAQ®)[3] reagents. These tags are suitable for use with any type of complex protein sample. Their usefulness for multiplexing, reducing overall experiment time and experimental variance, is one of their main attractions. Despite this, both precision and accuracy can be challenging to achieve in some circumstances.[4] For example, lack of quantitative precision is common for low-abundance peptide signals. A potentially more damaging problem with isobaric tagging is poor accuracy when working with complex peptide mixtures. This can occur for two main reasons:

- Presence of low *m/z* chemical interference ions that interfere with the peaks of reporter ions.
- Co-isolation of interferences during parent ion isolation leading to MS^2-generated reporter ions derived from several precursors.

Since peptides vary in their fragmentation efficiency, the co-isolated isobaric peptides can contribute differently to the reporter ion abundances, i.e. a lower-abundance isobaric precursor can generate a more abundant reporter ions, thus distorting their ratio. This is especially prominent when isobaric species differ in their charge states.

The issue of background ions can be addressed by scanning with high resolution. Resolution in excess of 20,000 FWHM at *m/z* 130 is often required to resolve reporter ions and isobaric interferences. The issue of co-isolation interference has been recently addressed by using a MS^3 experiment on an LTQ Orbitrap instrument[5,6] and by combining a narrow precursor isolation width and fragmentation at the apex of the LC peak[7].

In this note we evaluated the impact of the technological advances of hybrid instruments on overall experimental outcome in terms of protein/peptide identification and quantitation. The number of peptides and proteins identified with the new Orbitrap Elite™ instrument were compared to those produced by a Thermo Scientific LTQ Orbitrap Velos hybrid ion trap-Orbitrap MS. In addition, a detailed assessment of the quantitative performance of the Orbitrap Elite instrument for the analysis of TMT®-labeled samples was carried out. The focus was on assessing the percentage of quantifiable peptides, the quantitative precision, and the quantitative accuracy.

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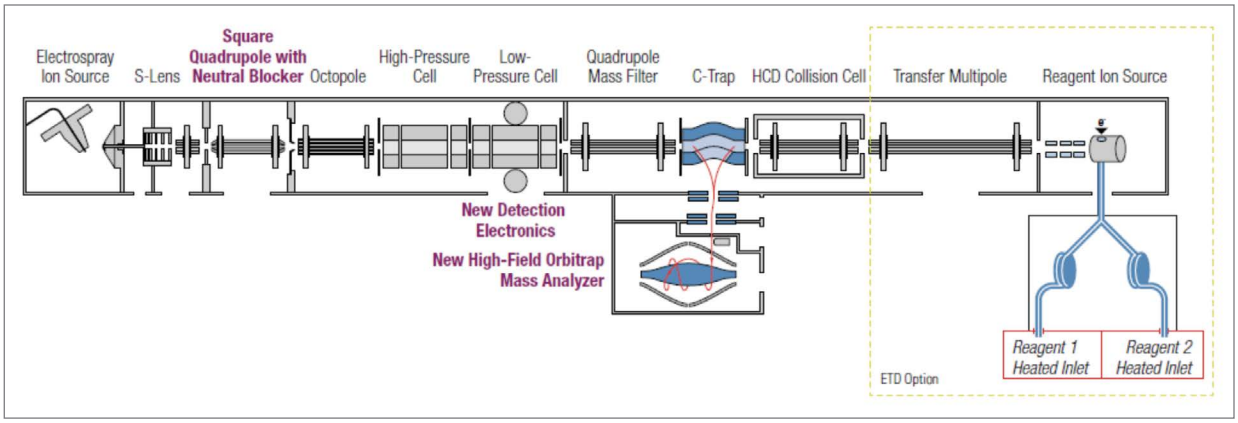


Figure 1. Instrument schematic of the Orbitrap Elite mass spectrometer. An optional electron transfer dissociation (ETD) module and major design improvements are highlighted in the figure.

Table 1. Innovations in the Orbitrap Elite instrument design and corresponding performance improvement

Innovation	Performance Improvement
Ion Trap	
New ion optics design	Prevents neutrals from entering the analyzer region
Improved electron multiplier	Better sensitivity, longer lifespan, wider dynamic range
Increased scan out speed (66 kDa/s)	Shortened cycle time and improved duty cycle
Orbitrap	
High-field Orbitrap mass analyzer	~1.8-fold increase in resolution over standard Orbitrap mass analyzer at constant transient acquisition time
High-field Orbitrap mass analyzer with new pre-amplifier	~30% higher sensitivity
Advanced signal processing	~1.7-fold increase in resolution

Experimental

Sample Preparation

E. coli cell lysate (BioRad) was reduced with 10 mM DTT for 1 h at 60 °C and alkylated with 25 mM iodoacetamide for 2 h at room temperature in 50 mM Tris-HCl, pH 8.6 with 0.1% sodium dodecyl sulfate (SDS) buffer. Reduced and alkylated proteins were precipitated overnight by the addition of five volumes of cold acetone containing 0.1 mM HCl (-30 °C) to remove chemicals and SDS. Proteins were pelleted by centrifugation (15 min at 10,000 *g* at 4 °C), air dried, and used for next steps. Each pellet (0.55 mg of protein) was digested with trypsin (ratio of trypsin to protein 1:50) in 85 mM triethyl ammonium bicarbonate (TEAB) buffer at 37 °C overnight. The enzymatic digest of reduced and alkylated *E. coli* proteins was divided into six aliquots and labeled with six TMT reagents according to the manufacturer's protocol. Samples were subsequently mixed at pre-defined ratios depending on the experiment, concentrated by Speed Vac and stored at -80 °C before further analysis.

For a set of comparative experiments on the Orbitrap Elite and the LTQ Orbitrap Velos™ platforms, the tagged lysates were mixed in equal-molar ratios (1:1:1:1:1:1). For experiments assessing the quantitative accuracy of the Orbitrap Elite instrument, this equimolar *E. coli* sample (200 ng) served as a background to which a total of 172.5 fmol (~5 ng) of a TMT-labeled digest of a standard protein mixture was added. This added mixture was a digest of 9 proteins (human serotransferrin, bovine beta-lactoglobulin, bovine serum albumin, horse cytochrome C, bovine alpha-lactalbumin, chicken ovalbumin, bovine carbonic anhydrase, bovine beta-casein, and bovine alpha-casein; Sigma) in equimolar amounts. It was aliquoted into six identical fractions labeled each with one of the TMTsixplex™ reagents, and mixed to obtain the final ratio (10:1:10:2:10:1.5).

Liquid Chromatography

Details of chromatographic settings are listed in Table 2. The total run time was 175 min.

Table 2. LC parameter settings

HPLC System	EASY-nLC II
Column	Spray Tip: 75 µm x 200 mm column packed with C_{18} 3 µm particles
Mobile Phases	0.1% formic acid in water (eluent A) 0.1% formic acid in acetonitrile (eluent B)
Gradient	5%–35% B in 150 min
Flow	300 nL/min

Mass Spectrometry

Details of the Orbitrap Elite and LTQ Orbitrap Velos acquisition methods used in experiments comparing these two instrumental platforms for identification and MS^2-based quantitation of TMT-labeled peptides are summarized in Table 3.

Table 3. Parameter settings used for comparing peptide identification performance of the Orbitrap Elite MS versus LTQ Orbitrap Velos MS

Parameter	Orbitrap Elite	LTQ Orbitrap Velos
Source	Nano-ESI	Nano-ESI
Instrument control software	Tune 2.7	Tune 2.6 SP3
Capillary temperature (°C)	250	250
S-lens RF voltage	55%	55%
Source voltage (kV)	2	2
Full MS mass range (*m/z*)	380–1600	380–1600
Full MS parameters		
Resolution settings (FWHM at *m/z* 400)	240,000	60,000
Target value	1×10^6	1×10^6
Max injection time (ms)	200	200
Dynamic exclusion	Repeat count 1	Repeat count 1
	Exclusion list size 500	Exclusion list size 500
	Exclusion duration 80 s	Exclusion duration 80 s
	Exclusion mass width relative to precursor ±10 ppm	Exclusion mass width relative to precursor ±10 ppm
MS^2 parameters		
Resolution settings (FWHM at *m/z* 400)	15,000	7,500
Target value	5×10^4	5×10^4
Isolation width (Da)	1.2	1.2
Minimum signal required	1000	1000
Collision energy (HCD)	40%	40%
Activation time (ms)	0.1	0.1
Top-N MS^2	15	15
Charge state screening on: 1^+ and unassigned rejected	Yes	Yes
Monoisotopic precursor selection enabled	Yes	Yes
Predictive AGC enabled	Yes	Yes
FT preview mass scan mode enabled	No	No
Lock mass enabled	No	No
Lowest *m/z* acquired	100	100
Max injection time (ms)	200	200

Acquisition method parameters for experiments assessing the quantitative accuracy using the MS^3 approach are summarized in Table 4. After acquiring a full scan at high resolution, a data-dependent rapid CID MS^2 scan is performed with detection in the ion trap mass analyzer. The most intense MS^2 fragment ion from a predefined mass range (*m/z* 400-800, corresponds to the region where most intense peptide fragments often appear) is then selected for higher-energy collisional dissociation (HCD) fragmentation followed by an Orbitrap detection. HCD step is carried out with an excess of collision energy effectively maximizing abundance of the reporter ions. In this case, the MS^2 CID scan is used for peptide identification, while the MS^3 HCD scan is used for relative quantitation only. Essentially, this method is very similar to the one published earlier[5] with some minor modifications. These are further discussed in Results section.

Table 4. Mass spectrometer parameter settings used for comparing a standard Top-10 HCD and MS^3-based method for assessing peptide quantitation accuracy with the Orbitrap Elite instrument

Parameter	Top-10 HCD	MS^3-based
Source	Nano-ESI	Nano-ESI
Capillary temperature (°C)	250	250
S-lens RF voltage	55%	55%
Source voltage (kV)	2	2
Full MS mass range (*m/z*)	380–1600	380–1600
Full MS parameters		
Resolution settings (FWHM at *m/z* 400)	60,000	60,000
Target value	1×10^6	1×10^6
Max injection time (ms)	100	100
Dynamic exclusion	Repeat count 1	Repeat count 1
	Exclusion list size 500	Exclusion list size 500
	Exclusion duration 80 s	Exclusion duration 80 s
	Exclusion mass width relative to precursor ±10 ppm	Exclusion mass width relative to precursor ±10 ppm
Top-N MS^2	10	10
MS^2 parameters rapid CID		
Target value	–	5×10^3
Max injection time (ms)	–	100
Minimum signal required	–	500
Isolation width (Da)	–	2
Collision energy	–	35%
Activation time (ms)	–	10
MS^n parameters HCD		
Resolution settings (FWHM at *m/z* 400)	15,000	15,000
Target value	3×10^4	3×10^4
Isolation width (Da)	1.2	4
Minimum signal required	500	200
Collision energy (HCD)	40%	50%
Activation time (ms)	0.1	0.1
MS^n mass range (*m/z*)	380–1600	400–800
Charge state screening enabled	Yes	No
Charge state rejection on: 1^+ and unassigned rejected	Yes	Yes
Monoisotopic precursor selection enabled	Yes	Yes
Predictive AGC enabled	Yes	Yes
FT preview mass scan mode enabled	No	No
Lock mass enabled	No	No
Lowest *m/z* acquired	100	100

Data Processing

Thermo Scientific Proteome Discoverer software version 1.3 with Mascot® 2.3 or SEQUEST® search engines was used for peptide/protein identification. The searches were performed against an *E. coli* taxonomy subset of Swiss-Prot® database (version 57.15). For samples containing the digest of the standard protein mixture, the search was performed against the *E. coli* database subset as above as well as against a database containing the 9 standard proteins. Resulting peptide hits were filtered for maximum 1% FDR using Percolator[8] for Mascot search or Peptide Validator for SEQUEST (Figure 2). Proteins were grouped by applying the maximum parsimony rule (i.e., the protein groups in the final report represent the shortest possible list needed to explain all confidently observed peptides). Database search parameters are detailed in Table 5.

Table 5. Database search parameter settings

Peak list generation conditions	
Total intensity threshold	1.5
Minimum peak count	1
Peptide mass range	500–7000 Da
Mascot search engine (version 2.3)	
Database	Swiss-Prot (version 57.15)
Taxonomy	*Escherichia coli*
Peptide/protein validation	Percolator
SEQUEST search engine	
Database	9 protein
Peptide/protein validation	Peptide Validator
Search parameters	
Mass tolerance (precursor)	10 ppm
Mass tolerance (fragment) HCD	20 mmu
Mass tolerance(fragment) CID	0.6 Da
Dynamic modifications	Deamidation (N, Q), Oxidation (M)
Static modifications	TMTsixplex (*N*-terminal, K), Carbamidomethyl (C)

The quantitation module within Proteome Discoverer™ software was used to assess the ratios for individually tagged *E. coli* cell lysate digest samples. The height of reporter ions detected with mass tolerance ±10 ppm was adjusted, taking into account the isotopic correction factors provided by the TMT kit manufacturer. Peptide spectra containing all six reporter ions were designated as "quantifiable spectra", and the ratios 127/126, 128/126, 129/126, 130/126, and 131/126 were calculated. A protein ratio was expressed as a median value of the ratios for all quantifiable spectra of the peptides pertaining to that protein. In a specific case when an even number of peptide spectra contributed to a given protein ratio calculation, a geometric average of the two middle values was used. For multiple analyses of the same sample, the final protein ratio was calculated as an arithmetic average of individual ratios of that given protein from replicate runs.

Quantitative precision represents the spread of the measurements and can be expressed as variability. For single-search reports, the protein ratio variability was calculated as a coefficient-of-variation (CV) for log-normal distributed data from the peptide ratios that were used, multiplied by 100 (%CV). For replicate analyses, the protein ratio variability was calculated as a CV from protein ratios in individual replicate runs. For details on statistical treatment of reporter ion quantitation data in Proteome Discoverer software, refer to the on-screen Help topic "Calculating ratio count and variability" or to the equivalent information in the user's manual.

Quantitative accuracy represents a deviation of a measured ratio value from the expected value. For assessing the quantitative accuracy, the reporter ion ratios for peptides originating from the digest of the standard protein mixture added into *E. coli* digest background were expressed relative to the 127 reporter ion. With data originating from the Top-10 HCD method, a precursor co-isolation filter of 25% was applied in Proteome Discoverer software. This eliminated peptides where contributions from co-eluting, nearly isobaric peptide species could interfere significantly with the reporter ion signals coming from the peptide of interest. The 25% threshold expresses the maximum allowed signal intensity within the isolation window that does not originate from the peptide precursor of interest. Data sets obtained with the MS^3-based method, which contained both CID spectra (MS^2 that were used for peptide identification) and HCD spectra (MS^3 that contained the quantitative information from reporter ions present), were processed with Proteome Discoverer software using the workflow outlined in Figure 2.

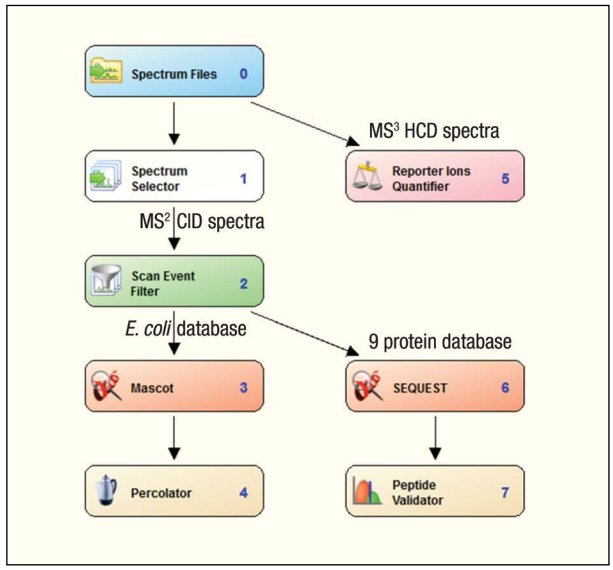


Figure 2. Proteome Discoverer workflow used to process data acquired with MS^3-based method

Results

The major design improvements (highlighted in Table 1) implemented on the Orbitrap Elite system allow for the acquisition of a full-scan mass spectrum at 240,000 resolution in less than 1 second. This represents about a 4-fold improvement compared to the resolution achievable within the same time period on an LTQ Orbitrap Velos system. Table 6 provides an overview of the mass resolution performance characteristics for these two instruments.

Table 6. Key performance characteristics, resolution (FWHM at *m/z* 400) and scan speed, of the LTQ Orbitrap Velos and the Orbitrap Elite mass spectrometers

LTQ Orbitrap Velos Resolution Settings	LTQ Orbitrap Velos Max. scan speed [Hz]	Orbitrap Elite Resolution Settings	Orbitrap Elite Max. scan speed [Hz]	Orbitrap Elite Transient time [ms]
		15,000	7.7	48
7,500	6.9	30,000	6.9	96
15,000	4.0	60,000	4.0	192
30,000	2.3	120,000	2.3	384
60,000	1.2	240,000	1.2	768
100,000	0.5	480,000*	0.5*	1536

*These settings can only be accessed using the developer's kit

The flexibility built into hybrid ion trap-Orbitrap systems allows the high resolution to be 'traded in' for a higher acquisition speed. As a consequence, the Orbitrap Elite permits the acquisition of up to 8 full-scan MS or 8 MS^2 spectra at mass resolution 15,000 within 1 second.[1] For TMT-labeled peptide analyses, instrument resolution settings of 15,000 at *m/z* 400 result in an effective mass resolution of over 27,000 for TMT reporter ions with their mass around *m/z* 130. In most cases, this is sufficient to resolve the reporter ion signals from chemical interferences, a prerequisite for maximizing the quantitative performance of this technique (see further text for discussion of quantitative analysis).

Comparison of Orbitrap Elite and LTQ Orbitrap Velos Instruments – Peptide/Protein Identifications

To determine whether the improvements in overall cycle time and sensitivity of the Orbitrap Elite would translate into higher numbers of peptide/protein identifications, we performed a set of experiments using an equimolar mixture of TMTsixplex-labeled digest of *E. coli* cell lysate. A series of varying sample loads in two technical replicates was used in this experiment. The rationale was that an instrument running under sample-limited conditions, common in real-world applications, would test the benefits of increased sensitivity and scan speed.

The same chromatographic conditions were maintained for the comparative analyses. Key acquisition parameters such as target values, maximum ion time, and precursor ion isolation width were kept identical on the LTQ Orbitrap Velos and Orbitrap Elite instruments (Table 3). The important differences were in the resolution settings used for full-scan MS analysis and for detection of HCD fragmentation spectra. Specifically, for all sample loads, the full-scan MS analysis was carried out at 60,000 FWHM on the LTQ Orbitrap Velos MS and 240,000 FWHM on the Orbitrap Elite MS. These settings were chosen so that the speed of full-scan MS acquisition for both instruments was practically identical (1.2 Hz in both cases, Table 6). The resolution for detecting HCD fragmentation spectra was set to 7,500 on the LTQ Orbitrap Velos MS and 15,000 on the Orbitrap Elite MS. Figure 3 provides a schematic representation of the Top-15 HCD method showing average cycle times achieved for 500 ng sample load. Adhering meticulously to the conditions and method settings above allowed us to perform a true head-to-head comparison between the two instruments.

At low sample loads (20 ng of *E. coli* cell lysate digest on column), fill times for both instruments reached the maximum injection time of 200 ms on almost all of the HCD fragmentation scans. This confirmed that the experiment was carried out under sample-limited conditions, and that the results obtained by the instruments reflect their sensitivity differences. The Orbitrap Elite system identified 785 protein groups compared to the LTQ Orbitrap Velos system's 600 protein groups, an increase of more than 30%. The numbers of unique peptides identified at a 1% FDR were 7663 and 6377 on the Orbitrap Elite and LTQ Orbitrap Velos instruments, respectively, an increase of 20%. At higher sample loads (80 and 500 ng), the Orbitrap Elite system also outperformed the LTQ Orbitrap Velos system in both the number of unique peptides and protein groups identified (Figure 4). It should be noted that the increase in HCD acquisition rates did not come at the expense of the quality of the spectra (Figure 5, see discussion further in the text).

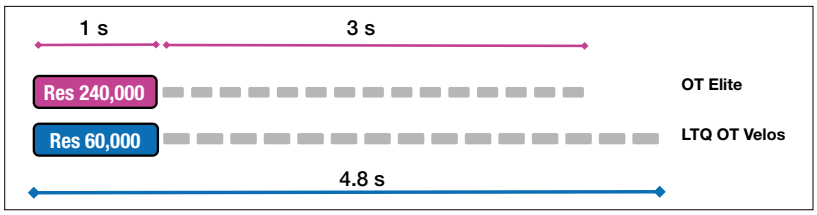


Figure 3. Graphical representation of Top-15 HCD method. The time needed for completion of individual scan events is scaled relative to each other.

Similar experiments performed using the Orbitrap Elite system at reduced MS resolution settings (120,000 or 60,000 for full-scan MS using the Top-15 HCD method) resulted in a 30%–35% increase in peptide identifications compared to the standard Top-15 HCD used with the LTQ Orbitrap Velos system (data not shown). The observed gain in the number of identified peptides on the Orbitrap Elite system can be ascribed, for the most part, to faster/more MS^2 acquisitions.

Quantitative Performance Figures of Merit

Quantifiable peptides

This figure of merit represents the number of peptides whose fragmentation spectra contain all six reporter ions, out of the total number of identified peptides. The percentages of quantifiable peptides were approximately 90% and 96% for 20 ng and 500 ng sample loads, respectively. These values were similar for both instruments. The Proteome Discoverer software version 1.3 is, nevertheless, capable of processing spectra with one or more reporter ion channels missing. This aspect of data processing is fully user-definable.

The quality of HCD fragmentation spectra generated by either the LTQ Orbitrap Velos or the Orbitrap Elite systems is highlighted in Figure 5. The spectra generated at 40% normalized collision energy contain both rich peptide sequence records and intense reporter ion signatures. Normalized collision energy values used for TMT-labeled peptides rarely require optimization, a setting of 35%–40% will provide well-balanced spectra for a majority of analyzed peptides.

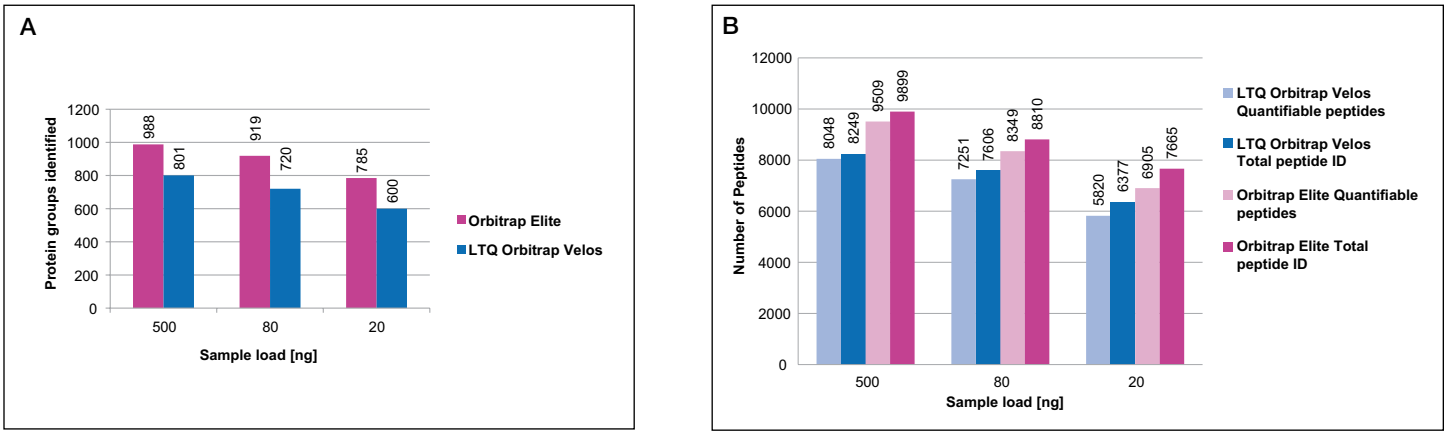
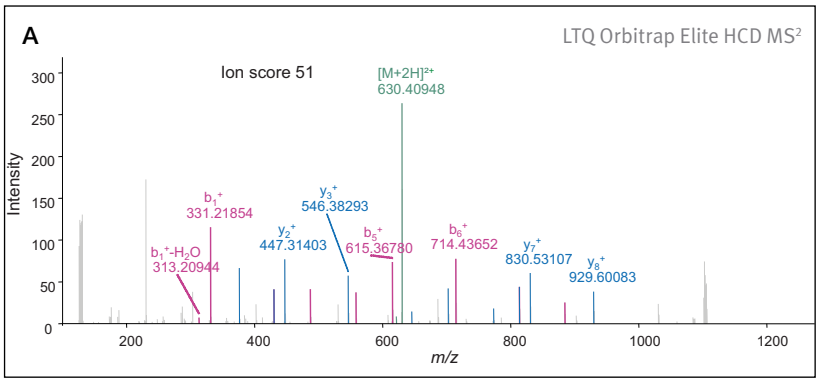


Figure 4. Comparison of identification and quantification results for the Orbitrap Elite and LTQ Orbitrap Velos instruments. Number of protein groups (A) and total peptide identifications (B) at 1% FDR obtained when analyzing TMT-labeled *E. coli* cell lysate digest is shown for various sample loads. The number of quantifiable peptides is also shown. Results represent an average of two replicate runs for each sample load.



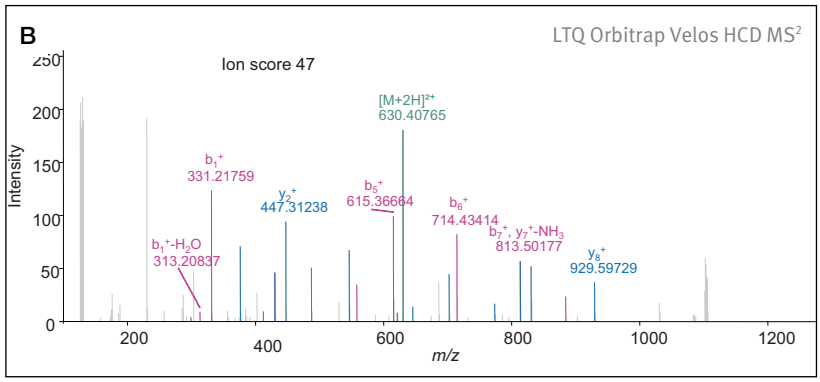


Figure 5. Quality of HCD spectra. A) HCD fragmentation spectrum for TVGAGVVAK peptide (elongation factor Tu 1 *E. coli* A7ZSL4) acquired on the Orbitrap Elite system. B) HCD fragmentation spectrum of the same peptide acquired with the LTQ Orbitrap Velos system. On the Orbitrap Elite system, the required high spectral quality is obtained with a considerably shorter analysis time (0.2 ms versus 0.33 ms for the LTQ Orbitrap Velos system).

Quantitative precision

Quantitative precision expressed as %CV is an important measure of the quality of quantitation experiments. Lack of quantitative precision is often a result of low signal-to-noise precursor ion measurements. Figure 6 summarizes the quantitative precision results for TMT-labeled *E. coli* cell lysate digest at various sample loads when analyzed with the Orbitrap Elite instrument. The expected dependence of protein ratio variability on signal intensity can be seen. The highest sample load, 500 ng, produced the greatest signal intensity and the lowest protein ratio variability. For the 500 ng sample, approximately 90% of the quantified proteins had variability below 10%. The remaining 10% had variability of 10%–20% or >20%. For the lowest sample load, 20 ng, approximately 70% of the quantified proteins had variability below 10% while the other 30% had variability of 10%–20% or >20%.

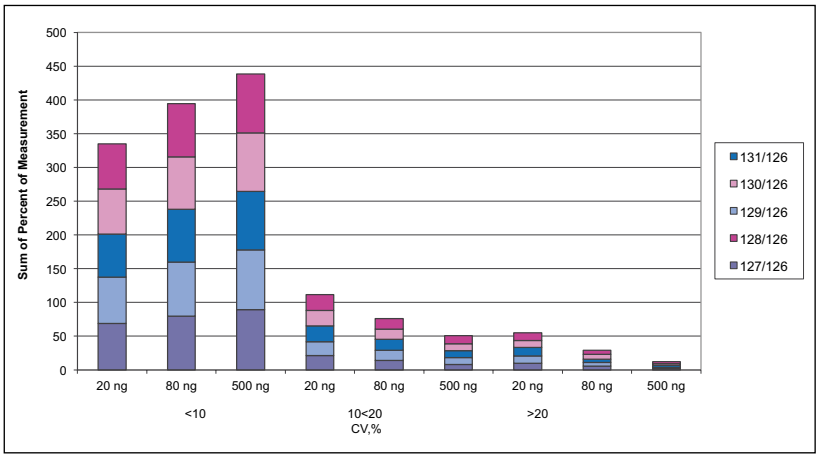


Figure 6. Quantitative precision of protein ratio measurements. Variability (%CV) of protein ratios was calculated for different sample loads (20, 80, and 500 ng) of TMTsixplex-labeled *E. coli* cell lysate digest. The values reflect results from a duplicate analysis of each sample.

Quantitative accuracy

Poor accuracy is considered a far more significant problem with isobaric tagging. Two main factors contribute to quantitative inaccuracy: isobaric interferences in the precursor and reporter ion regions. To assess the impact of these interferences, a sample containing a TMT-labeled digest of a standard protein mixture was added to the TMTsixplex-labeled *E. coli* cell lysate digest (Figure 7). The TMT-labeled digest of the standard protein mixture was prepared so that the ratio between the reporter ions should be 10:1:10:2:10:1.5. The fidelity of this ratio has been verified in a separate experiment.

A background ion/impurity can be close in mass to the reporter ion in the MS^2 spectrum, interfering with peak area estimation if the two peaks are not resolved. It is an inherent problem for all mass analyzers with inadequate mass resolution. It is a legitimate concern not only for unit resolution instruments such as ion traps or quadrupole-ion trap hybrids, but also time-of-flight instruments. Because the resolution of a time-of-flight analyzer drops considerably in the low-mass region, even the modern high-resolution time-of-flight analyzers achieve only modest resolution in the region of TMT/iTRAQ reporter ions. Orbitrap-based instruments, on the other hand, gain extra resolution in the low *m/z* region of the spectrum. Detection of HCD MS^2 spectra at a resolution setting of 15,000 at *m/z* 400 on the Orbitrap Elite system translates to an effective resolution of more than 27,000 for ions at *m/z* 126-131. Figure 8 captures a reporter ion region of a TMT-labeled peptide spectrum obtained using HCD fragmentation with detection at 15,000 resolution. The six TMT reporter ions are shown with their mass errors in ppm. The effective resolution in the region of TMT reporter ions approaches 28,000 and is enough to separate A+1/A-1 ions of 127/129 reporters ($C_7{}^{13}C_1H_{16}{}^{15}N_1$, 128.1281) from the reporter ion at *m/z* 128 ($C_6{}^{13}C_2H_{16}N_1$, 128.1344). At this resolution, an accurate ratio calculation for the 128 ion can be achieved using a mass tolerance window of up to 10 ppm without applying isotope correction factors (insert). Increasing the resolution to 30,000 for HCD scans would translate into a 56,000 resolution for TMT reporter ions. In that case, a complete separation of A+1(2)/A-1(2) isotopes potentially interfering with TMT reporter ions is obtained resulting

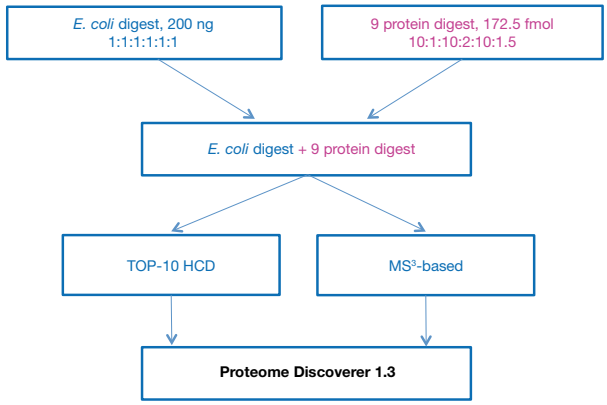


Figure 7. TMTsixplex-labeled digest of a standard protein mixture (172.5 fmol) was added to the background of TMTsixplex-labeled *E. coli* cell lysate digest. Top-10 HCD or MS^3-based data acquisition methods were used on the Orbitrap Elite instrument.

in increased dynamic range for isobaric labeling. Reliable accurate-mass measurement enables the filtering out of any potential interference or background ions by using a very tight mass tolerance setting. A mass tolerance ±10 ppm was used for reporter ion detection in all experiments described herein.

The second major issue with isobaric tagging is that of precursor co-isolation interference. Isolating other peptides together with the peptide of interest manifests itself by a ratio compression. Comparing ratios obtained for proteins of the standard digest analyzed alone and when added to the background of the TMT-labeled *E. coli* cell lysate digest should reveal the extent of potential ratio compression.

Quantitative accuracy for the peptides with a true ratio of 10 was 9.76 (Figure 9A) for the neat sample. As expected, significant compression of measured ratios was noted for the 9 proteins spiked into the complex matrix (Figure 9B). The ratio was reduced from 9.76 to 5.64 on average for 126, 128 and 130 reporter-ion channels.

Recently, Ting *et al.* proposed an MS^3-based approach addressing the co-isolation problem.[5] We employed a similar method outlined in Table 4. We found that the most intense fragments presented themselves within the *m/z* 400-800 range of the CID fragmentation spectra. We thus used that range for MS^3 precursor selection instead of the 110%-165% of precursor *m/z* as described in Ting's

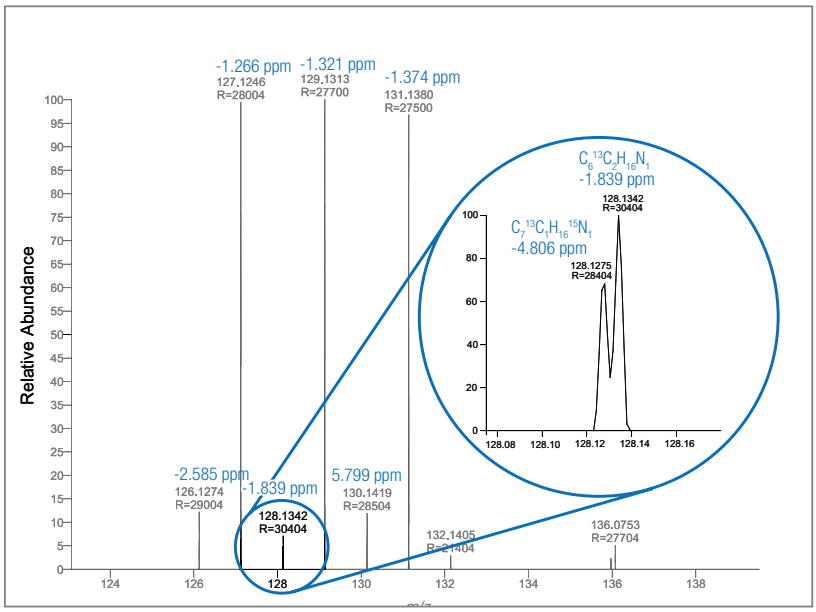


Figure 8. Resolving TMT isobaric interferences with the Orbitrap Elite system. The low *m/z* region of typical HCD fragmentation spectrum acquired for peptide labeled with tags at 1:10:1:10:1:10 ratios, is shown. A detail (insert) capturing 128 reporter ion demonstrates that effective resolution in excess of 28,000 is required to separate isotopes A+1 of 127 or A-1 of 129 reporter ions from the 128 reporter ion. Mass accuracy and resolution for each report ion are indicated.

We employed a Top-10 HCD method on the Orbitrap Elite instrument with resolution settings of 60,000 and 15,000 for full-scan MS and MS^2, respectively (Table 4). Before the statistical data processing was done, we used Proteome Discoverer software to filter out fragmentation spectra whose co-isolation interference exceeded 25% of the precursor ion current in the precursor isolation window. This filtering step was a precautionary measure used to remove those spectra whose ratios had been very likely significantly affected by co-fragmented peptide reporter ions. The filtering step removed 72 spectra out of total 323 quantifiable spectra assigned to the peptides from the standard proteins spiked into the mixture (FDR <1%).

paper. A comparative analysis employing both methods showed a 20% improvement in the number of quantified peptides using our modified settings (data not shown). The improved scan rate of the Orbitrap Elite instrument allowed us to keep the total cycle time for the MS^3 method under 4 seconds. As a result, no significant decrease in sequence coverage was observed for the standard proteins analyzed either neat or spiked into the *E. coli* digest background (Figure 10).

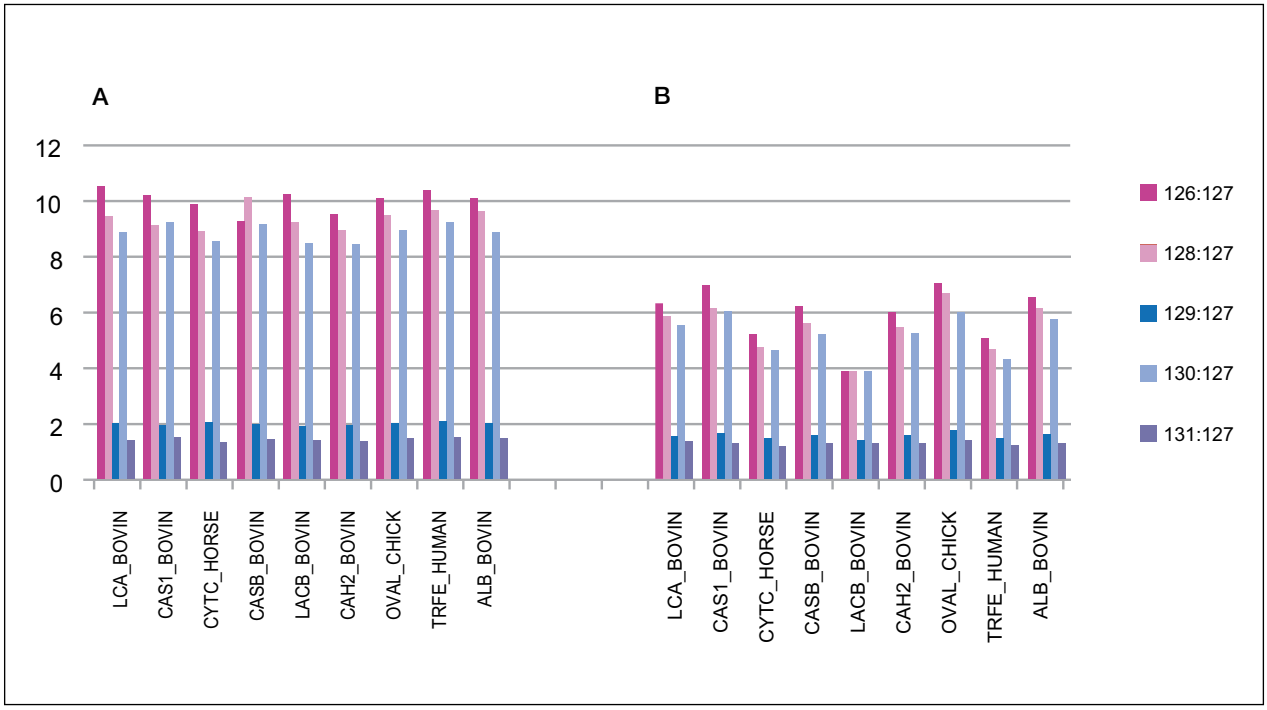


Figure 9. Ratios for TMTsixplex-labeled standard protein mixture digest. A) The expected ratio 10:1:10:2:10:1.5 for TMT-labeled standard protein mixture digest was verified in a separate analysis of the neat sample. B) Ratios for the same standard protein mixture digest when analyzed in the background of TMT-labeled *E. coli* cell lysate digest. Note a significant compression of ratios for TMT reporter ion channels 126, 128, and 130.

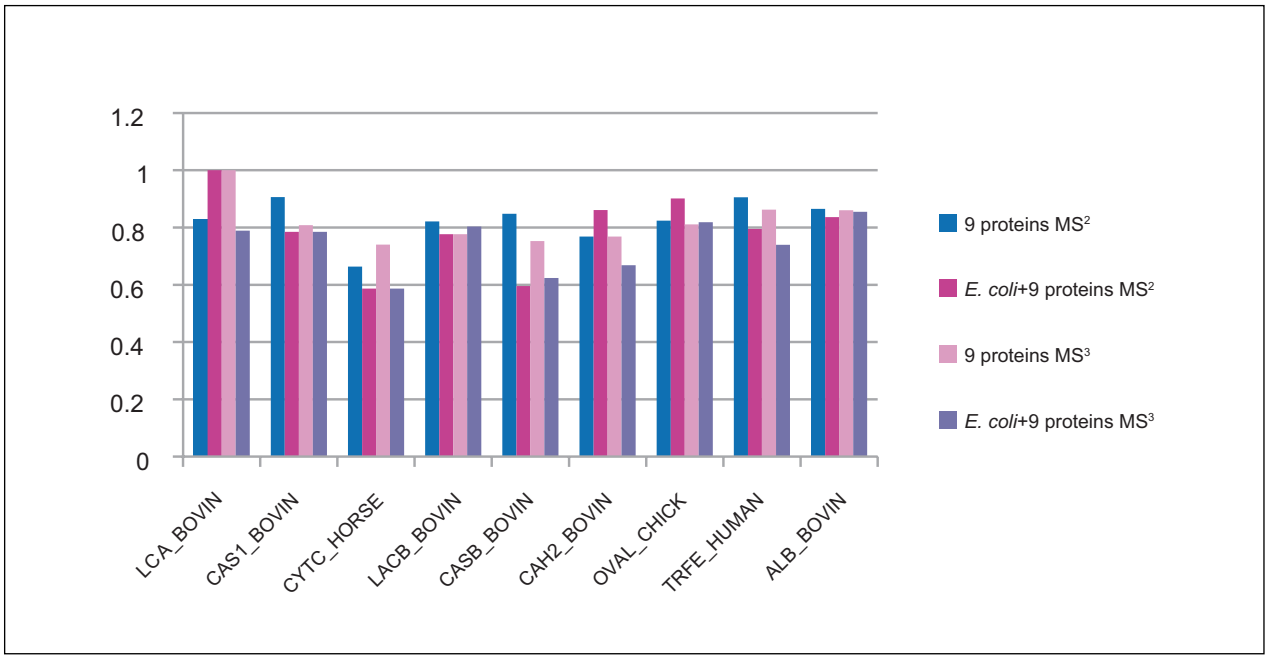


Figure 10. Sequence coverage for standard proteins analyzed neat and spiked into *E.coli* background as obtained with Top-10 HCD and MS^3 methods

Table 7. Quantitative accuracy of the Orbitrap Elite instrument for TMT-labeled complex sample analysis. Quantitative accuracy was calculated for peptides of TMT-labeled standard protein mixture digest spiked into a complex background of TMT-labeled *E. coli* cell lysate digest.

Value	Expected Ratio	Top-10 HCD No Background	Top-10 HCD *E. coli* Background	MS^3-based *E. coli* background
# quantified peptides		436	251	190
Median ratio spiked proteins TMT label: 126, 128,130	10	9.76	5.64	7.82
Median ratio spiked proteins TMT label: 129	2	1.96	1.52	1.70
Median ratio spiked proteins TMT label: 131	1.5	1.49	1.27	1.32

The quantitative accuracy of the Orbitrap Elite instrument for analysis of a TMTsixplex-labeled digest of standard proteins spiked into the complex background of a TMT-labeled *E. coli* cell lysate digest is summarized in Table 7. For higher ratios (theoretical ratio 10:1, reporter ion channels 126, 128, 130) the median peptide ratio increased from 5.64 to 7.82. The MS^3-based approach significantly improved quantitation results by reducing the interferences from co-isolated TMT-labeled peptides. Nevertheless, some ratio compression is still observed, especially for the largest ratios, most likely due to the matrix complexity and, in general, the very low amounts of proteins spiked into it. The improved quantitation accuracy achieved with the MS^3-based method comes at the price of a somewhat decreased number of quantifiable peptides (approximately 24%).

Conclusion

The number of proteins and peptides identified increased by 30% and 21%, respectively, when analyzing 20 ng of a TMTsixplex-labeled *E. coli* cell lysate digest using the Orbitrap Elite mass spectrometer compared to the results obtained with the LTQ Orbitrap Velos mass spectrometer.

The percentage of quantifiable peptides (with MS^2 spectra containing all six reporter ions) exceeded 90% for sample loads higher than 20 ng on column, and approached 97% for a 500 ng sample load for both systems.

Higher-resolution analysis resulted in better mass accuracy for the reporter ion measurements. This in turn allowed use of tighter mass tolerances for extraction of reporter ion peaks, thereby eliminating impurities and ensuring more accurate quantitation.

Quantitative precision for TMT-labeled samples analyzed on the Orbitrap Elite instrument, expressed as relative variability, was less than 10% for about 90% quantifiable peptide ratios for 500 ng sample load. 70% of peptide ratios met the same specification when a 25-fold lower sample load was used.

The co-isolation effect linked to the selection of additional peptides during precursor ion isolation was clearly demonstrated using a complex proteome as a background matrix. Our results show that this problem can be addressed to a large extent by relying on the reporter ion intensities extracted from MS^3 spectra.

The Orbitrap Elite instrument significantly outperformed the LTQ Orbitrap Velos instrument with respect to both peptide/protein identification and quantitation. The novel design of the Orbitrap Elite instrument combines a high-field Orbitrap mass analyzer, advanced signal processing, and improved ion optics. The result is increased sensitivity along with a fourfold increase in resolution that can be either used outright or “traded in” to some degree for an increased scan rate. These features are reflected in an overall performance enhancement of the Orbitrap Elite mass spectrometer, enabling more comprehensive identification and more precise relative quantitation of complex proteomes.

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