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

Terry Zhang¹, Rosa Viner¹, Seema Sharma¹, Vlad Zabrouskov¹, Jin Zou², Qiang Zhang², Guangdi Wang² ¹Thermo Fisher Scientific, San Jose, CA; ²Xavier University of Louisiana, New Orleans, LA

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 TMT⁶-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 expression⁴. Isobaric tags allow for accurate and precise relative quantitation of peptides in multiple samples simultaneously^{1,4}. Higherenergy collisional dissociation (HCD) has previously proved to be very effective for mass analysis of isobarically-labeled peptides⁵. 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 cell⁶. Together, they provided robust and sensitive relative quantification of TMT- and iTRAQ-labeled peptides. 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

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 experiments⁸. 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

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 17 β -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 C₁₈, 15 cm or 20 cm x 75 μ m 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.

Quantification Reproducibility Comparison



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.

tmt_ecoli_500ng_12iso_180min_80ng_ce45	12/6/2009 4:26:34 PM
--	----------------------

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.

Workflow Editor (TMTsa	mple_search_all_four)	× Administrati	on x TMT.	ss_clean4_clea	an3.msf*	×)own-	reaul	ation	at 24	hrs			
Proteins Peptides Se	earch Input Result Filters	Peptide Confidence	Search Summary	Quantitation S	Summary			rogai						
Drag a column headar	here to group by that co	olumn				/								
Sequence	127/126	130/126	128/126	131/126	Charge	129/126	m/z [Da]	MH+ [Da]	ΔM [ppm]	RT [min]	First Scan	Last Scan	MS Order	- 2
	સ્ લ		9			- /	-	-	-	1	-	-		
DQTEYLEER	0.871	0.912	1.021	0.998	2	1.053	770.86713	1540.72698	-3.84	124.38	14334	14334	MS2	_
DQTEYLEER	0.940	0.921	1.005	0.994	2	0.922	770.86719	1540.72710	-3.76	125.49	14473	14473	MS2	_
DSHPFDLGLYNEAVK	0.811	0.727	0.739	0.666	3 /	0.840	764.73413	2292.18784	-1.51	200.26	26096	26096	M52	
DSNLTLQEK	0.966	1.000	0.776	0.812	2/	0.991	817.94977	1634.89226	-4.85	122.52	13551	13551	MS2	
DSNLTLQEK	1.136	1.175	0.902	0.904	1	1.013	817.95068	1634.89409	-3.73	138.74	16313	16313	MS2	
EASDYLELDTIK	0.783	0.848	0.661	0.631	12	1.032	992.52667	1984.04607	-3.36	206.61	27067	27067	MS2	
EGVLTLWR	0.924	0.887	0.983	0.917	12	1.038	666.37891	1331.75054	-1.19	214.66	28277	28277	M52	
EIQSSISGVTAAYNR	0.985	1.080	0.975	1.029	3	0.844	652.00562	1954.00229	-2.90	178.89	22627	22627	MS2	
EIQSSISGYTAAYNR	1.172	1.048	0.686	0.592	2	1,204	977.50562	1954.00395	-2.05	178.78	22608	22608	M52	
EIOSSISGVTAAYNR	0.884	0.920	1.000	0.902	3	0.839	652.00641	1954.00467	-1.68	178.68	22592	22592	M52	
EIVYLPCIYR	0,996	0.998	0.526	0.558	2	1.067	842,44897	1683,89067	-4.23	224.50	29731	29731	MS2	_
ELSNVLAAMR	0.898	0.996	0.750	0.62	2	0.922	731.39966	1461,79204	-0.90	182.77	25651	25651	MS2	
ETOPPVALk	1,186	1.226	1,292	409	2	1.030	785,46204	1569,91680	-5.41	127.03	14485	14485	MS2	
FADSLGIPFLETSAK	0.700	0.767	0.235	0.245	2	1.025	1092,10120	2183,19512	-2.28	191.73	27411	27411	M52	
FGTNIk	0.985	0.905	0.738	0.745	2	1.013	633,87305	1266.73882	-5.55	128.04	14691	14691	M52	
FPTVPIVk	0.968	0.965	0.860	0.837	2	0.973	744 46344	1487 91960	-2.84	211.93	27875	27875	MS2	
	0.978	0.994	0.793	0.894	2	1.004	744.46381	1487.92034	-2.35	212.20	27916	27916	MS2	
ETMANIE .	0.570	0.934	0.775	0.659	2	1.017	736 42908	1471 85088	-3 56	156 44	20425	20425	MS2	_
EVTAEL	0.892	0.994	0.325	0.304	2	1.006	650 38562	1200 76306	-5.50	162 34	21620	21620	MS2	
SUTACL	0.072	0.030	0.470	0.304	2	1.000	650.00002	1200 76665	-3.61	162.07	21655	21655	MED	
100/100 Uni-hite. for	1 100/100 1	20/10/ Crush 1/	odi oć Usviskihu Po	1 1014	~ .	at land Count	101/102 0-0-6	1299.70005	-5.01	102.47	LIUSS	21055	POL	1
123/120 variability [70]	130/120 1		20/150 Aduidonin's E	- 131/1	- 20	51/120 COUR	151/120 Validui	mch [10]		Uc	scription			
	7.8 1.003	- 20		12.4	0.981	-30		17.9 Prote	in diculfide-icome	aca 14 precur	or Ing=645	# SD[647 E 25 E	1#	- 1
Sequence	127(126	130/126	128/126	131/126	Charge	129/126	m/z [Da]	MH+ [Da]	AM form]	RT [min]	First Scan	Last Scan	MS Order	
GEEVAOEk	0.903	0.918	0.705	0.668	2	1.012	756,92462	1512,84197	-2.87	159.43	21026	21026	MS2	
GEMTIK	0.870	0.964	0.840	0.872	2	0.987	633 35907	1265 71086	-5.32	122 51	13548	13548	MS2	- 1
GETHER	0.982	0.935	0.731	0.803	2	0.967	745 44421	1489 88115	-4 49	158 31	20797	20797	M52	_
GETHELK	0.917	0.893	0.751	0.000	2	1.034	745 44543	1489.88359	-7.85	185.23	23667	23667	MS2	_
GGELLIHTUR	0.517	0.050	0.005	0.500	3	1.001	533 32428	1597 95829	-2.82	190.36	27126	27126	MS2	
GGELLIHTUP	1 226	1 514	1 410	1 216	3	1.029	533 32477	1597 95975	-1 00	248 14	33000	33000	MS2	
GGI GPI NIPI LADVITE	1.061	0.944	0.938	0.976	2	1.036	982 57013	1964 13298	-2.50	193 73	27785	27785	MS2	
GGI GPI NIPI I ADVTD	1.034	1.047	0.950	1.014	2	0.020	982 57074	1964 13420	-1.82	193.73	27822	27822	MS2	
COSIDUTI TEOFATCH	0.920	0.972	0.919	0.061	2	0.909	731 74202	2103 21421	-1.00	210 (5	20022	20022	MCO	
	0.020	0.072	0.945	0.901	3	0.009	097 00000	1074 05450	-1.16	120.00	16076	16076	MCO	_
	0.791	0.955	0.917	1.002	2	0.900	937.90090	1874 05450	-7.00	130.03	16743	16742	MED	
CIELDEDEEEL	0.931	0.941	0.047	0.041	2	0.024	037 00240	1974 05050	-3.74	167.90	20747	20747	MCO	_
CLEIDEDEEEK	0.884	0.930	0.947	0,941	2	0.980	937.98340	1074.95952	-2.18	167.02	20747	20797	MS2	
	0.891	0.946	1.003	0.918	2	1.022	931,98328	1674.95989	-1.98	166.75	20/07	20/07	MSZ)
		ADEE JEADE D		Deside(s) From	1/50201-0	sale horizonal				_	-		1	

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. TMT⁶-labeled *E. coli* cell lysate digest was used as a sample.

HCD Parameters Optimization

Introduction of an integrated C-trap/HCD collision cell combination⁶ 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.





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



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

- Thompson, A.; Schafer, J.; Kihn, S.; Schwarz, J.; et al. (2003) Tandem mass tags: a novel quantitation strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal. Chem.* 75, 1895-1904.
- 2. Ross, P.I.; Huang, Y.L.N.; Marchese, J.N.; et al. (2004) Multiplexed protein quantitation in *saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 3, 1154-69.
- 3. Yang, Y.; Zhang, S.; Howe, K.; et al. (2007) A Comparison of nLC-ESI-Ms/MS and nLC-MALDI-MS-MS for GeLC-Based Protein Identification and iTRAQ-Based Shortgun Quantitative Proteomics. *J. of Biomolecular Techniques*, 18:226-237.
- 4. Ong, S. E. and Mann, M. (2005) Mass spectrometry-based proteomics turns quantitative. *Nat. Chem. Biol.* 1, 252-262
- 5. Olsen, J.; Macek, B.; Lange, O.; et al. (2007) Higher-energy C-trap dissociation for peptide modification analysis. *Nature Methods* 4, 709-712.
- 6. Olsen, J.; Schwartz, J.C.; Griep-Raming, J.; et al, (2009) A dual pressure linear ion trap Orbitrap instrument with very high

Window	FIOLEINS	replices	70 Quan rep	Unique rep
2.2	680	7267	80	4769
1.2	691	7229	77	4819

sequencing speed. Mol. Cell. Proteomics 8, 2759-2769.

iTRAQ is a registered trademark of Applera Corp. Tandem Mass Tag and TMT are registered trademarks of Proteome Sciences plc. Mascot is a trademark of Matrix Science Ltd. All other trademarks are the property of Thermo Fisher Scientific Inc. and its subsidiaries. This information is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others.



Part of Thermo Fisher Scientific