

# Relative Quantitation of iTRAQ Labeled Protein Mixtures Using High Energy Collisional Dissociation on an LTQ Orbitrap

Terry Zhang, Vlad Zabrouskov, Yingying Huang, Ken Miller

Thermo Electron, San Jose, CA, U.S.A.

**Thermo**  
ELECTRON CORPORATION

## Overview

**Purpose:** Simultaneous quantitative and qualitative analysis of iTRAQ™ labeled peptides with High Energy Collisional Dissociation (HCD) on the LTQ Orbitrap.

**Method:** 4-plex iTRAQ labeled peptides from a mixture of nine enzymatically digested standard proteins were analyzed using nano LC-MS/MS on an LTQ Orbitrap hybrid mass spectrometer with HCD functionality.

**Results:** The results demonstrate that HCD is a powerful tool for quantitation of iTRAQ labeled peptides. It is highly reproducible and yields accurate quantitative results. In addition, high resolution of iTRAQ reporter ions improves quantitation precision by separating reporter ion signals from isobaric interferences.

## Introduction

Accurate quantitation of differentially expressed proteins is a continuing challenge in proteomics research. Many analytical methods have been developed, including several isotope labeling techniques: ICAT<sup>®</sup>, SILAC<sup>™</sup>, <sup>13</sup>C<sub>6</sub>-AQUA<sup>™</sup> and iTRAQ. LC-MS/MS with linear ion trap (LIT) technology is a proven method for sensitive and robust protein identification in bottom-up experiments. Quantitation of low mass reporter ions created during MS/MS analysis of iTRAQ labeled peptides can be performed on an LIT system by MS<sup>n</sup> or Pulsed-Q Dissociation (PQD) methods. It can also be performed using High Energy Collisional Dissociation (HCD) on the LTQ Orbitrap. HCD is accomplished by raising the voltage offset between the LIT and the C-trap of the Orbitrap. As a result, ions accelerate as they leave the LIT and are fragmented using nitrogen collision gas in the C-trap. The resulting primary and secondary fragments are then measured in the Orbitrap mass analyzer. The fragmentation technique produces rich "triple quad" like fragmentation patterns including fragments in the low m/z range. High mass accuracy of the peptide fragments and high resolution of the iTRAQ reporter ions are ideally suited for simultaneous confident identification and quantitation in complex protein digests.

## Methods

**Sample:** Four separate 9 protein mixture digests with protein concentrations ranging from 4 to 600 fmol were labeled with the four iTRAQ mass tags of 114, 115, 116 and 117 (ratio of 1:1.31:1.31:1.31) individually. The labeled samples were then combined and cleaned with RP C18 trap column, followed by an LC/MS<sup>n</sup> analysis on LTQ Orbitrap with HCD functionality.

**LC/MS:**  
HPLC System: Surveyor™ MS Pump with a flow splitter  
Column: Agilent ZORBAX™ 300SB C18 trap column (0.3mm x 5mm)  
Mobile Phase: ProForm™ Biobasic™ C18 column (7µm 10 x 150mm)  
A: Water, 0.1% formic acid; B: Acetonitrile, 0.1% formic acid  
Gradient: 0-40% B in 90 minutes  
Flow Rate: 300nL/min, post split  
Mass Spectrometer: LTQ Orbitrap™  
Spray Voltage: 2.0 kV  
Capillary Temp: 160 °C  
Cilipatory Voltage: 42.0V  
Tube Lens: 150.0V

**MS/MS:**  
2: Labeled, 750 ms max ion time  
MS: 300-1200 m/z  
Top Three Data Dependent MS<sup>n</sup> acquisition  
Dynamic Exclusion: Repeat count 2, Duration 30sec, Exclusion duration 90sec  
Collision Energy: 50%, Activation q: 0.13

**Data Processing:** BioWorks™ 3.3 with SEQUEST™

Based for peptide ID based on protein probability 1e-003. Peptide probability 1e-002  
Quantitation based on iTRAQ reporter ions was accomplished by PepQuan software within BioWorks 3.3, only data points that fell within 2 standard deviations from the average ratio for each protein were considered.

## Results

Figure 1 shows the HCD-based experimental workflow.

After optimization of experimental conditions, 55% of collisional energy was used. An MS<sup>n</sup> target of SE5 with 2 microscans was used for improved MS/MS sensitivity and spectral quality. Six repeated runs were conducted to evaluate the method reproducibility. Figure 2 shows the base peak profiles from the six repeat runs. BioWorks 3.3 was used for data processing. Protein identification was conducted first taking into account iTRAQ modifications (K and N-termini) and 114-1026. Nine proteins were identified with a minimum probability of 1e-3 and with at least two peptides identified. The protein ratios were calculated by PepQuan (BioWorks 3.3).

FIGURE 1. HCD method setup workflow

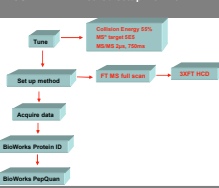


FIGURE 2. Base peak chromatograms of six repeat runs of protein mixture analysis

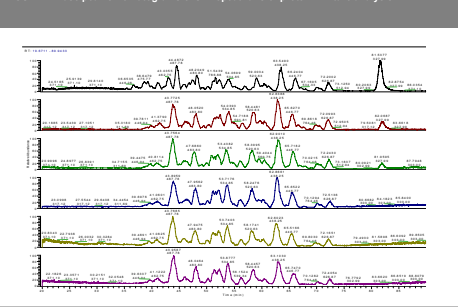


Figure 3 shows an example of Bovine Cytochrome C processed by BioWorks 3.3. The protein was identified with 7 peptides (RMS=5 ppm). The ratio of the reporter ions was calculated by the software from each HCD spectrum. The protein quantitation was achieved by taking the average of each peptide's iTRAQ reporter ion ratios (filtered with 2X standard deviation). The standard deviation ranged from 0.14 to 0.23 and CVs ranged from 10% to 16%. Figure 4 is an example of an HCD MS/MS spectrum and the iTRAQ reporter ion ratios, calculated by BioWorks 3.3.

FIGURE 3. Protein identification and iTRAQ reporter ions quantification using BioWorks 3.3

Protein	Peptide	MS	AM	ppm	z	114/114	115/114	116/114	117/114
Cytochrome C	114026	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114027	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114028	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114029	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114030	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114031	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114032	114	114	1.00	1.00	1.00	1.00	1.00	1.00
Serum Albumin	114033	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114034	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114035	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114036	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114037	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114038	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114039	114	114	1.00	1.00	1.00	1.00	1.00	1.00
G3P	114040	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114041	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114042	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114043	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114044	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114045	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114046	114	114	1.00	1.00	1.00	1.00	1.00	1.00
Ovalbumin	114047	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114048	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114049	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114050	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114051	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114052	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114053	114	114	1.00	1.00	1.00	1.00	1.00	1.00
Myoglobin	114054	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114055	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114056	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114057	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114058	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114059	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114060	114	114	1.00	1.00	1.00	1.00	1.00	1.00
Cytochrome C	114061	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114062	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114063	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114064	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114065	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114066	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114067	114	114	1.00	1.00	1.00	1.00	1.00	1.00
Lysozyme	114068	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114069	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114070	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114071	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114072	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114073	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114074	114	114	1.00	1.00	1.00	1.00	1.00	1.00
Carbonic Anhydrase	114075	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114076	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114077	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114078	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114079	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114080	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114081	114	114	1.00	1.00	1.00	1.00	1.00	1.00
β-Casein	114082	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114083	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114084	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114085	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114086	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114087	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114088	114	114	1.00	1.00	1.00	1.00	1.00	1.00
α-Lactalbumin	114089	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114090	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114091	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114092	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114093	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114094	114	114	1.00	1.00	1.00	1.00	1.00	1.00
	114095	114	114	1.00	1.00	1.00	1.00	1.00	1.00

	115/114	116/114	117/114
MEAN	1.39	1.46	1.21
STDEV	0.14	0.23	0.17
CV	10.9%	16.4%	15.0%

FIGURE 4. HCD Spectrum of 2+ peptide DJSPDPLK\* and the iTRAQ reporter ion quantitation ratio from BioWorks 3.3

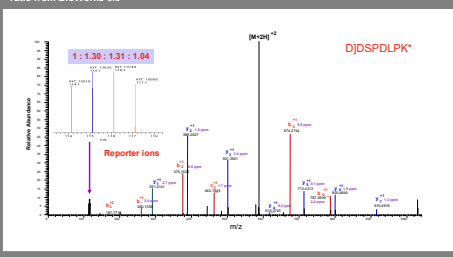


FIGURE 5. Peptide AJELFLR (2+) from Myoglobin iTRAQ quantitation with and without isobaric interferences

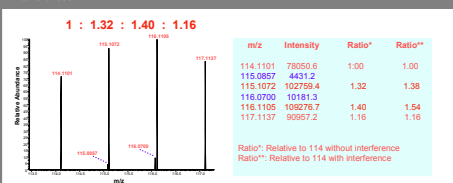


Table 1. Reproducibility of protein quantitation

Protein:	114/114	115/114	Std	CV%	116/114	Std	CV%	117/114	Std	CV%
Serum Albumin	1	1.34	0.18	13.1	1.39	0.04	2.82	1.11	0.03	2.82
G3P	1	1.35	0.03	1.96	1.48	0.03	1.97	1.16	0.03	2.60
Ovalbumin	1	1.36	0.05	3.66	1.43	0.03	1.96	1.14	0.06	5.27
Myoglobin	1	1.37	0.12	8.76	1.44	0.10	7.14	1.11	0.06	4.26
Cytochrome C	1	1.33	0.04	3.03	1.46	0.03	2.36	1.15	0.05	4.12
Lysozyme	1	1.39	0.09	6.64	1.42	0.07	4.97	1.15	0.05	3.90
Carbonic Anhydrase	1	1.33	0.05	3.47	1.41	0.04	2.81	1.12	0.04	3.56
β-Casein	1	1.33	0.02	1.70	1.39	0.02	1.66	1.12	0.02	1.43
α-Lactalbumin	1	1.25	0.20	16.06	1.47	0.06	3.79	1.09	0.04	3.31

Figure 4 shows that for this 2+ peptide from BSA, HCD generated a high quality MS/MS spectrum (20ppm mass accuracy) that matched all the b and y fragments except b<sub>2</sub>. Also the high resolution of iTRAQ reporters improves quantitation precision by separating reporter ion signals from isobaric interferences. Figure 5 is an example. The isobaric interference for this particular peptide could be up to 14%. Table 1 is the summary of protein mixture quantitation reproducibility studies. The analyzed protein's amount ranged from 10 fmol to 1µmol. The protein ID results were filtered with protein probability of 1e-003 and peptide probability of 1e-002. The individual ratios of identified peptides were calculated from the signature ion peak areas by BioWorks 3.3 (PepQuan). The average of the identified peptides ratio would then yield the "expressed" ratios for each individual protein, with averages for six runs reported in the table. Eighteen proteins of nine were identified and quantified in all six runs. The protein with lowest amount (<10 fmol), α-lactalbumin, was identified/quantified from three runs. The iTRAQ reporter ion's ratio was calculated using 114 amount as a reference. The results showed that for six proteins the CVs for iTRAQ reporters were less than 5%. Two proteins had CVs <10%. Relatively high CV for lactalbumin (16%) is related to its low concentration in the sample mixture. Table 2 is a summary of accuracy studies of protein quantitation. All nine proteins showed protein quantitation accuracy in the range of 1% to 15%.

Table 2. Accuracy of protein quantitation

	114/114	115/114	Ratio*	%Error	116/114	Ratio*	%Error	117/114	Ratio*	%Error
Serum Albumin	1	1.34	1.33	0.59	1.39	1.33	4.76	1.11	1	11.33
G3P	1	1.35	1.33	1.38	1.48	1.33	11.90	1.16	1	16.67
Ovalbumin	1	1.36	1.33	2.25	1.43	1.33	7.77	1.14	1	13.83
Myoglobin	1	1.37	1.33	2.76	1.44	1.33	7.89	1.11		