Application Note: 452

Relative Quantitation of Protein Digests Using Tandem Mass Tags and Pulsed-Q Dissociation (PQD)

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Key Words

- LTQ XL™
- PQD
- Proteomics
- Quantification
- TMT

Introduction

Quantitation of differentially expressed proteins is one of the most challenging areas in proteomics. A variety of quantitation methods have been developed, including isotope labeling approaches like ICAT®[1], SILAC[2], iTRAQ™[3], AQUA[4], and Tandem Mass Tag® (TMT®)[5]. In contrast to MS-based quantitation methods, iTRAQ- and TMT-labeled peptides are identified and quantitated by MS/MS. Pulsed-Q Dissociation (PQD)[6,7] has been developed to facilitate quantitating of the low-mass reporter ions in MS/MS spectra of iTRAQ- or TMT-labeled peptides. The PQD technique enables the detection of low-mass fragments in MS/MS mode including y1- and b1-type fragment ions, and also allows the quantitation of peptides using the TMT reporter ions which appear in the 100 *m/z* range.[8]

Goal

To demonstrate the benefits of the PQD-based quantitation of isobarically labeled peptides in protein digests.

Experimental Conditions

Preparation of TMT-labeled Peptides

A protein mixture containing ten standard proteins in various concentrations was denatured, reduced, alkylated and digested. After the digestion, six individual fractions of this ten-protein mixture were labeled according to manufacturer provided protocol with 126, 127, 128, 129, 130 and 131 tags. The contents of the labeled samples were then combined into one tube in a one-to-one ratio. The sample was then cleaned with a Thermo Scientific PepClean C-18 Spin column. The resulting sample mixture was used for both infusion and HPLC-MS/MS analyses.

LC Separation and MS Analysis

LC Separation

HPLC:	Thermo Scientific Surveyor equipped with Micro AS autosampler
Columns:	PicoFrit™ column (10 cm x 75 µm i.d.), (New Objective, Inc., Cambridge, MA)
Sample:	Inject 2 µL TMT-labeled digest mixture
Mobile Phases:	A: 0.1% Formic acid in water B: 0.1% Formic acid in acetonitrile
Gradient:	10% B 10 minutes, 10% – 30% in 120 minutes
Flow:	300 nL/min on column

MS Analysis

Mass Spectrometer:	Thermo Scientific LTQ XL equipped with a nanospray ion source
Spray Voltage:	2.0 kV
Capillary Temperature:	160 °C
Full MS:	300-1600 *m/z*
Isolation:	3 Da
MS^2:	AGC Target 4e4, 3 microscans
Collision Energy:	32% PQD
Data-dependent MS/MS:	Top 4

Database Search and Quantitation

Thermo Scientific Proteome Discoverer 1.0 software with SEQUEST® was used for data analysis. TMT modification of 229.16 on lysine and the peptide N-terminal amino acid were used for database searching. For high-confidence peptide identification, a peptide probability of middle, and peptide Xcorr vs charge (1, 2, 3) = 1.5, 2.0, 2.5 were used. Peptides that fell outside two standard deviations for relative expression quantitation ratio were removed for peptide reproducibility calculations.

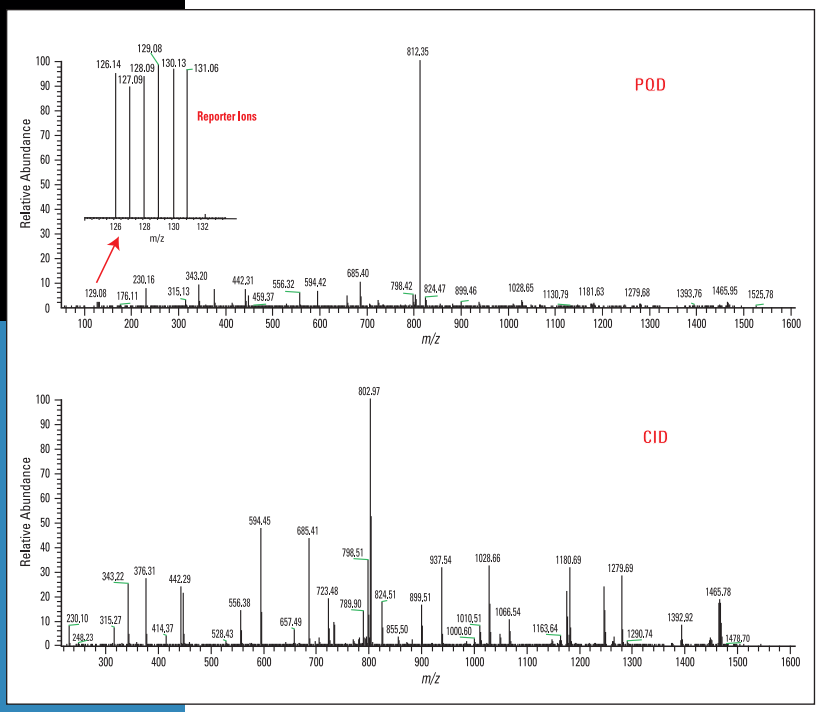

Figure 2: PQD and CID spectra of peptide L*VNELTEFAK from BSA (*Indicates TMT^6 label)

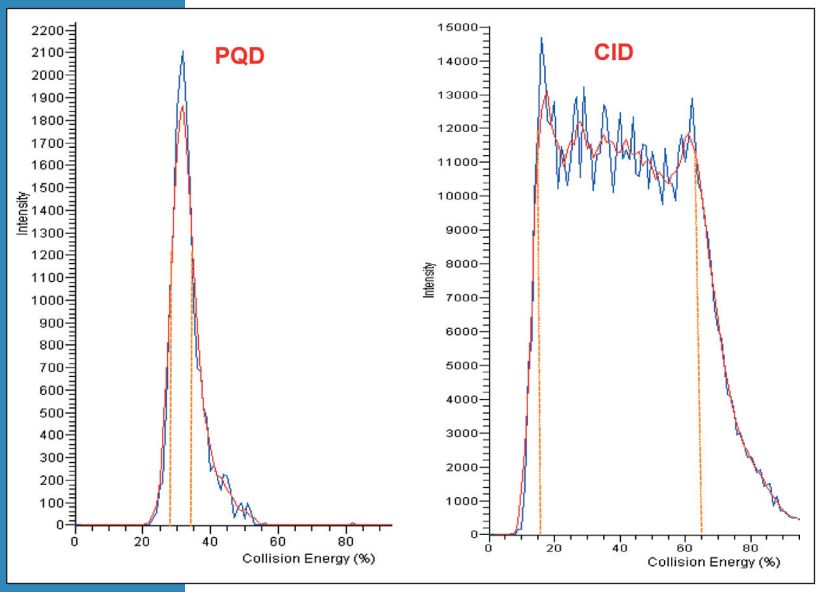

Figure 3: Collision-energy optimization for both PQD and CID using peptide F*ESNFNTQATNR from lysozyme (*Indicates TMT^6 label)

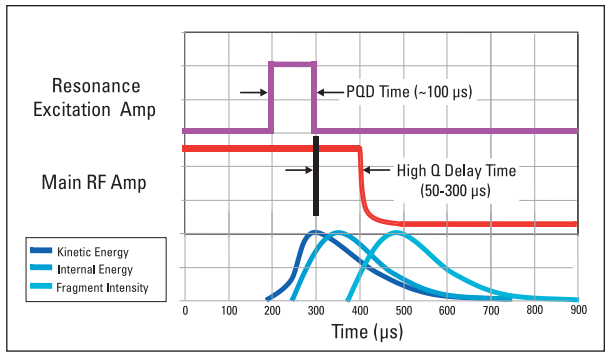

Figure 1: Schematic of dissociation process

Results and Discussion

PQD

PQD can be regarded as a three-step dissociation process involving the variation of key parameters such as the resonance excitation amplitude and the main RF amplitude. The first step involves putting the precursor ion at a high-q value (0.6 - 0.8), and using a short (~100 µs), high amplitude resonance excitation pulse as shown in the schematic in Figure 1. In this step, the ions with *m/z* resonant to this excitation pulse absorb energy and become kinetically excited. Next, ions are held at the high q value for a short delay time (~100 µs), which is long enough for the kinetic energy of the ions to be converted into internal energy through collisions, but not long enough for significant dissociation to occur. Subsequently, the precursor ions' q value is pulsed to a low value by rapidly dropping the RF amplitude and then allowing the precursor ions to undergo fragmentation at this low q value. The combination of activating at high q values (high energies) and collecting fragments at low q values (to trap low-*m/z* fragments) results in an information-rich spectrum including low-mass fragment ions.

PQD Applications

With the capability to trap and detect lower-*m/z* product ions, PQD has been applied to peptide quantitation using such methods as stable isobaric labeling, including TMT. An infusion experiment was done first to study the feasibility of the experiment. It has been shown that the fragment reporter ions generated by the labeled peptides and appearing in the *m/z* range of 126-131 can be identified and quantitatively measured. Figure 2 shows the CID and PQD spectra of a peptide (L*VNELTEFAK) from BSA. The peptide could be identified from both spectra, however, PQD also generated reporter ions in the *m/z* range of 126–131 with sufficient intensity for quantitation.

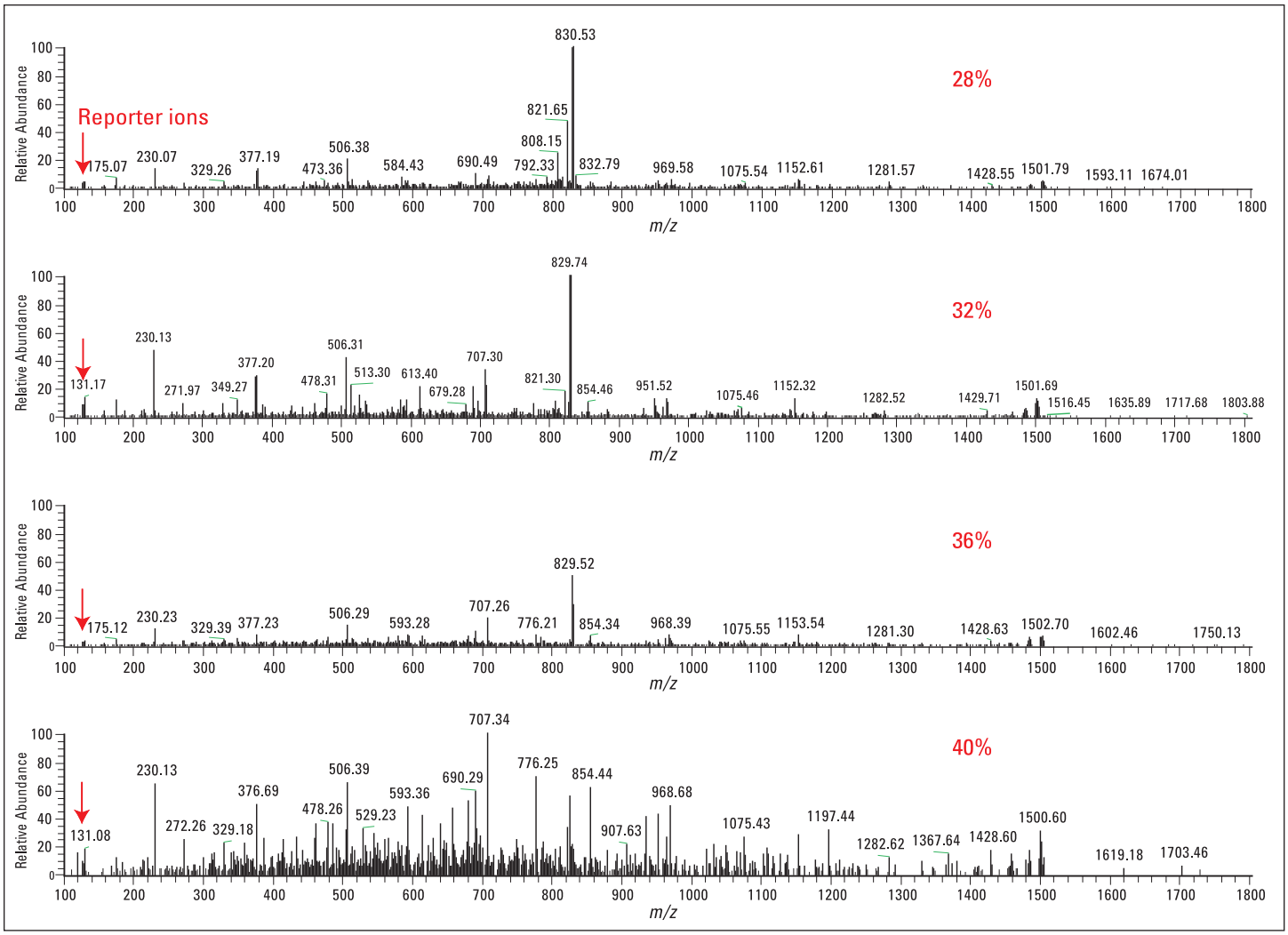

Figure 4: CE optimization for peptide F*ESNFNTQATNR from lysozyme (*indicates TMT⁶ label)

PQD Optimization

The PQD process is quite different than CID in that the dissociation kinetics have a significant effect on the performance of PQD and on the PQD parameters. The collision energy is normalized to a range similar to that used in CID. However, the actual voltage used for PQD is approximately seven to ten times higher than is used for CID. As a consequence, the range of working collision energies is much narrower for PQD than CID and needs to be optimized for compounds of interest. Figure 3 shows the PQD and CID collision-energy optimization profile for the peptide F*ESNFNTQATNR from the protein lysozyme. The peptides were identifiable from PQD spectra that used 28% to 48% collision energies. Among those, the peptides that were fragmented with 28% to 38% collision energies were quantitatable (i.e. had sufficient intensity of reporter ions present). Figure 4 displays PQD MS^2 spectra generated at various collision energies. The data show that 32% collision energy generated optimal qualitative and quantitative spectra. Therefore, 32% collision energy was chosen for later experiments. For CID, in contrast to PQD, the peptides were identifiable with a much wider optimal collision-energy range from 20% to 60%.

The PQD parameters activation q and activation time (high q delay time) should also be optimized for the compound of interest. As indicated in Figure 1, the activation q is only applied during activation and not during fragment ion accumulation. It directly affects the amount of kinetic energy the precursor ion obtains, and therefore the MS/MS spectrum. It was reported that changing q to a lower value (0.55) than the default setting (0.7) with a longer delay time would yield more reproducible reporter ions for quantitation.[9] Figure 5a compares the spectra using the two sets of parameter values. The default values generate slightly lower relative abundances of the reporter ions. Figure 5b contains the fixed-scale spectra generated from the two conditions. In fact, the spectrum generated using the default values has somewhat higher reporter ion intensities. The reproducibility of the reporter ions for quantitation is comparable for both settings. In the PQD process, using too much collision energy can eject precursor ions before they can fragment, thus lowering abundance of the fragments. Optimization of the PQD collision energy should be done by maximizing the fragment ion intensity, and not by minimizing the precursor intensity. Typically, a spectrum optimized for PQD efficiency contains significant precursor ion intensity. Therefore, in general, the default value should be sufficient for most analyses.

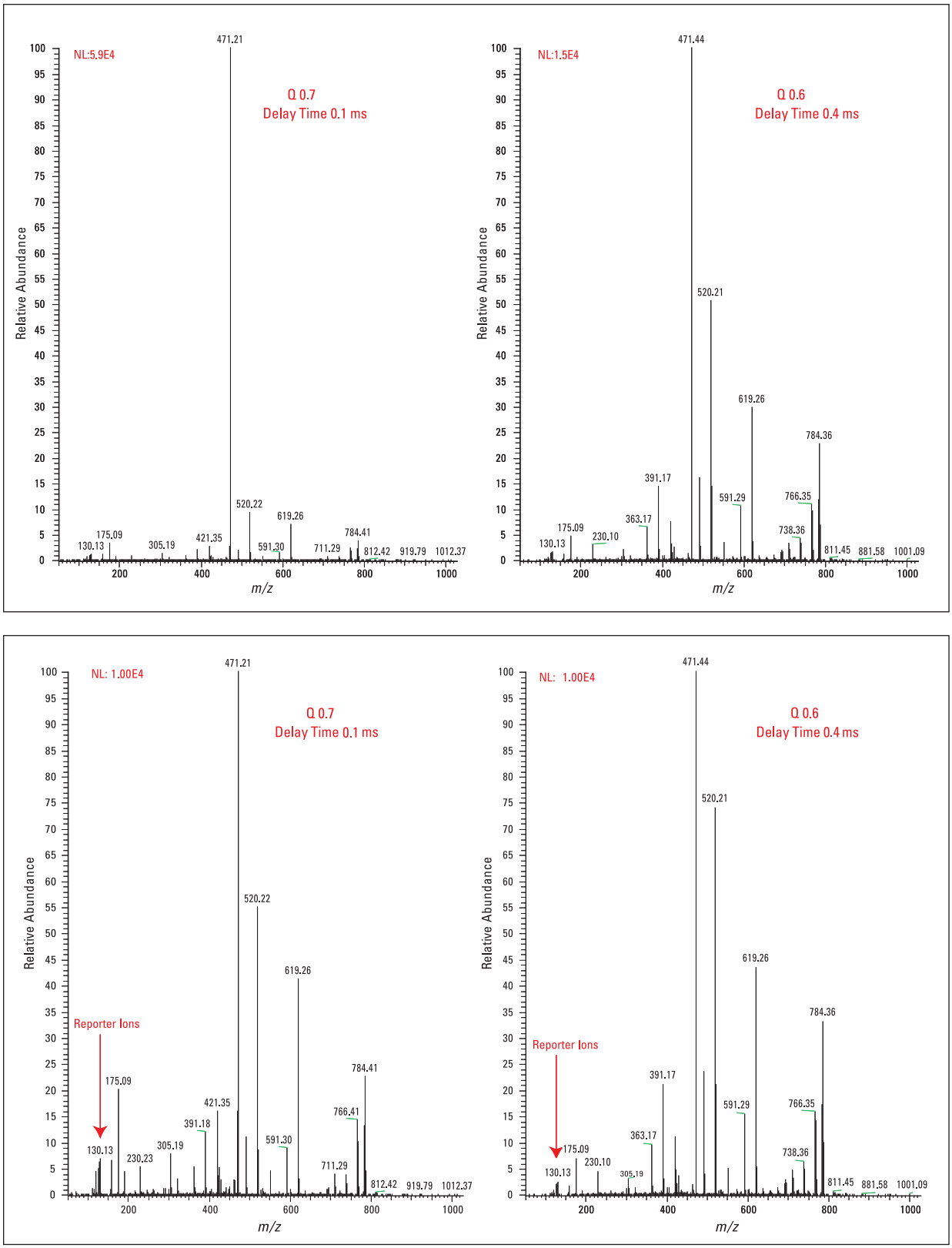

Figure 5, Top: PQD spectra acquired at different parameter values; Bottom: top normalized.

Experimental Workflow for Relative Quantitation of Isobarically Labelled Peptides and PQD

Figure 6 depicts an experimental workflow for analysis of TMT-labeled peptides by PQD. Based on an infusion experiment, 32% collision energy was found to be optimal. Since MS/MS data were used to quantitate the peptides, the MS/MS sensitivity and reproducibility were critical, and therefore the MS^n AGC target value was increased to four times the default AGC MS^n target value. Also, dynamic exclusion with a repeat count of 4 was chosen for LC/MS analysis allowing more data points for quantitation statistics. The ten-protein mixture was labeled with TMT tags and mixed 1:1, followed by LC/MS analysis using optimized experimental conditions. The data was analyzed in Proteome Discoverer using a SEQUEST search. Protein identification was conducted first, considering the TMT modification (K, +229; N-terminus, +229 and C, +58). Quantitative protein analysis was accomplished using the Reporter Ions Quantitizer as shown in Figure 7. The identified proteins were filtered with Xcorr vs charge (1, 2, 3) = 1.5, 2, 2.5, and peptide misidentification probability set at middle. Protein identification results, including protein coverage and relative ratios are reported for all ten identified proteins as shown in Figure 8. The individual reporter ion intensity with a specified mass tolerance window (0.6 Da) was used to compute the ratios of identified peptides. The average of the identified peptide ratios was then used to calculate the ratios for each individual protein. The protein quantitation results are summarized in Table 1. The quantitated proteins had an accuracy of better than 19%. Since the accurate peptide quantitation allows for protein quantitation, only unique peptides containing all six reporter ions were used for quantitation. Quantitative reproducibility on the peptide level is depicted in Figure 9, using myoglobin peptides as an example. Peptide quantitation was calculated after removing outliers outside of the range of two standard deviations. The remaining identified peptides were used for the reproducibility calculation. The relative standard deviation was in the range of 2% to 17%.

For comparison, the same experimental conditions were applied for an iTRAQ-labeled sample. Similar protein identification and quantitation results were achieved.

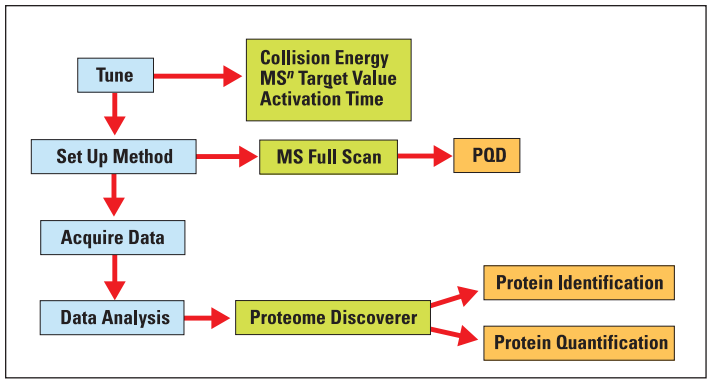

Figure 6: PQD experimental workflow

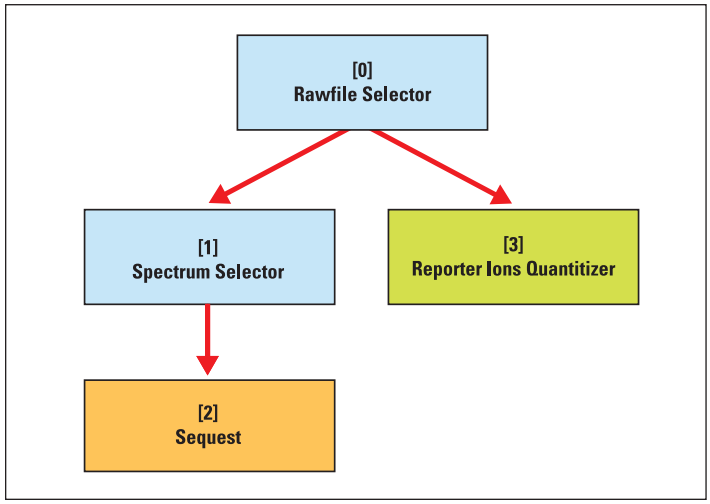

Figure 7: TMT quantitation workflow in Proteome Discoverer

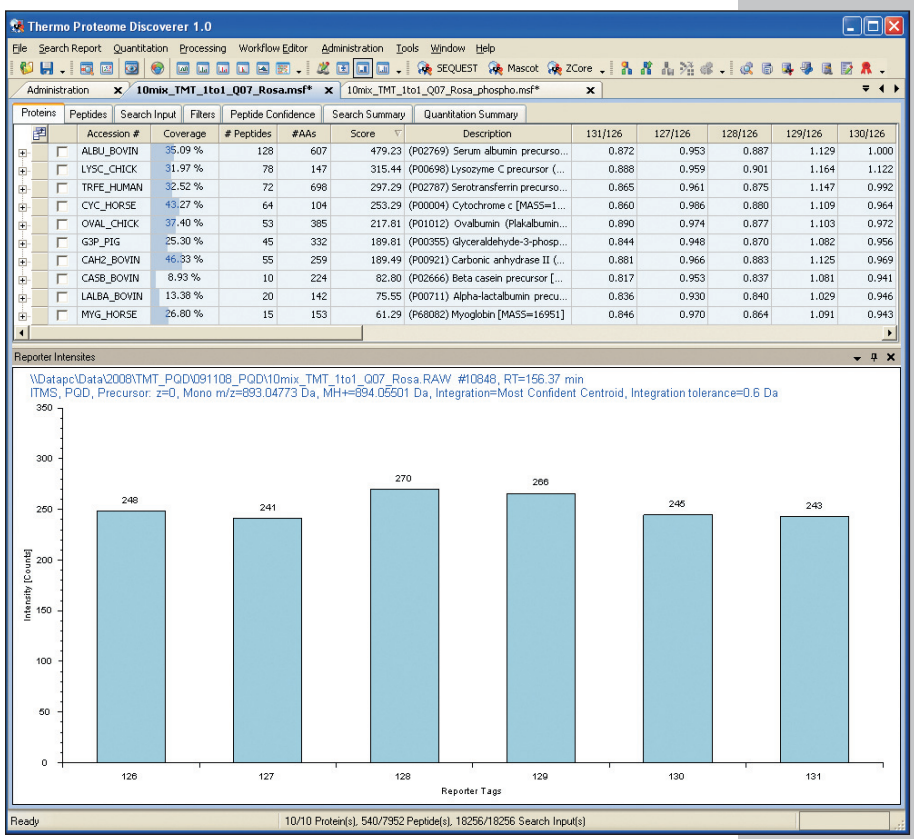

Figure 8: Protein identification and quantitation in Proteome Discoverer. Lower panel: Reporter ion intensities for G*YSLGNWVCAAK from lysozyme (*Indicates TMT^6 label)

Protein	127/126 Ratio	127/126 % Error	128/126 Ratio	128/126 % Error	129/126 Ratio	129/126 % Error	130/126 Ratio	130/126 % Error	131/126 Ratio	131/126 % Error
ALBU_BOVIN	0.986	-1.430	0.899	-10.135	1.140	13.956	0.979	-2.087	0.883	-11.748
LYSC_CHICK	0.978	-2.249	0.916	-8.353	1.127	12.744	1.049	4.919	0.866	-13.390
TRFE_HUMAN	0.980	-1.957	0.898	-10.171	1.153	15.347	0.968	-3.204	0.888	-11.200
OVAL_CHICK	1.013	1.271	0.898	-10.207	1.145	14.452	0.970	-2.970	0.871	-12.895
G3P_PIG	1.011	1.085	0.902	-9.796	1.133	13.287	0.947	-5.270	0.856	-14.361
CYC_HORSE	0.995	-0.494	0.877	-12.350	1.151	15.091	0.973	-2.697	0.870	-13.032
CAH2_BOVIN	0.999	-0.105	0.946	-5.449	1.167	16.716	0.999	-0.146	0.891	-10.912
CASB_BOVIN	1.005	0.452	0.887	-11.307	1.148	14.816	0.968	-3.214	0.852	-14.788
MYG_HORSE	0.998	-0.179	0.899	-10.072	1.136	13.563	0.981	-1.944	0.858	-14.159
LALBA_BOVIN	0.977	-2.318	0.866	-13.402	1.144	14.379	0.947	-5.341	0.868	-13.201

Table 1: Accuracy of protein quantitation

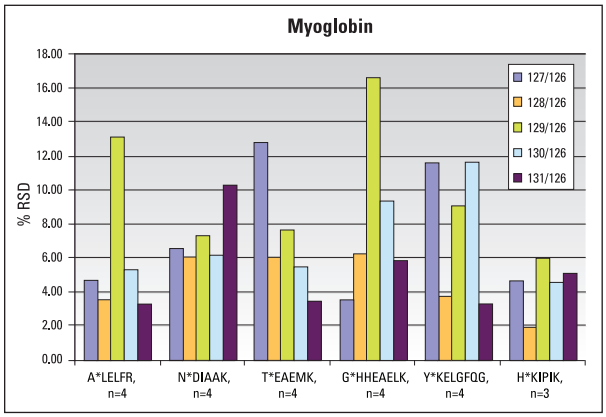

Figure 9: Quantitation reproducibility on peptide level for myoglobin (*Indicates TMT^6 label)

Conclusions

- Optimization of PQD parameters for LC MS/MS analysis is important for reproducible and accurate protein quantitation.
- The PQD fragmentation technique produces high quality MS/MS spectra with good signal to noise for TMT reporter ions.
- The accuracy of the TMT ratios was better than 19% on the protein level.
- Proteome Discoverer performs protein identification and protein quantitation using the MS/MS spectra generated by PQD. Reporter ion intensities of the MS/MS spectra were successfully used for peptide and protein quantitation.

References

1. Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H. and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nature Biotechnol.* 17, 994-999.
2. Ong, S.E., Blagoev, B., Kratchmarova, I., Kristensen, D.B., Steen, H., Pandey, A. and Mann, M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* 1, 376-386.
3. Mann, M. (2006) Functional and quantitative proteomics using SILAC. *Nature Rev. Mol. Cell Biol.* 7, 952-958.
4. Gerber, S. A., Rush, J., Stemman, O., Kirschner, M.W., Gygi, S.P., (2003) Absolute quantitation of proteins and phosphoproteins from cell lysates by tandem MS. *Proc Natl Acad Sci USA.*, 100(12), 6940-5.
5. Thompson, A., Schafer, J., Kuhn, 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.
6. Schwartz, J. C., Syka, j.E.P., Quarmby, S.T., (2005) Improving the fundamentals of MS^n on 2D ion traps: new ion activation and isolation techniques. *53rd ASMS conference on Mass Spectrometry.*
7. Schwartz, J., United States Patent, US 7, 102, 129, B2, Sep. 5, 2006
8. Schlabach, T., Zhang, T., Miller, K. Kiyonami, R., (2006) Protein identification and quantitation using an ion-trap with enhanced detection in the low mass range. *ABRF conference proceedings.*
9. Bantscheff, M., Boesche, M., Eberhard, D., et al. (2008) Robust and sensitive iTRAQ quantitation on an LTQ-Orbitrap mass spectrometer. *Mol. Cell. Proteomics* 7, 1702–1713.

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